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Genetic Variation in PTPN22 Corresponds to Altered Function of T and B Lymphocytes

Mary Rieck,* Adrian Arechiga,* Suna Onengut-Gumuscu,†§¶# Carla Greenbaum,‡ and Jane H. Buckner**

A variant of the PTPN22 gene, 1858C/T, is associated with an increased risk for the development of a wide array of autoimmune disorders. It is known that the protein tyrosine phosphatase Lyp encoded by this gene has an inhibitory effect on the proximal TCR signaling pathways. However, the consequences of carrying this variant and the mechanism by which it contributes to the development of autoimmunity are poorly understood. In this study, we demonstrate that homozygosity for this variant results in a profound deficit in T cell responsiveness to Ag stimulation. Heterozygosity for the variant allele is associated with reduced responsiveness of CD4+ memory T cells, characterized by diminished calcium mobilization, expression of CD25, and IL-10 production upon TCR stimulation. Additionally, the presence of the variant allele is associated with an increase in circulating memory T cells. We further demonstrate that these effects are not limited to the T cell compartment. Individuals with the variant allele have fewer memory B cells and these cells display a reduced response to stimulation via the BCR indicative of a B cell intrinsic defect. By identifying an immunologic phenotype in healthy subjects which correlates with the PTPN22 1858C/T genotype, we can now explore specific hypotheses regarding pathogenesis of diseases associated with the PTPN22 1858T variant. The Journal of Immunology, 2007, 179: 4704–4710.

The gene PTPN22 encodes the protein tyrosine phosphatase N22 also referred to as Lyp. Lyp is an inhibitor of TCR signal transduction and is known to function by dephosphorylating the autophosphorylation sites on the protein tyrosine kinases: Lck, Fyn, and Zap70 (1). Recently, a variant of PTPN22 (1858T) has been associated with an increase in risk for the development of multiple autoimmune diseases including type 1 diabetes (T1D)2–5, rheumatoid arthritis (6), systemic lupus erythematosus (7), and Graves’ disease (8). This variant is a single nucleotide change at residue 1858 from C to T, which results in a single amino acid substitution from arginine to tryptophan at position 620 of the Lyp protein (Lyp 620W).

The PEST domain phosphatase (PEP) is the murine homolog of Lyp (1). Mice that lack PEP demonstrate several alterations in lymphocyte function and phenotype including an increase in the sensitivity of memory T cells to activation via TCR, an increase in circulating memory T cells, and enlarged germinal centers with an increase in GL-7+ B cells and CD23+ follicular B cells (9). The inhibitory function of Lyp on TCR transduction is enhanced when it interacts with the tyrosine kinase CSK (10). This interaction occurs in the region of Lyp which includes residue 620. Variation at this position might be expected to alter the inhibitory effect on TCR signal transduction mediated by this complex. Initial reports suggested that the Lyp 620W isoform interacted less strongly with CSK. Thus, it was hypothesized that the PTPN22 1858T variant would result in a loss of TCR inhibition and T cell hyperresponsiveness (11), but more recently studies using transfected Jurkat cells have shown that the Lyp 620W variant has an enhanced inhibitory effect on TCR signaling (12). However, the role Lyp plays in autoimmunity may extend beyond T cells, since Lyp is expressed in B and other hematopoietic cells, raising the possibility of other yet uncharacterized effects of this variant on the immune response.

The clear link between PTPN22 and lymphocyte signaling pathways and the association of the 1858T variant with multiple autoimmune diseases suggests that it may influence a pivotal and common pathway leading to the development of autoimmunity. Identifying the functional consequences of the PTPN22 1858T variant has the potential to elucidate a “common” immune phenotype linking the pathogenesis of these diseases. In this article, we use lymphocytes from subjects homozygous for the variant and confirm that the 1858T variant results in a gain of inhibitory function by Lyp. We then examine the function and profile of lymphocytes taken from healthy homozygous subjects and show that altered phenotype and function is also present in individuals without autoimmunity. We then extend this work to demonstrate that the presence of this variant alters not only the function and character of the T cell compartment, but also has a direct impact on B cell function. Thus, the role this variant plays in the development of autoimmunity likely extends beyond the T cell compartment to multiple compartments and the interactions between them.

Materials and Methods

Subjects

Samples for this study were obtained from participants in the Benaroya Research Institute Immune Mediated Disease Registry and Juvenile Diabetes Research Resources Center for Translational Research. The control population was selected based on a lack of personal or family history of diabetes, autoimmune diseases, and other chronic inflammatory conditions.
diabetes, autoimmunity, or asthma. The typing of 167 control subjects for the 1858T variant resulted in no subjects homozygous for the 1858T allele (T/T), 31 subjects heterozygous for the 1858C and 1858T alleles (C/T) and 136 subjects homozygous for the 1858C allele (C/C).Typing of subjects with TID, rheumatoid arthritis, relapsing polychondritis, autoimmune thyroiditis, and their first-degree relatives found 11 subjects homozygous for the 1858T variant. All 1858T/T samples used in this study were matched with an 1858C/C subject of the same age and diagnosis for research. Analysis protocols were approved by the Benaroya Research Institute Institutional Review Board. Subjects and/or their parents provided written informed consent before participation in the study.

Genotyping

The 1858 C/T SNP was genotyped using the MGB-Eclipse System (Nanogen). The genotyping assay was performed using 10 ng of genomic DNA, 0.3 U JumpStart Taq (Sigma-Aldrich) in a 5-μl reaction volume according to the manufacturer’s protocol and analyzed on an Applied Biosystems HT7900.

FACS analysis

In T cell population studies, PBMC were stained with CD45RA-FITC (eBioscience), CD3-PE (eBioscience), CD4-PerCP (BD Pharmingen), and CD45RO-allophycocyanin (BD Pharmingen). For B cell studies, PBMC were stained with CD19-FITC (eBioscience), IgD-PE (BD Pharmingen), CD27-allophycocyanin (BD Pharmingen), and biotin-labeled IgM (eBioscience), secondarily labeled with anti-biotin-PerCP (BD Pharmingen). The flow cytometry was conducted on a BD Biosciences FACS Calibur. Analysis of FACS data was done by CellQuest (BD Biosciences) and FlowJo (Tree Star) software.

Calcium flux assays

CD4+ T cells were isolated from PBMC via negative selection using a human CD4+ T cell isolation kit II (Miltenyi Biotec) The CD4 T cells were stained with fluo-4 acetoxymethyl ester (Molecular Probes) at 37°C for 1 h as previously described (14). CD4+ samples were then flushed with anti-CD3 at 1–100 μg/ml (UCHT clone; eBioscience) or ionomycin at 1 μg/ml (Sigma-Aldrich) and read for a period of ~30 min on a Wallac Victor plate reader (PerkinElmer) after a 2-min basal read. CD4+ samples analyzed for memory subset studies were further stained with either CD4-PerCP and CD45RO-allophycocyanin or fura red (Molecular Probes) and CD45RO-allophycocyanin. Samples were assessed by FACS at 37°C at basal fluorescence for 1 min and then assessed for 9 min after addition of either anti-CD3 or ionomycin. B cells were isolated from PBMC via negative selection using a Miltenyi Biotec human B cell isolation kit. B cells were labeled with fluo-4 and fura red for 1 h at 37°C, followed by surface staining for CD27. Calcium mobilization was plotted as a ratio of Fluor-4:fura red (FL1:FL3) using the kinetics suite of FlowJo software.

Cytokine secretion and proliferation

For the analysis of proliferation and cytokine production, CD4+ T cells were isolated from PBMC via negative selection using a human CD4+ T cell isolation kit II (Miltenyi Biotec) The CD4 T cells were stained with fluo-4 acetoxymethyl ester (Molecular Probes) at 37°C for 1 h as previously described (14). CD4+ samples were then flushed with anti-CD3 at 1–100 μg/ml (UCHT clone; eBioscience) or ionomycin at 1 μg/ml (Sigma-Aldrich) and read for a period of ~30 min on a Wallac Victor plate reader (PerkinElmer) after a 2-min basal read. CD4+ samples analyzed for memory subset studies were further stained with either CD4-PerCP and CD45RO-allophycocyanin or fura red (Molecular Probes) and CD45RO-allophycocyanin. Samples were assessed by FACS at 37°C at basal fluorescence for 1 min and then assessed for 9 min after addition of either anti-CD3 or ionomycin. B cells were isolated from PBMC via negative selection using a Miltenyi Biotec human B cell isolation kit. B cells were labeled with fluo-4 and fura red for 1 h at 37°C, followed by surface staining for CD27. Calcium mobilization was plotted as a ratio of Fluor-4:fura red (FL1:FL3) using the kinetics suite of FlowJo software.

Results

CD4 T cells from subjects homozygous for the 1858T variant display a profound reduction in calcium mobilization in response to TCR stimulation

To address the impact of the 1858T variant of PTPN22 on TCR signal transduction in unmanipulated T cells, we evaluated the ability of CD4+ T cells from individuals homozygous for the 1858T variant to mobilize Ca2+ upon activation with anti-CD3 Ab. To do these studies, we paired samples from 1858T/T subjects with samples from 1858C/C individuals, matching samples for age and disease. All 1858C/C samples showed robust responsiveness to activation with anti-CD3, whereas calcium mobilization was profoundly diminished in all of the 1858T/T subjects studied. Fig. 1 shows a representative pair of samples from individuals with TID, an 1858T/T (Fig. 1A) as compared with an 1858C/C subject (Fig. 1B). This defect in calcium mobilization was present over a range of activation stimuli (0.1–100 μg/ml anti-CD3) with a response in the 1858T/T CD4+ T cells only seen at the highest levels of activation. When ionomycin was used to stimulate the CD4 T cells of 1858 T/T subjects in these assays, calcium mobilization was similar to that of 1858C/C subjects (data not shown). This demonstrates that the defect in the response to TCR stimulation in 1858T/T homozygotes is in the proximal TCR signaling pathway and suggests that the product of the 1858T allele, Lyp 620W, results in a gain of inhibitory function of Lyp.

To further investigate the immunologic consequences of the PTPN22 1858T allele, we focused our studies on unaffected individuals with no family history of autoimmune disease. This was...
Altered TCR signal transduction induced by the PTPN22 1858T allele result in a decrease in the early activation marker CD25, but not a decrease in proliferation

To address whether the diminished calcium mobilization seen in response to TCR stimulation of CD4 T cells of 1858T carriers results in alterations in the downstream responses to activation, we examined the expression of the early activation marker CD25. CD25 expression typically peaks in activated CD4+CD25− T cells 24 h after of stimulation (15). We found a significant decrease in the percentage of CD4+ T cells expressing CD25 from PTPN22 1858T carriers when compared with C/C subjects after activation (Fig. 3A). The decrease was seen with activation in the presence or absence of APCs. These studies were done in the presence of exogenous IL-2; therefore, the decrease in CD25 expression was not secondary to a decrease in IL-2 production. However, when proliferation was measured, we found no differences in proliferation between PTPN22 1858 C/T and PTPN22 1858 C/C control subjects when [3H]thymidine incorporation was measured at day 3 (Fig. 3B) or day 6 (data not shown), even over a range of anti-CD3 concentrations (0.1–100 μg/ml). Further studies were performed with FACS-sorted CD4+, CD4+CD45RA− naive T cells, or CD4+CD45RO− memory cells and again we found no difference in proliferation between the two groups of subjects at day 3 or day 6 after activation (data not shown). In addition, studies using CFSE to examine proliferation on day 2, 4, or 6 did not demonstrate any

done to minimize the impact of other risk factors, either genetic or environmental, and to avoid any alterations in immune function related to disease or immune modulatory therapies. Within this control population, no individuals homozygous for the 1858T variant were identified. Thus, further studies were done in control individuals with either the 1858C/C or 1858C/T genotype. When 1858C/C and 1858C/T control subjects were studied, calcium mobilization was decreased in 1858C/T controls, although not to the same extent as that seen in the 1858T/T subjects (Fig. 2A), suggesting a gene dose effect of the variant. When calcium mobilization was examined simultaneously for the naive and memory populations, the defect in calcium mobilization within heterozygous subjects was most pronounced in the memory T cell population (Fig. 2A).

FIGURE 2. CD4 T cells from control subjects heterozygous for the PTPN22 1858T variant demonstrate reduced TCR-mediated calcium mobilization, which is most profound in the memory T cell compartment. CD4 T cells were purified from 1858C/C, T/T, or T/C control subjects and labeled with fluo-4 and fura red, stained with Abs against CD4 and CD45RO, then stimulated with anti-CD3. A, Representative plots of calcium flux over time assessed for total CD4 T cells, where calcium flux was measured by taking the ratio of the change in fluorescence between fluo-4 and fura red. B, Representative samples of naive and memory T cell response to anti-CD3 measured simultaneously, C/C subject is depicted by a solid line, and the C/T subject by a dotted line. C, Percent maximal calcium flux for either CD4+CD45RO− memory T cells or CD4+CD45RO− naive T cells, stimulated with anti-CD3 at 100 μg/ml. Plotted values were determined by dividing the peak calcium flux value for either the memory or naive subset by the maximal flux value as determined by ionomycin. Filled symbols are shown for the C/C genotype and open symbols represent the C/T genotype. D, A representative example of the similarity between C/C and C/T samples in Ca++ mobilization with ionomycin stimulation.
significant differences between the CD4⁺ T cells of 1858C/C and 1858C/T subjects (data not shown). These findings suggest that while carrying the 1858T allele is associated with initial inhibition of activation as measured by Ca⁺ mobilization and expression of CD25 on the cell surface, the effect does not extend to proliferation with in vitro cultures where the effect may be overcome due to persistent stimuli, costimulation, and/or the presence of cytokines in the cell culture.

PTPN22 1858T variant is associated with alterations in IL-10 production

To address the effects of Lyp620W on the functional consequences of T cell activation, we examined the production of cytokines by CD4⁺ T cells carrying the variant allele. Supernatants taken 24 h postactivation were assayed for IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α. When the results were examined based on genotype, we found that IL-10 was reduced in PTPN22 1858C/T samples relative to 1858C/C samples, and this difference was most pronounced in cultures activated with anti-CD3/anti-CD28 coated beads (Fig. 3B; p = 0.04). These same samples showed no PTPN22-associated difference in proliferation nor secretion of IFN-γ or TNF-α (Fig. 3B). Of the additional cytokines analyzed in these assays; decreases in IL-4 (p = 0.06), IL-2 (p = 0.4), and IL-5 (p = 0.2) were seen but did not reach statistical significance. These findings suggest that the presence of the 1858T variant does not result in a global decrease in cytokine production, but is associated with an alteration in the balance of cytokines produced upon activation.

CD4⁺ T cell profiles are altered in subjects with the 1858T variant

To address the question of whether altered CD4⁺ T cell responses in vitro correspond to altered T cell function and survival in vivo, we examined the T cell profile of subjects based on genotype. PBMC from control subjects were stained with the T cell markers CD3, CD4, CD45RA, and CD45RO and analyzed via FACS for the relative number of naive and memory T cells. When T cell profiles based on the PTPN22 genotype were compared, we found the percentage of CD4⁺ memory cells in C/C controls ranged between 14 and 70%, with the greatest variation in younger individuals, whereas a more limited range and higher number of memory cells was found in C/T subjects (48–65%), resulting in a significant increase in the mean percentage of memory T cells in C/T subjects as compared with C/C subjects (Fig. 4A; p = 0.04). These numbers reflect a numerical increase in total memory T cells with a decrease in naïve T cells as the percentage of CD4⁺ T cells relative to total mononuclear cells is similar between all groups. Because memory T cells increase with age (16), an analysis of the relationship between age, percent memory, and PTPN22 genotype was also performed using linear regression analysis. This confirmed the association between the C/T genotype and an expanded CD4 memory T cell pool (Fig. 4B; p = 0.03).

B lymphocyte profiles are altered in subjects with the 1858T variant

The 1858T variant is associated with a group of autoimmune diseases characterized by the development of autoantibodies, potentially implicating Lyp620W in the dysregulation of the B cell response. In addition, the PEP⁻/- mouse has an altered B cell population (17). To address the question of whether the PTPN22 1858T variant influences the function or repertoire of B lymphocytes, we examined B cell profiles of PTPN22 1858C/C or C/T control subjects. We found no difference in the number of CD19⁺ cells present in PBMC between homozygotes and heterozygotes. However, the CD27⁺ memory population within CD19⁺ B cells was decreased in individuals with the 1858T allele relative to subjects without the variant (Fig. 5A; p = 0.025); furthermore, the association of genotype with diminished memory B cells was significant when the analysis was adjusted for age by use of regression analysis (p = 0.028). Further examination of the memory B cell subsets showed no difference in the number of IgM⁺IgD⁺ or
PTPN22 1858T allele results in altered lymphocyte function

IgM^−/IgD^− B cells, nor any differences in the number of CD27^{high} plasmablasts between the two subject groups (data not shown). None of these results was influenced by recent immunizations.

**FIGURE 4.** Memory T cell profiles are altered in individuals who carry the PTPN22 1858T allele. Previously frozen PBMC from control 1858C/C and C/T subjects were analyzed by FACS. Percent memory in CD4^+ T cells was characterized as CD4^+CD3^+CD45RO^−CD45RA^−. A, Memory T cell relative to the total number of CD4^+CD3^+ lymphocytes is plotted based on genotype with solid triangles representing C/C subjects (n = 29) and open squares for C/T subjects (n = 22). A significant difference is present between the control 1858C/C and 1858C/T subjects (p = 0.04). B, Percent memory CD4^+ T cells plotted against age, with triangles for C/C and squares for C/T subjects. Trend lines are plotted as a solid line for C/C subjects and dots were used for C/T subjects. Linear regression analysis showed a significant difference between the C/C and C/T populations irrespective of age (p = 0.03).

These findings demonstrate an association between Lyp 620W and a reduction in the size of the circulating memory B cell pool. The mechanism by which Lyp 620W may contribute to this is unclear, but may be secondary to alterations in B cell survival or maturation.

To address whether the decrease in circulating memory B cells in subjects carrying the 1858T variant is due to a direct effect on B cell response to activation or is solely a consequence of the T cell defects we had previously characterized, we examined the Ca^{2+} mobilization of CD19^+ B cells in response to BCR stimulation by cross-linking the BCR with anti-IgM. A diminished Ca^{2+} mobilization response was seen in the B cells of 1858C/T subjects in comparison to 1858C/C subjects. This decrease was most pronounced in the CD27^+ memory B cell populations (Fig. 5, B and C), as the CD27^-naive populations peak calcium flux populations are not significantly different (Fig. 5, B and D). These findings implicate B cell intrinsic factors in the decrease of memory in the B cells in peripheral blood.

**FIGURE 5.** Memory B cells are decreased in subjects with the variant 1858T allele and demonstrate reduced BCR-mediated calcium mobilization. A, Previously frozen PBMC from subjects were analyzed by FACS. Control C/C subjects (n = 15) are represented by solid triangles and control C/T subjects (n = 15) by open squares. Memory B cells are shown as a percentage of total CD19^+ B cells and distinguished by the presence of CD27. 1858C/C controls showed a significantly greater percentage of memory B cells in comparison to either C/T controls (p = 0.025), as determined by the t test. Total B cells from 1858C/C or T/C subjects were purified and labeled with fluo-4 and fura red, stained with Abs against CD27, then stimulated with anti-IgM. B, Representative plots of calcium flux over time assessed for naive B cells or memory B cells. Calcium flux was measured by taking the ratio of the change in fluorescence between fluo-4 and fura red. Peak calcium response for CD27^+ cells is shown (C; n = 4, p = 0.005) or naive CD27^- B cells (D; n = 4).

These findings identify a unique immune phenotype that is associated with the PTPN22 1858T variant in healthy heterozygous individuals. Furthermore, we show that these features are more pronounced in individuals homozygous for this variant in the setting of autoimmunity.
We have used unmanipulated cells from subjects who are heterozygous or homozygous for the PTPN22 1858T allele. We observed a reduced T cell responsiveness to anti-CD3 stimulation in healthy heterozygous subjects possessing the Lypr620W isoform and a profound deficit in the ability of T cells from individuals homozygous for the 1858T variant to mobilize calcium in response to TCR stimulation. These results are similar to observations in transfected Jurkat T cell lines where overexpression of the Lypr620W isoform, in comparison to Lypr 620R, resulted in a reduced calcium flux in response to anti-CD3 and decreased phosphorylation of TCRζ, Erk2, Lck Y394, and LAT (17). Thus, our findings in unmanipulated human T cells, support the hypothesis that the PTPN22 1858T variant is a gain of function mutation resulting in enhanced inhibition of TCR signaling.

We further hypothesize that the risk of autoimmunity conferred by this variant may not arise directly from its effects on T cell responses to Ag but rather to the effects of this phenotype on the character and composition of the T cell pool. We observed that subjects with the 1858T genotype have an increased CD4+ memory T cell compartment. There are several plausible mechanisms whereby a muted response to TCR stimulation could lead to an increase in memory T cells. A decrease in thymic negative selection could result in increased responses to self in the periphery or the expansion of the memory T cell compartment could be due to an increased resistance of T cells, particularly high-affinity self-reactive T cells, to activation-induced death. Examination of CSFE and annexin V-labeled T cell proliferation on days 4 and 6 provide no evidence to support the latter in the setting of activation with anti-CD3.

Our findings and those of Vang et al. (18) implicate altered cytokine production as a potential pathogenic mechanism by which the 1858T variant may contribute to the development of autoimmunity. In both studies, cytokines were measured from total cytokine production as a potential pathogenic mechanism by anti-CD3.

Whether alterations in the levels of IL-10 secretion reflect an effect of Lypr at the proximal TCR signaling cascades or as part of another pathway is unclear. T cell commitment could be skewed by alterations in signal strength or Lypr may interact with other pathways which directly influence the production of IL-10. Recent studies suggest that Lypr may have other binding partners (20). Nevertheless, this finding suggests a role for PTPN22 in immune regulation and links alterations in this gene to a phenotype associated with the development of autoimmunity.

Our findings also suggest that the 1858T variant may contribute to autoimmunity via B cells. We demonstrate that the B cell compartment is altered in subjects who carry the 1858T variant and further show that B cells from these subjects have a decreased ability to respond to stimulation via the BCR. Although B cell differentiation and proliferation are influenced by T cell function, the association of the 1858T variant with a diminished response to BCR stimulation indicates that B cell intrinsic processes are directly altered by the 1858T variant. A diminution of the BCR signal could result in the escape of autoreactive B cells into the periphery and, in addition, the relative decrease in memory B cells seen in subjects with the 1858T variant may be the result of a shunting of B cell into the plasma cell pool. In each case, these changes could enhance the subsequent development of autoantibodies and is notable in light of the fact that the diseases associated with this variant have the production of autoantibodies as a common characteristic.

Taken together, our findings demonstrate that the PTPN22 1858T variant is associated with a dampened response of both the T and B cell Ag receptors, furthering the hypothesis that the Lypr 620W isoform has enhanced inhibitory function in human lymphocytes. On the surface these results seem paradoxical since autoimmunity would seem more likely to arise from hyper-responsive T and B cells. However, we demonstrate that the variant is associated with a change in the cytokine profile resulting from T cell activation as well as a shift in the T and B cell memory populations. These global changes may provide a more fertile environment for the development of autoimmunity, either by favoring the development and survival of autoreactive lymphocytes or by a loss of the regulatory mechanisms by which they are held in check. Although these findings are associated with this specific genetic variant, the data suggest that the attenuation of Ag receptor signaling may be a common mechanism that underlies the pathogenesis of the autoimmunity.

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**Disclosures**

The authors have no financial conflict of interest.

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