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Genetic Variation in PTPN22 Corresponds to Altered Function of T and B Lymphocytes[1]

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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)[3](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 $GL\text{-}7^+$ 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 hemopoietic 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 heterozygous 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

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[3] Abbreviation used in this paper: T1D, type 1 diabetes; PEP, PEST domain phosphatase.

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 T1D, 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 analysis. Research 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 FACSCalibur). 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 fluxed 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 at 100 μg/ml or ionomycin. B cells were isolated from PMBC 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 Fluo-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 Miltenyi Biotec human $CD4^+$ T cell isolation kit II. In assays using naive and memory subsets, CD4 cells were further sorted using a FACSVantage cell sorter (BD Biosciences), based upon staining with CD45RA-FITC, CD3-PE, CD4-PE-Cy7 (eBioscience), and CD45RO-allophycocyanin. Cells (10^6/well) were cultured in triplicate in 96-well round-bottom plates (Costar; Corning) that had been partially precoated with 50 μl/well anti-CD3 at concentrations of 0.01–100 μg/ml for 2 h at 37°C. Soluble anti-CD28 Ab at 1 μg/ml (eBioscience) was added to the wells containing anti-CD3. Additional wells containing beads coated with anti-CD3 and anti-CD28 (Excite) were used at a 1:1 bead:cell ratio, and wells with PMA at 400 ng/ml (Sigma-Aldrich) and ionomycin at 40 ng/ml were added as a control to cells in uncoated wells. At 24 and 48 h supernatant was removed from each well and analyzed for cytokines using a cytometric bead array system (BD Immunocytometry Systems). [^{3}H]Thymidine was then added after 48 h (1 μCi/well), and cells were cultured for an additional 20 h before being harvested and having their incorporation determined using a Wallac Microbeta Plus liquid scintillation counter (PerkinElmer).

Experiments using CFSE were performed as above, but before activation cells were washed and resuspended in PBS (5×10^6/0.5 ml). CFSE was added at a final concentration of 0.8 μM (Molecular Probes). The cells were harvested at 2, 4, and 6 days after stimulation and analyzed using flow cytometry.

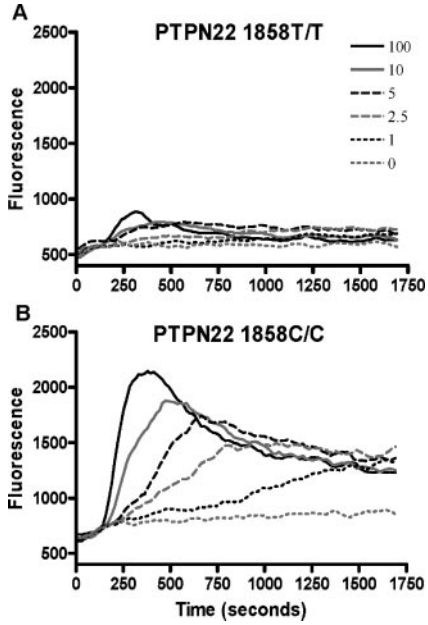

FIGURE 1. CD4 T cells homozygous for the *PTPN22 1858T* variant demonstrate poor calcium mobilization. Freshly isolated $CD4^+$ T cells were stained with fluo-4 and activated with a range of anti-CD3 concentrations (1–100 μg/ml). *A*, A representative sample of calcium flux from an *1858T/T* subject ($n = 4$) showing a response to TCR stimulation only at the highest levels of activation. *B*, Data from an *1858C/C* subject matched for age and disease status to the *1858T/T* subject shown above ($n = 5$).

Statistical analysis

Data were analyzed by unpaired two-tailed *t* tests, assuming unequal variances using GraphPad Prism version 4.02 for Windows (www.graphpad.com) or linear regression with robust SEs using Stata statistical software, version 8.0. Values of $p < 0.05$ were considered significant.

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 Ca^+ 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 T1D, an *1858T/T* (Fig. 1*A*) as compared with an *1858C/C* subject (Fig. 1*B*). 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

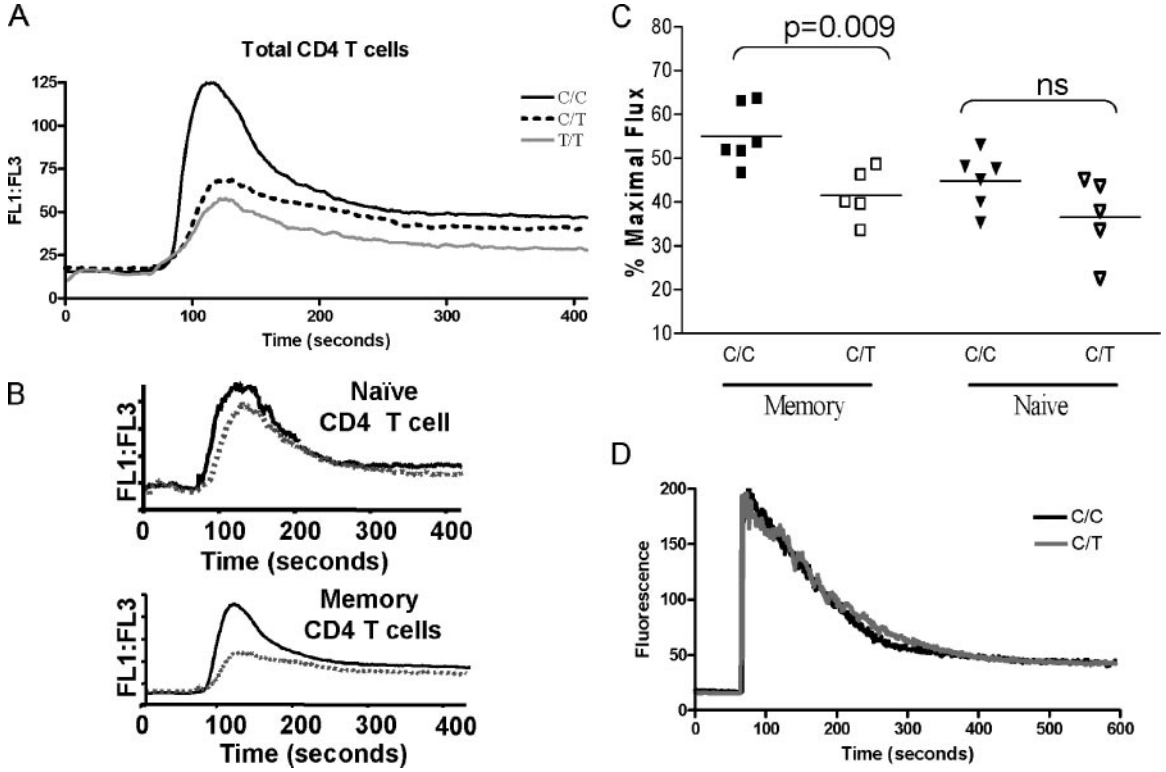

FIGURE 2. CD4 T cells from control subjects heterozygous for the *PTPN22 1858T* variant demonstrate reduced TCR-mediated calcium mobilization, which is most profound in the memory T cell compartment. CD4 T cells were purified from *1858C/C*, *T/C*, or *T/T* control subjects and labeled with fluo-4 and 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.

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 individual 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. 2*A*), 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 prominent in the memory T cell population (Fig. 2, *B* and *D*). In comparison to *PTPN22 1858 C/C* subjects, calcium mobilization was significantly lower in the $CD4^+$ memory T cells of subjects with the *PTPN22 1858C/T* genotype (55% $\pm$ 7.5 vs 42% $\pm$ 5.9, respectively; $p = 0.0074$), whereas no significant differences were observed between the naive T cell populations in these subjects (Fig. 2*C*). The time to peak fluorescence did not differ between the two groups of subjects (data not shown). No difference in peak calcium mobilization was seen when TCR was bypassed by use of ionomycin (Fig. 2*D*). These findings indicate that the Lyp 620W variant inhibits T cell activation even in the presence of the Lyp 620R and that the decrease in the signal transduction via TCR is most pronounced in the memory T cell compartment.

Alterations in 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 72 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. 3*A*). 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 [^{3}H]thymidine incorporation was measured at day 3 (Fig. 3*B*) 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

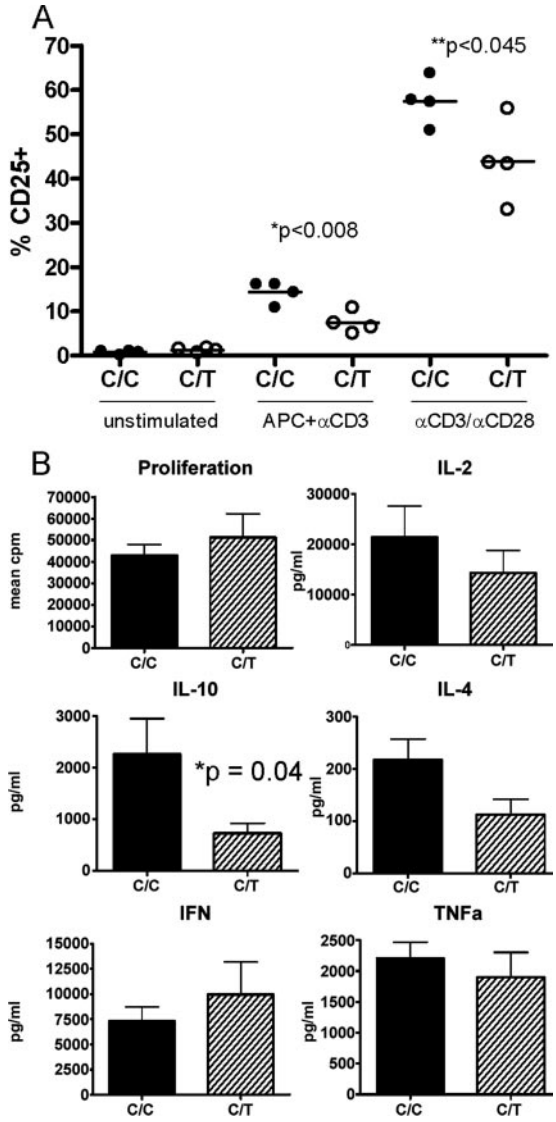

FIGURE 3. Activation profiles of CD4 T cells from subjects with and without the 1858T variant. *A*, Freshly isolated $CD4^+CD25^-$ T cells were isolated from control subjects and activated with either irradiated APCs or anti-CD3/anti-CD28 beads, then evaluated by FACS on day 3 for expression of the activation marker CD25. $CD4^+$T cells from 1858T carriers demonstrate a diminished level of CD25 expression with both forms of activation ($p = 0.008$ and $p = 0.045$, respectively). *B*, $CD4^+$ cells from control subjects matched for age (20–25 years) were isolated from previously frozen PBMC activated with anti-CD3/anti-CD28-coated beads at a cell:bead ratio of 1:1. Proliferation as measured by [^{3}H]thymidine incorporation at 72 h and cytokine levels in supernatants taken after 24 h are shown. IL-10 secretion is significantly decreased among *C/T* relative to *C/C* controls ($p = 0.04$). A trend toward decreased IL-4 ($p = 0.06$) in *C/T* control subjects was also observed. No difference was found in 72-h proliferation, IFN-γ or TNF-α secretion between groups. No correlation was found between the percent memory T cells in the starting population and cytokine production at 24 h. ■, *C/C* controls ($n = 6$); ▨, *C/T* control subjects ($n = 6$).

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.

The 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. 3*B*; $p = 0.04$). These same samples showed no *PTPN22*-associated difference in proliferation nor secretion of IFN-γ or TNF-α (Fig. 3*B*). 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. 4*A*; $p = 0.04$). These numbers reflect a numerical increase in total memory T cells with a decrease in naive 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. 4*B*; $p = 0.03$).

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. 5*A*; $p = 0.025$); 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

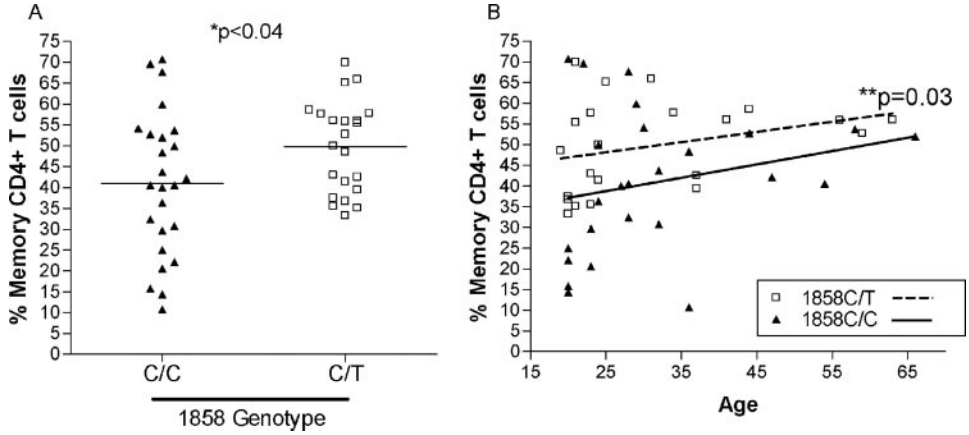

FIGURE 4. Memory T cell profiles are altered in individuals who carry the *PTPN22 1858T* allele. Previously frozen PBMC from control *1858C/C* and *C/T* subjects were analyzed by FACS. Percent memory in $CD4^+$ T cells was characterized as $CD4^+CD3^+CD45RO^+CD45RA^-$. *A*, Memory T cell relative to 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 ($p = 0.03$).

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.

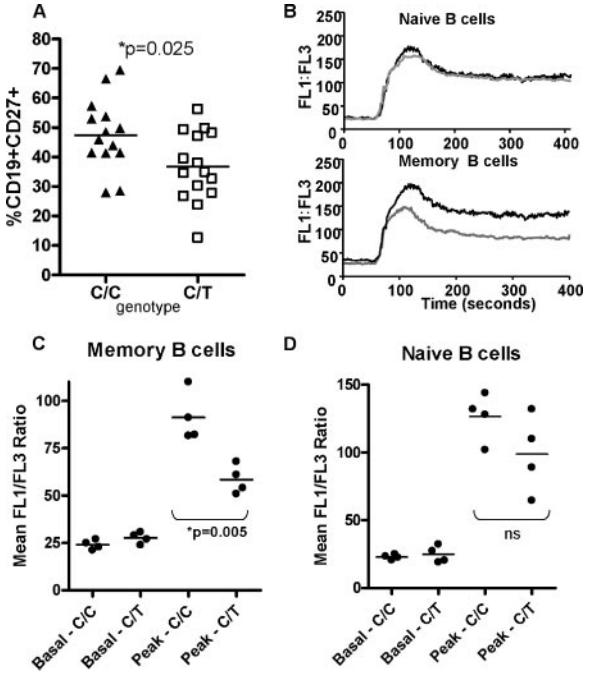

FIGURE 5. Memory B cells are decreased in subjects with the variant 1858T allele and demonstrate reduced BCR-mediated calcium mobilization. *A*, Previously frozen PBMC from subjects were analyzed by FACS. Control *C/C* subjects ($n = 15$) are represented by solid triangles and control *C/T* subjects ($n = 15$) 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 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^+ mobilization of $CD19^+$ B cells in response to BCR stimulation by cross-linking the BCR with anti-IgM. A diminished Ca^+ 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.

Discussion

The functional consequences of the recently described *1858C/T* variant in the *PTPN22* gene are of considerable interest because this variant is increased in frequency in subjects with several autoimmune disorders. Identifying the immunologic consequences of polymorphic alleles at individual risk loci associated with an autoimmune disease is a significant challenge because these diseases arise from the actions of multiple genetic loci and environmental exposures. The immune mechanisms involved in the development of autoimmunity are likely to be similarly complex. To reduce this complexity, we focused our studies on unaffected control individuals who were carriers for the risk allele. This minimized the impact of other disease-associated risk factors, either genetic or environmental, and avoided the influence of therapies that might also act to alter the immune profile. Using this approach, we have identified 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 Lyp620W 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 cell lines where overexpression of the Lyp 620W isoform, in comparison to Lyp 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 $CD4^+$ cells. The increase in memory T cells that we see in the *1858C/T* control subjects in itself could influence cytokine production because memory T cells produce greater amounts of cytokines, particularly IL-10. However, in the samples tested for cytokine production, we found no correlation between IL-10 production and the composition of the T cell compartment, but rather a decrease in IL-10 production by CD4 T cells of 1858 C/T subjects. Vang et al. (17) describe a diminished production of IL-2 by CD4 T cells of individuals with T1D, whereas we only see a trend toward decreased IL-2 production in healthy subjects with the *1858T* variant. These differences may be a result of our use of controls as compared with T1D subjects or the manner of activation. IL-10 production was not addressed in the publication by Vang et al. (17). However, this group has informed us that they did observe a significant decrease in IL-10 production in 1858C/T T1D subjects as compared with *1858C/C* T1D subjects and a similar trend in their control population (M. Congia, F. Cucca, and N. Bottini, personal communication). IL-10 is required to keep the immune response in check. IL-$10^{-/-}$ mice develop autoimmunity due to an enhancement in homeostatic proliferation of the autoreactive repertoire and skewing of the CD4 T cell response to a Th1 profile (18, 19). Thus, a generalized decrease in the production of IL-10 could result in the loss of regulation, expansion of the memory T cell pool, and a predisposition toward autoimmunity among individuals with the *PTPN22 1858 C/T* variant. This possibility is supported by our finding that production of IL-10 upon TCR stimulation was decreased, whereas no differences were seen in the production of the proinflammatory cytokines IFN-γ and TNF-α. Whether alterations in the levels of IL-10 secretion reflect an effect of Lyp 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 Lyp may interact with other pathways which directly influence the production of IL-10. Recent studies suggest that Lyp 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 Lyp 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- rather than hyporesponsive 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

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