Altered B Cell Homeostasis Is Associated with Type I Diabetes and Carriers of the PTPN22 Allelic Variant

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Human autoimmune diseases are genetically complex, arising from the combined impact of environmental factors and multiple polymorphic alleles with varying disease risk (1). Among the genetic variants associated with autoimmunity, the PTPN22 1858C/T variant stands out because of its association with multiple autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), type 1 diabetes (T1D), Graves disease, and myasthenia gravis (2, 3). PTPN22 encodes the lymphoid tyrosine phosphatase (Lyp) protein, which is expressed in multiple hematopoietic cell types. The disease-associated single-nucleotide polymorphism is a missense mutation at position 1858 (C→T; Arg620→Trp), which results in a gain-of-function manifest by blunting of TCR and BCR signaling (3, 4). In previous studies, we demonstrated that healthy individuals who carry the PTPN22 1858T allele exhibit alterations in BCR signal transduction characterized by diminished phosphorylation of proximal-signaling effectors, impaired BCR-driven proliferation, and a decrease in the size of the memory B cell compartment (5, 6).

Multiple tolerance checkpoints censor autoreactivity during B cell maturation in the bone marrow and the periphery (7). Each of these checkpoints relies, in part, on AgR-controlled mechanisms for counterselection of autoreactive cells and include clonal deletion, receptor editing, or anergy (8–10). The BCR-signaling threshold is pivotal to the ultimate fate of autoreactive B cells, and aberrations in proximal BCR signaling during B cell development have the potential to result in a loss of tolerance, as well as increased survival of autoreactive cells (11–14). In individuals with SLE and RA, the development of autoantibodies and perturbations in the B cell compartment parallel an increased frequency of polyreactive new autoreactive B cells in the mature naive compartment, implying defects in central and peripheral tolerance in these diseases (15–18). However, the mechanisms that permit escape of autoreactive B cells during human B cell development and the role for such changes in human autoimmunity remain unclear.

In this study, we sought to address the role of Lyp620W on B cell tolerance with respect to the composition of the transitional and naive B cell compartments and its direct impact on BCR signaling and B cell survival. We also extended our studies to T1D, a human autoimmune disease strongly associated with the PTPN22 1858T variant. We demonstrated that Lyp620W is associated with signaling defects in both transitional and naive B cells in healthy subjects and an increased resistance to BCR-driven apoptosis in these cell populations. In addition, PTPN22 1858C/T subjects
displayed alterations in the composition of the developing B cell pool, including an increased frequency of transitional and naive IgD+IgM- (BND3) anergic B cells, a recently defined peripheral reservoir of autoreactive cells (19). Further, we observed nearly identical alterations in the B cell compartment and BCR signaling in T1D subjects. Our combined findings suggested a mechanism by which Lyp620W contributes to a loss of B cell tolerance and implicate a range of as-yet-uncharacterized, analogous B cell-signaling deficits in T1D.

Materials and Methods

Subjects
Peripheral blood and frozen PBMC samples for this study were obtained from control and T1D participants in the Benaroya Research Institute Immune Mediated Disease Registry and Juvenile Diabetes Research Foundation Center for Translational Research. The control population was selected based on a lack of personal or family history of diabetes, autoimmunity, or asthma. Research protocols were approved by the Benaroya Research Institute Institutional Review Board. All experiments were performed in a blinded manner without prior knowledge of disease state, and each analysis consisted of carriers and noncarriers of PTPN22 185T.

FACS analysis and cell sorting
The following Abs were used for B cell immunophenotyping: PE Cy7–anti-CD19, allophycocyanin Cy7–CD27, allophycocyanin–CD10 (BioLegend); FITC-IgM (Southern Biotech); PE–CD24, PerCP Cy5.5–CD38, PE Cy5–CD21, biotin–IgD (BD Biosciences); and Alexa Fluor 700-streptavidin (Invitrogen). Flow cytometry was performed using FACSaria or LSR II flow cytometers, and analysis was performed with FlowJo software (Tree Star, Ashland, OR). Cell sorting of naive mature and transitional B cells for survival and iRS–KDE analysis was performed by labeling freshly isolated PBMCs with PE–Cy7–CD19, allophycocyanin–CD27, PE–CD24, and PerCP Cy5.5–CD38 (BD Biosciences) or with PE–Cy7–CD19 (BioLegend), allophycocyanin–CD27 (eBiosciences), PE–CD24, PerCP Cy5.5–CD38, and biotin–anti-human α (BD Biosciences), Alexa Fluor 700-streptavidin (Invitrogen), respectively. Sorting was performed using FACSVantage or FACSaria sorters with FACSdiva software (BD Biosciences).

B cell purification, activation, and survival
Total B cells were enriched from PBMC by negative selection using the Human B cell isolation kit II (Miltenyi Biotec). Naive mature B cells depleted of transitional B cells were enriched from PBMC by negative selection using the Human Naive B cell Isolation Kit II (Miltenyi Biotec) supplemented with 0.5 μg anti-CD10–biotin (eBioscience) per 20 million PBMC. Cells were rested in RPMI 1640 medium supplemented with 1% human serum (MP Biomedicals) and then stimulated with F(ab′)2 fragment goat anti-human κ (20 μg/ml) or F(ab′)2 fragment goat anti-human κ plus F(ab′)2 anti-human λ (anti–κ/λ) (20 μg/ml; Southern Biotech) for 5 min. Immediately thereafter, cells were fixed with BD Fix Buffer I, permeabilized with BD Perm Buffer III (BD Biosciences), and surface stained with anti-CD27–allophycocyanin and CD20–Alaqua Fluor 488 in combination with PE–conjugated anti–phospholipase C–γ2 (PLC–γ2) (Y759) (BD Biosciences) for intracellular phosphoprotein staining, according to the manufacturer’s instructions. To quantify naive and transitional B cell apoptosis, sorted naive CD19+CD27–CD38hiCD24int B cells (≥90% pure) and transitional CD19+CD27–CD38hiCD24hi B cells (≥80% pure) were immediately plated in complete RPMI 1640 media (10% FCS with 1-glutamine, penicillin-streptomycin) in the presence or absence of 20 μg/ml F(ab′)2 anti-IgM. Cells were fixed with BD Cytofix and permeabilized as above after 0, 12, 18, and 24 h in culture for naive B cells and after 18 h in culture for transitional B cells; thereafter, they were stained with anti–cleaved caspase–3–Alexa Fluor–488 (Asp 175; Cell Signaling) as per the manufacturer’s instructions. Flow cytometry was performed using a BD FACSCalibur flow cytometer. To detect Bcl-2 and Bim proteins, PBMC were first surface stained with anti-CD27–allophycocyanin, anti-CD38–PerCP Cy5.5, and anti-CD24–FITC mAbs (for subsequent Bcl-2 or isotype-control Ab staining) or with anti-CD27–allophycocyanin, anti-CD38–PerCP Cy5.5, and anti-CD24–PE (for subsequent Bim or isotype control Ab staining), in combination with the fixable dead cell discrimination dye violet (Invitrogen) for 5 min at room temperature. Cells were then fixed and permeabilized with BD Cytofix/Cytoperm reagent, as per the manufacturer’s protocol (BD Biosciences). Intracellular staining was performed simultaneously with anti-CD20–PE–Cy7 (BD Biosciences), using the anti-human Bcl-2–PE kit (BD Biosciences), anti-Bim, or anti-rabbit IgG–Alexa Fluor 488 isotype control Abs (Cell Signaling), in 1× Perm/Wash buffer (BD Biosciences) for 30 min at 4°C, followed by incubation of Bim-stained samples with secondary Alexa Fluor 488 F(ab′)2 fragment of goat anti-mouse IgG (Invitrogen) in 1× Perm/Wash reagent. Flow cytometry was performed using an LSR II flow cytometer. Bcl-2 expression in gated naive and transitional B cells is expressed as the Bcl-2 mean fluorescence intensity (MFI) divided by the MFI of the corresponding isotype control Ab for each sample. The ratio of Bcl-2/Bim expression is expressed as (Bcl-2 MFI/isotype-control MFI)/Bim MFI/isotype control MFI) for each sample. All figures generated using the above methods consist of data combined from more than one experiment, in which separate donor groups of mixed genotype were processed and analyzed sequentially in the same manner.

Inhibitor studies
The Lyp–specific inhibitor L-C11 was synthesized, as described previously (20). Total B cells purified from previously frozen PBMC were pretreated with 10 μM L-C11 or 0.2% DMSO vehicle and stimulated as previously described (5).

Lentiviral transduction
Wild-type (WT) PEST domain–enriched tyrosine phosphatase (PEP) or R619W PEP was cloned into the lentiviral (LV) vector pRL–SFV–IRES–mCherry, where mCherry serves as a marker for the flow cytometry–based identification of LV–transduced cells. LV transfections were performed, as previously described (21). Ramos cells were infected with lentivirus in RPMI 1640 media (10% FCS, 4 μg/ml gluta mine, 50 μM 2-ME, 10 mM HEPEs, and antibiotics) in the presence of 8 μg/ml polybrene. Two days posttransduction, cells were harvested, and mCherry expression was analyzed on an LSR II flow cytometer (BD Biosciences), and protein-expression levels of WT or R619W PEP were analyzed by Western blot, as previously described (22). The following Abs were used: anti–PEP (provided by Dr. Andrew Chan); anti–HA (6E2, Cell Signaling). Anti–mouse–β actin (A-2066; Sigma) was used to verify equal protein loading.

Calcium-flux assays
LV–transfected Ramos cells were incubated with AM ester Indo-1 (Invitrogen) at room temperature for 30 min. The Indo-1 fluorescence ratio was acquired as a function of time on an LSR II flow cytometer. For each experiment, collection of a 1-min baseline measurement was followed by stimulation with anti–IgM F(ab′)2 (Jackson Laboratories) or 500 ng/ml ionomycin, as indicated. Freshly isolated PBMC were incubated with Indo-1 AM at 37°C for 30 min in room temperature and anti-CD19 and anti-CD27 to detect naive B and memory B cells. Collection of a 1-min baseline was followed by stimulation with F(ab′)2 fragment anti–κ/λ or 500 ng/ml ionomycin. The Indo-1 fluorescence ratio was acquired as a function of time, and kinetics curves were generated using FlowJo software.

Quantitative PCR
cDNA was prepared from sorted naive mature, memory, and transitional B cell subsets (>95% pure) using random primers (Applied Biosystems), and message levels of PTPN22/Lyp were quantified using Invitrogen primers, TaqMan Gene Expression Assays, and TaqMan Gene Expression Master Mix (Applied Biosystems). Reactions were run in real-time in duplicate on a StepOne Plus Sequence Detection System (Applied Biosystems). Amplification was carried out in a total volume of 20 μl for 15 cycles of 15 sec at 95°C, 1 min at 60°C. Relative expression was determined using the ΔΔCt method by normalizing expression to 18S rRNA.

Quantitative assay for RS rearrangement
Genomic DNA was isolated from sorted transitional (CD19+CD27–CD10+CD24+CD38+) and mature (CD19+CD27+CD24+CD38+) B cells separated by L chain isotype using the Gentra Puregene Tissue Kit (Qiagen). Quantitative PCR (60 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s) was performed on 15–50 ng template DNA in a 20-μl reaction mix containing 1× LightCycler 480 Probes Master Mix (Roche), 0.5 U LightCycler UraCIL–DNA Glycosylase (Roche), 0.5 μM forward primer, 0.5 μM reverse primer, and 0.2 μM hydrolysis probe using a LightCycler 480 real-time PCR system (Roche). Recombining sequence-specific deletion element (IRS–KDE) rearrangement frequencies were determined by absolute quantification using a standard curve generated from serial dilution of a cloned IRS–KDE rearrangement resuspended in 100 ng human fibroblast genomic DNA. Genome copy number was calculated from β–actin quantification using a standard curve generated from serially diluted hu
man fibroblast genomic DNA. All reactions were performed in duplicate, and samples with inconsistent replicates or β-actin cycle numbers $>35$ were excluded.

**BAFF immunoassay**

Sera from control and T1D subjects were harvested and stored at $-80^\circ$C. BAFF serum levels were measured using an ELISA with the Quantikine Human BAFF/BLyS/TNFSF13B Immunoassay (no. DBLYS0; R&D Systems), following the manufacturer’s instructions. Samples were analyzed in duplicates based on the use of a monoclonal anti-BAFF Ab and corresponding irrelevant IgG as negative control. The absorbance values in the isotype-control wells were subtracted from the corresponding anti-BAFF capture wells.

**Statistical analysis**

Differences between healthy control PTPN22 1858T carrier and noncarrier populations were analyzed for statistical significance with a two-tailed Student $t$ test using GraphPad Prism version 4.02 for Windows. Paired $t$ tests were used to analyze vehicle-treated versus I-C11–treated populations. Group comparisons between healthy control and T1D populations were performed using a one-way ANOVA (Dunnett multiple comparison test) and Student $t$ tests. The $p$ values $<0.05$ were considered significant.

**Results**

**Altered B cell compartment in healthy subjects heterozygous for PTPN22 1858T**

We previously demonstrated that there is a significant reduction in memory (CD19$^+$CD27$^+$) B cells in individuals who carry the PTPN22 1858T variant and that such memory B cells exhibit an impaired response to BCR engagement (5, 6). Although these findings likely reflect a direct impact of the variant allele on B cell activation, our previous data could not rule out a role for altered T cell function in the generation of this memory B cell population. To address whether the PTPN22 variant might directly impact B cell development, we first performed a detailed flow cytometric analysis of the B cell compartment of healthy subjects. Although the frequency and absolute number of peripheral CD19$^+$ B cells did not differ between the two groups (Supplemental Fig. 1A), the percentage of transitional B cells, defined as CD19$^+$CD27$^+$CD10$^+$CD24$^+$CD38$^+$ cells (23, 24) (Supplemental Fig. 1B), was significantly increased in individuals heterozygous for PTPN22 1858T compared with age-matched PTPN22 1858C/C healthy adult control subjects ($p < 0.006$) (Fig. 1A). When normalizing the transitional B cell frequencies to the percentage of CD19$^+$ B cells in total PBMC, we found a significant increase in the frequency of transitional B cells from 1858C/T control subjects compared with 1858C/C controls, suggesting that our findings were not due to differences in the size of the B cell pool overall (data not shown). We also determined the frequency of a recently identified population of autoreactive, anergic B cells defined as CD19$^+$CD27$^+$IgD$^+$IgM$^-$ (B$_{ND}$) B cells (19) (Supplemental Fig. 1B). This population was also significantly increased among individuals heterozygous for the PTPN22 1858T variant compared with 1858C/C controls ($p < 0.05$) (Fig. 1B). Thus, Lyp620W expression is associated with altered B cell homeostasis leading to an expansion of transitional and anergic B cells; these findings suggested that larger numbers of autoreactive B cells enter and/or survive within the peripheral B cell compartment in individuals who carry the risk variant.

**Naive PTPN22 1858C/T B cells exhibit intrinsic signaling defects that are recapitulated in PEP R619W-expressing Ramos cells**

BCR signal strength and BAFF are two factors that play crucial roles in B cell maturation in the periphery. BAFF is a survival factor for peripheral B cells, and it plays a pivotal role in peripheral B cell tolerance (25, 26). To determine whether the increase in the transitional and B$_{ND}$ populations in individuals who carry the PTPN22 1858T variant was due to an increase in BAFF levels, we evaluated a large, age-matched cohort of control and PTPN22 C/T subjects. We observed no correlation between BAFF levels and PTPN22 1858 genotype that would explain the expansion of the transitional and B$_{ND}$ B cells in healthy controls (Supplemental Fig. 1C).

Next, we examined whether B cell signal transduction was altered by Lyp620W expression in transitional or mature naive B cells. In previous studies, we demonstrated that LypR620W expression results in altered B cell signal transduction in mature B cells following anti-IgM/IgG cross-linking (5, 6). We hypothesized that if the diminished BCR signaling caused by Lyp620W extends to the transitional and naive B cell compartments, then censoring of autoreactive B cells might be inefficient in individuals who carry the PTPN22 1858T variant. We first tested whether Lyp620W altered the BCR-signaling threshold in naive mature B cells from 1858C/T subjects following cross-linking with anti-κ F(ab')$_2$ Abs in an effort to simulate low-affinity self-Ag engagement. Using flow cytometric analysis, we observed a significant decrease in phospho–PLC-γ2 (P–PLC-γ2) in naive CD20$^+$CD27$^+$ B cells upon activation with anti-κ (Fig. 2A). Treatment of 1858C/T B cells with the Lyp-specific inhibitor I-C11 (5, 20) prior to
BCR stimulation led to a rescue in anti-κ-mediated P-PLC-γ2 (p = 0.01) compared with paired vehicle-treated B cells (Fig. 2B), resulting in a mean fold change in P-PLC-γ2 equivalent to that of I-C11-treated 1858C/C control B cells. Each dot represents the fold change in MFI relative to the unstimulated control for a unique individual. B, Total B cells from 1858C/C (n = 6) and 1858C/T (n = 7) subjects were purified from previously frozen PBMC, rested overnight, treated with vehicle or 10 μM I-C11, and stimulated as in A, followed by intracellular staining for P-PLC-γ2. Fold change in MFI for P-PLC-γ2 in the presence of vehicle or I-C11 is shown in gated naive B cells from healthy noncarriers (closed symbols) and carriers (open symbols) of 1858T.

C, Generation of WT and variant PEP-expressing Ramos cells. Flow cytometric and Western blot analyses of control (nontransfected) and HA-tagged WT PEP and PEP-R619W depicting similar expression levels of WT and variant PEP following LV transduction of the IgM-expressing B cell line Ramos. D, The Lyp620W-associated BCR-signaling defect is recapitulated in Ramos cells expressing PEP-R619W. Ramos cells infected with SFFV-HA–WT PEP or PEP-R619W were loaded with the indo-1 calcium-binding dye. Kinetics profiles are shown comparing the mean indo-1 ratio (violet/blue) of mCherry+ (red trace) and mCherry− (blue trace) as a function of time before and after stimulation with anti-IgM F(ab′)2 (left panels) or ionomycin (right panels).

FIGURE 2. Blunted BCR signaling is an inherent defect of PTPN22 1858C/T naive B cells and PEP-R619W–expressing Ramos cells. A, Total B cells from healthy control 1858C/C and 1858C/T subjects (n = 8) were purified from previously frozen PBMC, rested overnight, and then stimulated with soluble anti-κ F(ab′)2 for 5 min. P-PLC-γ2 (Tyr759) was quantified by intracellular flow cytometry in the naive CD20+CD27− B cell population. Each dot represents the fold change in MFI relative to the unstimulated control for a unique individual. B, Total B cells from 1858C/C (n = 6) and 1858C/T (n = 7) subjects were purified from previously frozen PBMC, rested overnight, treated with vehicle or 10 μM I-C11, and stimulated as in A, followed by intracellular staining for P-PLC-γ2. Fold change in MFI for P-PLC-γ2 in the presence of vehicle or I-C11 is shown in gated naive B cells from healthy noncarriers (closed symbols) and carriers (open symbols) of 1858T.

Because the maturation of transitional cells to naive B cells is greatly influenced by BCR signal strength, we also determined whether the Lyp variant impacted transitional B cell signaling. Biochemical studies using primary transitional human B cells pose a technical challenge because of their low frequency in the peripheral circulation. Therefore, we carried out initial signaling studies using an IgM-expressing human B cell line. Ramos B cells were infected with LV constructs containing either WT or mutant phosphatase in association with a cis-linked, internal ribosomal entry site-mCherry marker gene (Supplemental Fig. 2A). However, limited cell numbers impaired our ability to directly quantify expression in transitional cells.

In contrast, anti-IgM–induced calcium flux was significantly impaired in B cells expressing PEP-R619W, as shown by comparison of the response in mCherry+ (red trace) versus mCherry− cells (blue trace; Fig. 2D, lower left panel). Control responses to the calcium ionophore (ionomycin) were equivalent in all populations (Fig. 2D, right panels). These data recapitulated the impaired calcium signal previously observed in Lyp620W-expressing B cells (6) and T cell lines (4) and support the idea that the increase in transitional B cells in subjects heterozygous for PTPN22 1858T is mechanistically linked to an altered BCR-signaling threshold.
PTPN22 1858C/T B cells exhibit reduced BCR-triggered apoptotic signaling

The attenuated BCR signals exhibited by naive and transitional B cells expressing the Lyp620W and PEP-R619W variants, respectively, suggested that apoptotic pathways coupled to the AgR might be altered. Apoptosis is the major tolerogenic program in transitional cells in the periphery (11, 13). Moreover, the BCR-driven proapoptotic signal in immature and mature B cells is dependent upon caspase-3 activation (27) and is triggered by strong BCR cross-linking (11, 28). Therefore, we directly measured caspase-3 activity in response to BCR cross-linking with F(ab′)2 anti-IgM in PTPN22 1858C/T subjects versus 1858C/C subjects. Sorted, purified naive B cells (Supplemental Fig. 2B) were cultured in the presence or absence of anti-IgM for up to 24 h, and the frequency of B cells positive for active caspase-3 were quantified by intracellular flow cytometry. In the absence of BCR engagement, we observed a trend toward fewer cleaved caspase-3+ cells in 1858C/T subjects compared with 1858C/C subjects (Fig. 3A, left panel). Most notably, the percentage of active caspase-3+ cells was significantly reduced following anti-IgM cross-linking in 1858C/T cells (Fig. 3A, 12 h, middle panel), with a similar trend toward reduced caspase activity compared with 1858C/C control B cells after 18–24 h of culture (data not shown). Similarly, the mean fold change in active caspase-3 was also reduced in unstimulated versus stimulated 1858C/T cells (Fig. 3A, right panel). As previously described (24), purified transitional B cells (Supplemental Fig. 2B) exhibited increased basal apoptosis compared with naive mature B cells (Fig. 3B versus Fig. 3A, media, 18 h). However, the relative frequency of active caspase-3+ in transitional cells from 1858C/T individuals was significantly less compared with C/C controls at 18 h in media alone; this population also exhibited a trend toward reduced caspase activity following anti-IgM stimulation (Fig. 3B).

The decrease in BCR-mediated apoptosis, as measured by cleaved caspase-3, was consistent with the blunted BCR signal in B cells expressing Lyp620W. However, the enhancement in survival in the absence of BCR activation suggested that Lyp620W also impacts basal signals mediated by the BCR and/or other receptors. To further assess such basal-signaling differences, we examined expression levels of key pro- and antiapoptotic BCL family proteins (Bim, Bcl-2) in naive mature and transitional B cells from 1858C/C and 1858C/T healthy control individuals by intracellular staining and flow cytometry. As previously reported (24), significantly higher expression of Bcl-2 protein levels were present in naive versus transitional B cells (p < 0.0001; Fig. 3C). However, when comparing the expression in each subset by PTPN22 genotype, basal levels of Bcl-2 were significantly higher in 1858C/T versus 1858C/C transitional B cells (p = 0.003), but it did not differ significantly between 1858C/T and 1858C/C naive B cells (Fig. 3C, left panel). Basal expression of proapoptotic Bim protein was detected at similar levels in naive and transitional B cell subsets from both noncarriers and carriers of PTPN22 C/T (data not shown), consistent with a more favorable ratio of anti- to proapoptotic proteins in transitional B cells from 1858C/T subjects versus 1858C/C subjects (p = 0.002; Fig. 3C, right panel).

Collectively, these data demonstrated increased survival of Lyp620W-expressing transitional and mature B cells and implicated blunted BCR signaling as one mechanism by which the variant allele may contribute to inefficient negative selection.

PTPN22 1858T B cells exhibit no evidence for altered receptor L chain editing

The altered naive B cell compartment and reduced BCR signaling observed in PTPN22 1858C/T subjects suggested that Lyp620W might impact central tolerance mechanisms operative within the bone marrow compartment and bypass or reduce editing at L chain loci important for expression of an innocuous, nonself-reactive receptor (8). To evaluate this idea, we used a quantitative PCR-based assay for iRS-KDE, a marker for receptor editing (29). The iRS-KDE assay measures the overall level of κ L chain gene rearrangement in a B cell population. The level of L chain gene rearrangement is correlated with receptor editing (29). We found
no significant difference in the frequency of iRS-KDE rearrangements per genome copy in highly purified, Igκ* or Igλ* transitional and naive B cells isolated from healthy control 1858C/C subjects compared with 1858C/T subjects (Supplemental Fig. 3). These data suggested that L chain receptor editing is largely intact in PTPN22 1858C/T subjects.

Alterations in the B cell compartment in T1D subjects phenocopy PTPN22 1858T/T controls

Previous work implicated B cells in the initiation of T1D and as precursors for relevant autoantibody-producing cells (30–34). However, little is known regarding defects in peripheral B cell tolerance that may favor development of T1D. Our observations with respect to the B cell compartment in control subjects with the autoimmune-associated variant of PTPN22 led us to hypothesize that analogous alterations might be present in individuals with T1D. Thus, we examined the B cell profiles of T1D subjects by FACS for relative frequencies of transitional, naive, anergic, and memory B cells. Although we found no difference in total CD19+ B cell numbers between subjects with T1D and healthy controls (data not shown, (29)), the frequency of both CD19+CD10+CD27−CD24hiCD38hi transitional and CD19+CD27−IgD+IgM− BND B cells were significantly increased in T1D subjects compared with 1858C/C healthy controls (Fig. 5A, 5B). These differences were most significant when control PTPN22 1858C/C and T1D 1858C/C subjects were compared. In contrast, comparing paired T1D subjects by PTPN22 genotype, we found that heterozygosity of the 1858T risk allele was not associated with further expansion of transitional and BND anergic B cells (Fig. 4A, 4B), indicating that PTPN22 1858T is not the sole factor leading to this overlapping phenotype in T1D. Similarly, the naive and memory B cell profiles of T1D subjects paralleled 1858C/T healthy controls, because we observed a significant expansion in the naive B cell subset and a reduction in the memory B cell pool in T1D subjects, again irrespective of PTPN22 genotype (Fig. 4C).

However, upon examination of a separate small cohort of age-matched T1D 1858C/C and T1D 1858T/T subjects, we found a modest trend toward increased transitional and BND B cells in homozygous carriers of 1858T, suggesting that Lyp620W contributes to this phenotype in T1D subjects who carry the variant (Supplemental Fig. 4A). We also measured serum BAFF levels in all subjects and found no correlation between BAFF levels and disease or PTPN22 1858 genotype that would explain the expansion of the transitional and BND B cells in T1D (Supplemental Fig. 1C).

Based on our observation that blunted BCR signaling is correlated with expansion of the transitional and BND B cell subsets in control 1858C/T subjects, we investigated proximal BCR signaling in T1D subjects using both Ca2+ flux and intracellular staining. We found impaired peak Ca2+ mobilization in mature B cells from T1D subjects following BCR cross-linking with anti-κ+κ− (Fig. 5A). These differences correlated with a significant decrease in P–PLC-γ2 levels and total phosphotyrosine in purified total B cells from T1D PTPN22 1858C/C subjects (p < 0.05) relative to healthy PTPN22 1858C/C controls, with a similar trend present in T1D PTPN22 1858C/T B cells (Fig. 5B, 5C). No additional impairment was observed in association with the 1858C/T genotype. The impairment in activation of T1D B cells mirrored that seen in control PTPN22 1858C/T subjects with the corresponding stimulation. When the naive (CD20+CD27−) and memory (CD20+CD27+) populations were independently evaluated, we observed a significant decrease in P–PLC-γ2 levels in both subsets from T1D PTPN22 1858C/C individuals and in the naive population of T1D PTPN22 1858C/T B cells (Fig. 5D). Moreover, our analysis of a separate cohort of age-matched T1D 1858C/C and T1D 1858T/T subjects revealed further blunting of BCR signaling in naive B cells from T1D 1858T/T individuals (Supplemental Fig. 4B). This phenotype appeared equally pronounced in control 1858C/T and T1D 1858T/T patients, suggesting that Lyp620W-dependent mechanism(s) may largely account for the enhanced phenotype in T1D 1858T/T individuals (Supplemental Fig. 4C).

These combined data revealed a striking similarity between T1D and the homeostatic alterations in the B cell compartment in control PTPN22 1858C/C subjects. Most notably, these findings suggested that blunted proximal BCR signaling and an altered transitional/ergic B cell compartment represent a common feature of T1D subjects, irrespective of the presence of Lyp620W.
In this study, we identified a unique immune phenotype in the B cell compartment of healthy individuals who carry the PTPN22 1858T variant. Together with the decreased memory B cell subset previously observed (6), we showed that altered B cell homeostasis in subjects who express the variant is characterized by an increase in transitional and CD19+CD27- IgD-IgM- B<sub>ND</sub> B cells, as well as blunted BCR signaling in naive mature B cells. Prior characterization of B<sub>ND</sub> B cells revealed that they are functionally anergic and exhibit anti-DNA and anti–HEp-2 reactivity, yet they exhibit no evidence of somatic mutation or class-switch recombination, indicating that they are likely naïve and naturally autoreactive (19). Interestingly, T cell-dependent stimulation can rescue B<sub>ND</sub> function ex vivo and drive development of plasmablast-like cells (19). Similarly, using a double-transgenic HEL-Ig mouse model, Goodnow et al. (35) demonstrated in vivo that functionally anergized, self-reactive B cells can be reactivated to produce Abs in the absence of continuous exposure to tolerizing Ag and with the provision of T cell help. Thus, the presence of a potentially reversible population of anergic B cells at higher frequency in 1858C/T individuals may represent an autoreactive B cell pool with the potential to produce autoantibodies upon reactivation. Importantly, our data suggested that these Lyp<sub>620W</sub>-associated effects on B cell homeostasis observed in healthy carriers of PTPN22 1858T mimic a more complex array of potential changes in B cell signaling that underlie a major phenotypic characteristic of autoimmune T1D and provide a clear demonstration of a human autoimmune disease associated with an altered anergic B cell compartment.

To gain further insight into possible mechanisms that promote the altered transitional and naive B cell populations in healthy 1858C/T individuals, we pursued the hypothesis that Lyp<sub>620W</sub>-mediated effects on the BCR-signaling threshold, which impact B cell selection, lead to an increased proportion of naive self-reactive B cells in the periphery. We showed that expression of Lyp<sub>620W</sub> is associated with diminished PLC-γ2 phosphorylation and reduced calcium signaling in mature naive B cells, and this phenotype is reversed by inhibition of Lyp. Similar biochemical studies using primary human transitional B cells have been challenging because of their low frequency in the periphery. Therefore, we showed in this study that enforced expression of the murine variant ortholog, PEP-R619W, resulted in impaired calcium flux following anti-IgM stimulation in a human B cell model, whereas WT PEP expression did not. These alterations in proximal BCR signaling are consistent with a role for Lyp<sub>620W</sub> in modulating B cell selection in the periphery. Previous studies unequivocally demonstrated that BCR signals play a crucial role in shaping the naive mature B cell repertoire through positive and negative selection (7, 12–14, 36, 37). In situations in which BCR signaling is reduced via inefficient costimulation and/or through deficits in intracellular signaling molecules, self-reactive B cells can escape deletion at critical tolerance checkpoints (36–40). In the periphery, the current model defines the early transitional stage as a crucial juncture at which maturing B cells with high affinity for self Ags can escape deletion at critical tolerance checkpoints (36–40). In the periphery, the current model defines the early transitional stage as a crucial juncture at which maturing B cells with high affinity for self Ags can escape deletion at critical tolerance checkpoints (36–40).
for self-Ag are most sensitive to BCR-driven negative selection via apoptotic cell death (7, 14, 41). The potential for the Lyp620W gain-of-function phosphatase to increase the signaling threshold that governs deletion in the periphery suggests that self-reactive transitional B cells that carry the variant allele may exhibit a selective advantage.

In support of this idea, we provide evidence for the increased survival of naive and transitional B cells from healthy individuals heterozygous for PTPN22 1858T. To directly evaluate proapoptotic signaling, we measured anti-IgM–induced activation of caspase-3 in PTPN22 1858C/C and 1858C/T naive B cells and found a significantly lower frequency of cleaved caspase-3 in 1858C/T B cells after 12 h of culture. By 24 h, we observed a similar trend, albeit more modest, toward decreased apoptosis in the naive 1858C/T B cell subset. Thus, it is likely that a subtle antiapoptotic phenotype was not previously detected in long-term cultures (24–72 h) of memory and naive 1858C/T B cells in response to IgM/IgG cross-linking (5). In contrast, transitional B cells from subjects who carry the variant exhibited reduced basal and BCR-triggered caspase activation, suggesting that altered negative selection may mechanistically contribute to expansion of this B cell population. Our additional observations that levels of BAFF are not altered in the sera of PTPN22 1858C/T subjects, together with the increased survival of transitional and mature B cells and demonstrated alteration in the balance of BCL family proteins, suggested that expression of Lyp620W promotes increased survival in these populations.

The increase in BND cells observed in our studies might reflect an increase in autoreactive B cells that escape central deletion and enter the periphery as anergic cells or, alternatively, blunted BCR signaling in transitional/naive B cells may permit an increase in this population. Because BND cells are CD10+CD23hi, a phenotype most consistent with a naive B cell, and we did not identify alterations in receptor editing, we speculated that the most likely explanation for the increase in this anergic population is relaxed peripheral tolerance (e.g., a relative shift away from deletion toward anergy induction [events that are likely further enhanced in the setting of a more autoreactive new emigrant B cell population]). However, because BCR signal strength uniquely impacts B cell fate, an additional (but not mutually exclusive) possibility is that BND cells develop and persist in the mature B cell compartment independently of selection events operating at the transitional stage. Blunted BCR signals in mature B cells may directly or indirectly alter the half-life of the anergic population and/or their response to survival factors, such as CD40L or BAFF.

Aberrations in B cell homeostasis were reported in cases of immunodeficiency or autoimmunity, such as common variable immunodeficiency (CVID), X-linked lymphoproliferative disease, SLE, and RA (17, 18, 20, 42–44). The potential role for B cells in the initiation of T1D and as precursors of autoantibody-producing cells is clear (28–32), yet little is known about defects in peripheral B cell tolerance that may favor development of autoreactivity in this disease. We hypothesized that the global changes observed in the B cell compartment of healthy control 1858C/T subjects may predispose to the development of autoimmunity and, thus, overlap with abnormalities exhibited by T1D subjects who carry the variant allele. Surprisingly, we found that the alterations in the transitional, mature, and memory B cell subsets associated with the variant in healthy subjects are characteristic of nearly all T1D subjects, irrespective of PTPN22 genotype. Functionally, we demonstrated impaired proximal BCR signaling in both naive and memory B cells from T1D subjects and parallel homeostatic alterations in the periphery (i.e., increased transitional and BND B cells and decreased memory B cells). These phenotypes appeared more pronounced in T1D 1858T/T patients, suggesting that, although altered homeostasis and blunted BCR signaling are common features of T1D individuals, Lyp620W can further alter the BCR-signaling threshold and potentially impact the development of autoreactive B cells. Our observations invoked the possibility that additional molecular mechanisms likely impact B cell selection and signaling in the setting of T1D and support a model in which a range of signaling deficits in BCR- and/or costimulatory (CD19, TLR, CD40) or coinhibitory (CD22, FcγRIIB1)–signaling pathways influence the BCR-signaling threshold and lead to a similar phenotypic outcome in individuals predisposed to T1D. Consistent with this idea, a recent report showed that the ITIM receptor CD22 [part of the Lyn–Src homology phosphatase-1 inhibitory axis in mature B cells (45)] is aberrantly expressed on CD21lo B cells that exhibit an attenuated Ca2+ response in patients with class Ia CVID, a group of individuals more likely to develop autoimmune syndromes. Interestingly, these patients display a high frequency of CD21−/− B cells with an anergic, autoreactive phenotype that also develops in a subset of RA patients (44, 46).

Further, emerging data from genome-wide association studies in human autoimmune diseases now implicate multiple variant alleles that may impact B lymphocyte signal transduction, including Blk, BANK1, FCRL3, and FcγRs (5, 47–50). Blk, a Src family kinase that phosphorylates ITAMs on BCR Igα/Igβ-chains, is of particular interest given the recent association of a variant allele of Blk with both T1D and SLE (48) and its potential to influence activation of Syk, a target of Lyp620W (5). Thus, multiple risk factors may fall into key overlapping pathways that target similar biochemical processes in T1D and other human disorders associated with autoantibody production.

Current studies also suggested that many risk loci for T1D exert their effects at multiple levels in the immune system (1). We previously reported that healthy carriers of 1858T have a blunted response to TCR stimulation and expansion of the memory T cell compartment; such alterations could contribute to a loss of tolerance through reduced negative selection of autoreactive T cells or impaired T cell regulation or T cell help. In addition, Lyp is expressed in multiple other immune cell types, including myeloid, dendritic cells, and NK cells, suggesting that the Rs620W polymorphism could also affect the differentiation or function of these cell populations. Thus, B cell extrinsic factors that modify B cell function may facilitate the progression to autoimmunity. Other approaches, such as the use of animal models in which Lyp620W is expressed ubiquitously, will be required to address whether specific immune phenotypes are causative and/or a consequence of disease.

Our combined data suggested that in T1D, both Lyp620W-dependent and -independent alterations in BCR signaling enable autoreactive B cells to escape one or more key tolerance checkpoints, leading, ultimately, to an expanded pool of autoreactive, anergic, and naive B cells. In humans, immature B cells expressing auto- and/or polyreactive Abs account for a high frequency (55–75%) of the bone marrow repertoire (18). Based upon murine models, central tolerance is achieved predominantly via receptor editing, with clonal deletion serving as a default mechanism when editing fails to silence self-reactive cells (8, 44, 51). Notably, in our analyses of receptor editing, we did not observe a significant difference in the relative level of RS rearrangement in either transitional or mature B cells purified from healthy 1858C/C versus 1858C/T individuals. These data suggested either that central tolerance is largely intact in 1858C/T individuals or, alternatively, that although sufficient to drive an equivalent proportion of editing, blunted signaling may shift the overall target repertoire, thereby permitting escape of a larger number of low-affinity autoreactive
cells. As discussed above, additional selective events operating within the transitional B cell compartment, including clonal deletion and anergy, further restrict the repertoire of naïve human B cells (7), and the altered BCR-driven apoptotic program observed in individuals with the PTPN22 variant implicates alterations in these processes. Interestingly, in contrast to our finding with healthy individuals, decreased receptor editing (RS levels) was reported in both κ and λ B cells isolated from patients with SLE or TID (27). In this study, the level of RS rearrangement did not correlate with PTPN22 status. Thus, as suggested by our data, the events leading to altered tolerance in TID are likely more diverse than in healthy individuals who carry the PTPN22 variant allele. Finally, consistent with our data implicating defective peripheral tolerance, recent studies using receptor profiling clearly demonstrated a significant increase in autoreactive B cells within the peripheral B cell compartment of healthy individuals who carry PTPN22 1858T (52).

Our novel finding that an expanded subset of transitional and naïve B cells is present in the periphery of healthy carriers of PTPN22 1858T, combined with the alterations in BCR signaling and survival associated with this variant, provide new insight into the mechanisms by which Lyp620W could potentially compromise peripheral tolerance. Importantly, our data also define similar, previously uncharacterized alterations in B cell homeostasis and function in TID subjects. The identification of developmental checkpoints that are impacted by Lyp620W may have predictive value for predisposition to autoimmune diseases associated with this variant, as well as the response to B cell-directed therapies.

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Disclosures

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Supplemental Figure 1. (A) The frequency and absolute number of peripheral CD19+ B cells are similar between PTPN22 1858C/C and 1858C/T subjects. The frequency of CD19+ B cells as a percentage of total lymphocytes (left, n=40 C/C; n=39 C/T) and absolute number (cells/ml X 10^3) of CD19+ B cells (right, n=13 C/C; n=10 C/T) are compared between healthy control donors, where each symbol represents a unique individual and bars represent the mean. (B) Representative FACS staining in one healthy control and one T1D patient (PTPN22 C/T). B cell subset analysis (upper panels) showing specific B cell developmental populations within the peripheral blood CD19+ B cell gate defined based on CD24 and CD38 surface expression. Transitional B cells comprise the CD19+CD38hiCD24hi CD10hi subset; Mature naïve B cells are defined as CD19+CD38intCD24lo CD10lo/neg, and Memory B cells as CD19+CD38loCD24hi. Representative gating strategy for BND B cells (lower panels), defined as the CD19+CD27IgD+IgM- gate. Naïve peripheral blood B cells (CD19+) were first gated according to the absence of CD27 (lower left panels) and then separated according to relative IgM vs. IgD expression. BND B cells comprise the CD27IgD+IgM- gate (middle panels). Using this approach, gated BND B cells primarily exhibit a surface phenotype consistent with cells within the mature naïve
B cell gate, based on relative CD24 and CD38 expression (lower right panels). Consistent with previous reports, B\textsubscript{ND} B cells comprised ~2.5% of the total peripheral B cell population in normal controls, and this population was consistently increased in TID patients. B\textsubscript{ND} B cells were also assessed by an alternative gating strategy (not shown) – by relative IgD/IgM expression in gated CD19\textsuperscript{+} cells followed by absence of CD27 expression (CD19\textsuperscript{+}IgD\textsuperscript{+}IgM\textsuperscript{-}CD27\textsuperscript{-}; Duty et.al, JEM. 206: 139-151). The % of the B\textsubscript{ND} B cells was found to be similar using both gating strategies, and the gating method shown here was utilized.

(C) Serum BAFF levels are similar between healthy control and T1D subjects. Individual serum BAFF levels in 53 healthy control subjects (n=28 1858 C/C; n=25 1858 C/T, range 19-45 years) and 64 age-matched T1D donors (n=46 1858 C/C; n=14 1858 C/T; and n=4 1858 T/T), as measured by ELISA. Statistical significance was determined using a one-way analysis of variance.
Supplemental Figure 2. (A). Real-time Q-PCR analysis of PTPN22 message in naïve (CD19^+CD27^neg) and memory (CD19^+ CD27^+) B cells FACS-sorted from fresh PBMC. Relative levels of Lyp mRNA (PTPN22/18S mRNA ± SEM) are shown for healthy control 1858C/C and C/T subjects (n=5), using 18S measurements to normalize. (B). Representative gating strategy is shown of sorted naïve (CD19^+CD27^negCD24^intCD38^int) and transitional (CD19^+CD27^negCD24^hiCD38^hi) B cells following culture in the presence and absence of anti-IgM, and intracellular staining with anti-cleaved caspase-3 (right).
Supplemental Figure 3. Receptor editing is intact in PTPN22 1858T heterozygous transitional and mature B cells. (A and B) iRS-KDE rearrangement frequencies as quantified in peripheral Igκ⁺ transitional (CD19⁺CD27⁻CD10⁺CD24hiCD38hi) and mature (CD19⁺CD27⁻CD24intCD38int) B cells, respectively. Data represent the iRS-KDE levels in healthy control 1858 C/C (filled circles, n=36 for immature subset, n=39 for mature subset) and 1858 C/T (open circles, n=27 for transitional subset, n=30 for mature subset) subjects. (C and D) iRS-KDE rearrangement frequencies as quantified in peripheral Igλ⁺ transitional and mature B cells, respectively. Data represent the iRS-KDE levels in healthy control 1858C/C (filled circles, n=16 for transitional subset, n=18 for mature subset) and 1858C/T (open circles, n=12 for transitional subset, n=17 for mature subset) subjects. Data are depicted as iRS-KDE rearrangement frequency per genome copy. Mean values are depicted as horizontal line.
Supplemental Figure 4. *B cells from T1D 1858T/T subjects display enhanced homeostatic alterations.* (A) Previously frozen PBMC from 10 age- and gender-matched T1D 1858C/C and T1D 1858T/T subjects (ages 27-57 years) were stained as in Figure 1 and analyzed by FACS for the relative frequencies of transitional and B\textsubscript{ND} B cells. (A) Transitional B cells (CD19\textsuperscript{+}CD27\textsuperscript{neg}CD10\textsuperscript{+}CD24\textsuperscript{hi}CD38\textsuperscript{hi}) and B\textsubscript{ND} B cells (CD19\textsuperscript{+}CD27\textsuperscript{neg}IgD\textsuperscript{+}IgM\textsuperscript{neg}) are further increased in T1D 1858 T/T individuals. (B) Proximal BCR signaling in gated naïve (CD20\textsuperscript{+}CD27\textsuperscript{neg}) B cells of subjects shown in (A). Total B cells from T1D 1858C/C and T/T subjects were purified from previously frozen PBMC, rested overnight, then stimulated with 20 μg/ml soluble anti-κ F(ab’)\textsubscript{2} for 5 minutes and P-PLC\textsubscript{γ2} (Tyr759) was quantified by intracellular flow cytometry in gated naïve CD20\textsuperscript{+} CD27\textsuperscript{neg} B cells. Bars show mean fold change in MFI. Statistical significance was determined using a Student’s *t* test. (C) Proximal BCR signaling in enriched naïve mature (CD20\textsuperscript{+}CD27\textsuperscript{neg}CD10\textsuperscript{neg}) B cells. Naive mature B cells from T1D 1858C/C, T1D T/T, and control T/T subjects were enriched from previously frozen PBMC, rested for 1 hour, then stimulated with 20 μg/ml soluble anti-κ+λ F(ab’)\textsubscript{2} for 5 minutes and P-PLC\textsubscript{γ2} (Tyr759) was quantified by intracellular flow cytometry in gated naïve CD20\textsuperscript{+} CD27\textsuperscript{neg} B cells.