BCR and co-receptor crosstalk regulates B cell positive selection in autoimmunity

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Abstract

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A common genetic variant in the gene encoding the protein tyrosine phosphatase nonreceptor type 22 (*PTPN22-C1858T*) has been linked to a wide range of autoimmune disorders. Although a B cell-intrinsic role in promoting disease has been reported, the mechanism(s) through which this variant functions to alter the pre-immune B cell repertoire remains unknown. Using a series of polyclonal and transgenic self-reactive models harboring the analogous mutation in murine *Ptpn22,* we show evidence for enhanced BCR, BAFFR and CD40 co-receptor programs, leading to broadly enhanced positive selection of B cells at two discrete checkpoints in the bone marrow and spleen. We further identified a bias for selection of self-reactive B cells into the follicular (FM) vs. marginal zone (MZ) B cell compartment. Using a biomarker to track a self-reactive heavy chain in peripheral blood, we found evidence of similarly enhanced positive selection in human carriers of the *PTPN22-C1858T* variant. Our combined data supports

a model whereby the risk variant augments the BCR and co-receptor programs throughout B cell development to bias self-reactive specificities into the FM compartment, thereby likely increasing the risk for seeding of autoimmune B cell responses.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1:

Introduction

Factors Influencing Autoimmune Pathogenesis

Autoimmune diseases are among the most prevalent disorders in developed countries, with 7.6 - 9.4% of the population estimated to be affected worldwide[1]. Over 80 autoimmune disorders have been identified thus far[2], each broadly defined by the erroneous targeting of healthy human tissues by the immune system. It is increasingly clear that many diseases are worsened by both innate and adaptive immune responses gone awry, yet activation of self-reactive B and T lymphocytes that have escaped self-tolerance appears particularly relevant[3]. The specific causes of autoimmunity remain elusive, due in large part to the vast clinical heterogeneity and multifactorial contributions of environmental triggers and (poly)genetic risk factors.

Environmental Triggers

Microbial agents (viral or bacterial) may serve as environmental triggers for autoimmunity, particularly under conditions of genetic predisposition. Consistent with this, type 1 diabetes (T1D) has been associated with a number of viruses[4]. The mechanisms thought to promote autoimmunity in this context include molecular mimicry, bystander activation, and epitope spreading. Each mechanism shares a common feature of self-reactive lymphocyte activation as a by-product of normal immune system function. As one example, the shared immunologic epitopes (molecular mimicry)

between *Streptococcus pyogenes* and myosin likely promote cross-reactive antibody secretion during infection, which can then target both pathogen and heart tissue[5]. In contrast, the general inflammatory milieu arising from infections can sometimes promote the bystander activation of self-reactive lymphocytes. And finally, epitope spreading is thought to require persistent microbial infection, which can lead to damage towards self-tissues. The self-antigens released as a result of this damage (for example, nucleic acids from dying cells) may be taken up and presented by host antigen presenting cells (APCs) to activate self-reactive lymphocytes[6,7].

Genetic Risk Factors: *PTPN22-C1858T*

Genome-wide association studies (GWAS) have recently made great strides in uncovering the genetic components of risk or protection associated with autoimmune disease. Identifying loci with functional significance and the mechanism(s) responsible for risk/protection has proven much more difficult, due to the polygenic nature of autoimmune pathogenesis. Nevertheless, one genetic variant in particular stands out for being the leading non-HLA genetic predictor of a growing number of diseases– *PTPN22-C1858T.* A better understanding for how this risk variant modulates immune cell homeostasis and function is expected to yield invaluable insights into common autoimmune pathways, allowing for more targeted future therapeutics.

The PEST family protein tyrosine phosphatase nonreceptor type 22 gene (*PTPN22,* encoding Lyp in humans and Pep in mice) is widely expressed across hematopoetic lineages (reviewed extensively here[8,9]). *PTPN22* is comprised of a catalytic N-terminal

PTP domain, an interdomain, and a protein-binding C-terminal domain with four highly conserved proline-rich motifs (P1, P2, P3, and P4). It is best characterized as a potent negative regulator of T cell receptor (TCR) signaling, mediating its effects through dephosphorylation of activating tyrosine residues on Src family kinases and substrates[10,11]. *PTPN22's* inhibitory function is further augmented by binding to C-terminal Src kinase (Csk) via its P1 region, resulting in a phosphatase:kinase complex that together inactivates Lck kinase[11,12]. Unlike T cells, its function in B lymphocytes is not as well understood, due to the intact B cell development and B cell receptor (BCR) signaling observed in *Ptpn22*-deficient mice[11].

While a potential role for *PTPN22* in B cells was not immediately clear from these studies, the later identification of an autoimmune risk variant (*C1858T;* encoding R620W) and subsequent follow-up studies eventually confirmed the importance of *PTPN22* activity in regulating B cell homeostasis and function. In 2004, a missense mutation in *PTPN22* (*C1858T*; encoding R620W) was first identified as a major risk factor for TID[13]. Additional studies quickly followed, further linking the *PTPN22-C1858T* variant to rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Graves' disease, Vitiligo, and others[14-18]. Meta-analysis comprising multiple independent studies across varied populations revealed odds ratios ranging from 1.2 to 2.0 (roughly translating to *PTPN22-C1858T* carriers being 1.2 to 2 times more likely to develop disease compared to non-carriers)[19].

The high risk conferred by this single variant is no doubt influenced by the widespread expression of *PTPN22*. Not surprisingly, numerous studies in human carriers and murine knock-in (KI) models harboring the analogous risk variant have identified dysregulated innate and adaptive immune cell functions. Its function in lymphocytes appears to be particularly relevant. In T cells, *PTPN22* variant binds less well to Csk, likely due to the mutation's location in the P1 region[20]. Whether this reduced binding leads to a *gain-* or *loss-of-function* activity on TCR signaling is controversial, given the *hypo-* or *hyper-*responsive signals identified in human carriers and murine models, respectively. Despite these differences in TCR signaling, the overall similarities between human and murine phenotypes[21], as well as the high sequence homology in the catalytic domain of *PTPN22/Ptpn22*[22], argues for a conserved function.

Notably, unlike in *PTPN22* KO models, alterations in BCR as well as TCR signaling have been identified, providing important evidence of a role for *PTPN22* activity in B cells as well[23,24]. Evidence of altered B cell tolerance in a number of studies further implicates B cells as playing a key role in potentiating autoimmune risk. A summary of these existing studies and our attempt to reconcile and expand on them further will be discussed in Chapter 2. It is clear that given the current emphasis on B cell-directed therapies for autoimmunity, a better understanding for how *PTPN22* variant impacts B cell biology will be crucial for assessing potential therapeutic benefits and pitfalls.

B Cells In Autoimmunity

B Cells Drive Disease

Human and murine studies show a clear role for B cells in promoting autoimmunity, and in some cases (notably in diseases associated with the *PTPN22* variant), they may even be required. For instance, the non-obese diabetic (NOD) murine model of T1D shows a clear dependence on B cells for disease progression, using mechanisms that require both antibody secretion and APC function[25,26]. In addition, B cell-restricted expression of *Ptpn22* risk variant expression is sufficient to trigger autoimmunity in the mixed autoimmune-prone 129/B6 background, further suggesting that in combination with other genetic modifiers, the *Ptpn22* variant modulates B cell function to drive disease[27]. In addition to these murine studies, many autoimmune-associated variants identified by GWAS studies are expressed in B cells and predicted to alter cellular function in myriad ways[28], although the exact molecular mechanisms underlying these pathways have not been completely defined. Finally, with regards to *PTPN22* variant, there is a striking correlation between disease association and presentation of high-titers of pathogenic autoantibodies[18,29], providing important (albeit indirect) evidence of dysregulated B cell signals in promoting these diseases.

B Cell-Directed Therapies

Autoimmune disorders have traditionally been treated with non-specific immunosuppressive drugs meant to dampen an over-active immune response[30,31]. More recently, the identification of relevant cell types and common molecular pathways in

autoimmunity has paved the way for a more targeted approach. Further underscoring the importance of B cell-driven pathogenesis, a number of B cell-directed therapies have shown therapeutic benefit in the treatment of T1D, RA, SLE, and multiple sclerosis (MS)[32-35].

Depleting B cells from patients using a chimeric anti-CD20 depleting antibody, rituximab, has proven effective at modulating autoimmunity in T1D, RA and MS[32,33,35]. Unfortunately, this approach has not been as successful in treating lupus, despite marked B cell depletion[36,37]. Providing some clue as to why this may be, SLE relapse was found to be correlated both with elevated B cell-activating factor (BAFF) serum levels[38], and higher anti-DNA autoantibody titers[39]. It's been proposed that the escape of plasma cells (which do not express CD20), combined with a surge in BAFF levels (driven by B cell depletion), together promotes the expansion of self-reactive plasmablasts responsible for secreting autoantibodies[40]. Therapies targeting serum BAFF levels in SLE patients have thus proven more effective[34]. Additional approaches are currently being investigated to help resolve these issues, including strategies targeting rituximab-resistant populations, as well as dual B cell and BAFF-depletion therapies[31]. Overall, the clinical outcomes observed in B cell-directed therapies lend strong support for the role of B cells in autoimmunity, warranting future studies to better understand their role in disease.

One final study worth mentioning is a recent follow-up of the T1D patients previously found to benefit from anti-B cell therapy. Despite an initial halt in β-cell destruction, after two+ years of therapy these patients were found to have resumed β-cell dysfunction

and disease pathology[41]. From additional studies carried out by Chamberlain *et al.,* it was discovered that rituximab failed to reset early B cell tolerance checkpoints, leading to a resurgence of autoreactive specificities upon eventual B cell reconstitution[42]. This study makes clear that removal of problematic B cells from the repertoire is not enough for sustained clinical benefit – successful future therapies will need to prevent them from developing in the first place. To accomplish this, a thorough understanding of the molecular mechanisms governing B cell tolerance is needed.

B Cell Self-Tolerance

The ongoing generation of B and T lymphocytes with unique AgRs specificities is necessary for protection against diverse pathogens. This diversity is achieved via random assortment of germline encoded V(D)J genes in developing lymphocytes. An inherent tradeoff within this process, however, is creation of receptors that are specific for self-tissues. As described previously, such self- or autoreactive lymphocytes have the potential to elicit a dangerous autoimmune response if they escape self-tolerance mechanisms and become aberrantly activated. Thus, a major challenge for the immune system is how to selectively promote the development of lymphocytes with AgRs that will recognize pathogens over self-tissues.

It may appear that simply removing all lymphocytes with self-reactive AgRs would be effective, but this is not the case. Although each lymphocyte typically expresses a single AgR structure, each receptor may bind multiple antigens with varying affinities.

Therefore, a single lymphocyte could conceivably react to both pathogenic and autoreactive antigens, albeit to varying extents. Any repertoire capable of responding to diverse pathogens will then inevitably contain some degree of self-reactivity. This is especially important for protection against pathogens that may share structural similarities to self-antigens (for instance, viral nucleic acids). How does the immune system decide which self-reactive lymphocytes are worth the risk? In other words, what is the ideal balance of negative selection (*against* self) and positive selection (*both for* self and non-self) that will achieve maximal repertoire diversity with minimal autoreactivity?

For B lymphocytes, establishing a diverse yet 'safe' repertoire in the follicular mature (FM) and marginal zone (MZ) compartments is achieved through regulated exposure to self-antigens and competition for limited survival factors at key developmental checkpoints in the bone marrow (BM) and periphery. While the BCR signal serves as the master regulator of survival and tolerance, its integration with key co-receptor pathways activated by these various survival signals – BAFFR, CD40, and TLRs – ultimately determines the fate of an individual B cell. The molecular mechanisms surrounding this crosstalk and how they regulate B cell tolerance (termed "central" in the bone marrow, and "peripheral" in primarily the spleen) will be discussed in detail below. (Fig. 1-1)

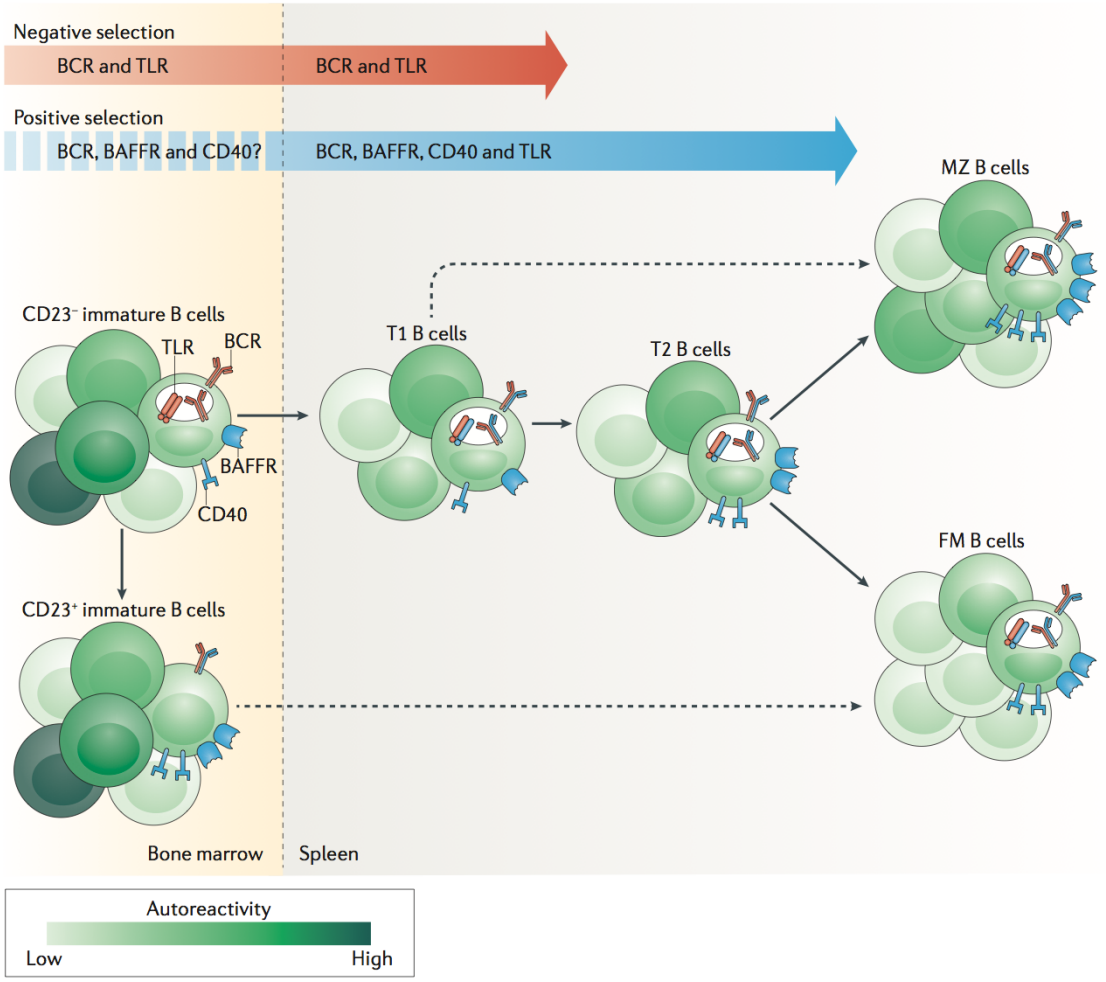

FIGURE 1-1. B cell receptor and co-receptor signaling govern B cell selection and maturation. Self-reactive B cells are subject to positive or negative selection throughout their development in the bone marrow and periphery (spleen). The selective fate of an individual B cell clone depends on multiple factors, including the location and form of self-antigen encounter, the strength of the B cell receptor (BCR) signal and synergy with co-receptor pathways. Negative selection mechanisms (such as deletion, receptor editing and anergy) are mediated primarily by BCR signaling, with potential input from specific Toll-like receptors (TLRs). By contrast, positive selection through survival and/or clonal expansion occurs primarily in transitional B cells in the periphery, and is driven by a complex interplay between BCR signaling and co-receptor signaling mediated by B cell-activating factor receptor (BAFFR), CD40 and TLRs. As the developing repertoire is fine-tuned, transitional B cells mature and populate the follicular mature (FM) compartment or the marginal zone (MZ) compartment. Although the majority of autoreactive BCR specificities are purged by negative selection, a proportion of mature naive B cells exhibit self-reactivity and/or polyreactivity, particularly within the MZ compartment. The dashed arrows indicate ongoing research regarding nonlinear routes for B cell development. T1, transitional type 1; T2, transitional type 2.

Negative Selection of Autoreactive B Cells

Antigen-mediated BCR

Antigen-mediated BCR signaling removes or segregates the majority of autoreactive B cells from the developing repertoire during the process of negative selection. Immature B cells with newly rearranged BCRs are first tested for reactivity towards self-antigens in the bone marrow. At this stage, self-reactive B cells have the opportunity to edit their receptor specificity, typically through additional light chain VJ rearrangements[43-45]. Receptor editing is considered successful if it sufficiently reduces the BCR signal, allowing such cells to egress into the periphery as transitional type 1 (T1) and type 2 (T2) cells for continued development. For immature B cells that remain strongly self-reactive (such as those binding membrane-bound self-antigen, which can crosslink receptors[46]), programmed cell death removes them from the repertoire. In contrast, those eliciting a more moderate BCR signal (for instance, B cells engaging with soluble self-antigen[47-49]) exit the BM yet are rendered functionally nonresponsive, or anergic. Even with these forms of negative selection, up to 40% of recent bone marrow human B cell emigrants exhibit some level of autoreactivity[50]. Splenic murine transitional B cells continue to undergo negative selection to further cull self-reactivity before maturation into the FM and MZ compartments[51-53], and studies of human peripheral blood B cells have identified a similar checkpoint for negative selection[50] (Fig. 1-2A)

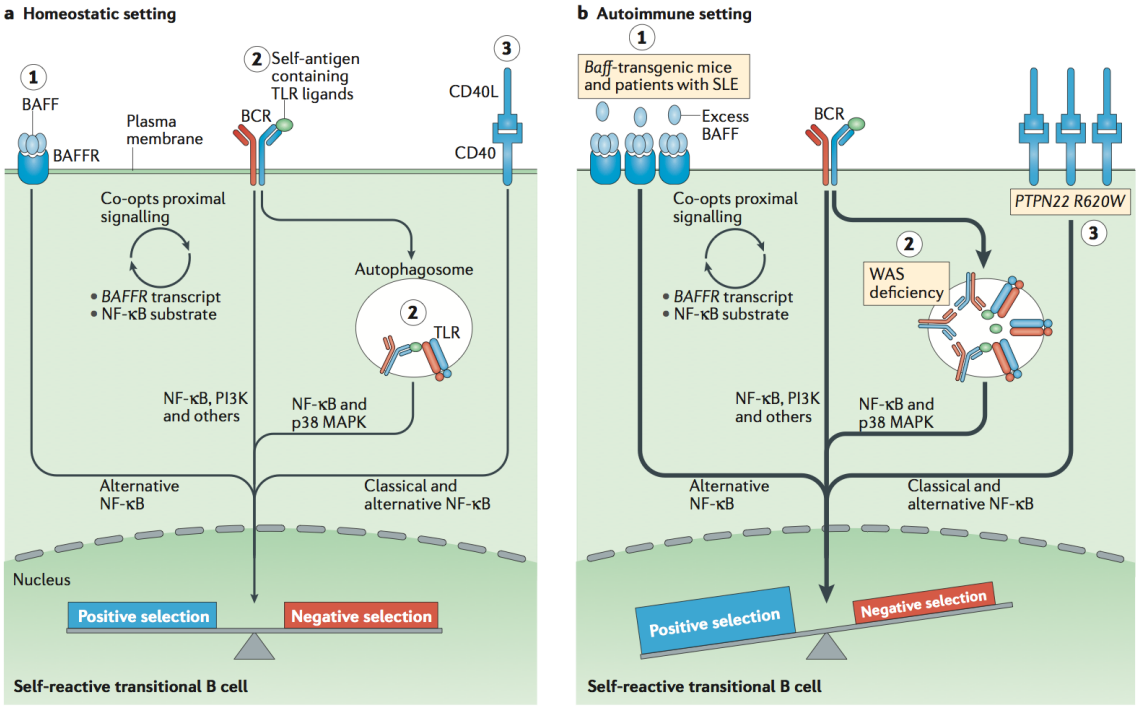

FIGURE 1-2. Altered B cell and co-receptor signaling promotes increased autoreactivity within the naïve repertoire. (A) Under homeostatic conditions, self-reactive B cells are subjected to both positive and negative selection mechanisms as they transit into the naive B cell pool and establish the naive repertoire. Whereas tonic B cell receptor (BCR) signaling and BCR engagement with self-antigen primarily regulate these events, synergy between the BCR and co-receptors fine-tunes the tolerance program within a given B cell. Among these co-receptors, B cell-activating factor receptor (BAFFR) signaling (1) synergizes with BCR signaling during late bone marrow and transitional development through a series of complex events, including proximal biochemical crosstalk and the downstream transcriptional regulation of both receptor and substrate expression. Dual BCR and Toll-like receptor (TLR) signaling (2) is mediated by internalization and delivery of self-antigens that contain TLR ligands to autophagosomes, which contain endosome-resident TLRs. CD40 signaling (3), which is triggered by interaction with CD40 ligand (CD40L) on T cells and possibly other cell types, also integrates with the BCR signaling pathway. Although BCR signaling can modulate CD40 expression, other biochemical or transcriptional events that affect this crosstalk are less well understood. **(B)** In genetic (or environmental) settings that promote an increased risk of developing autoimmunity, the homeostatic signaling thresholds are modulated, and self-reactive B cells exhibit greater positive selection and/or reduced negative selection, leading to a naive repertoire that is skewed towards autoreactivity. For example, excess amounts of B cell-activating factor (BAFF; 1) in the *Baff*-transgenic mouse model rescue low-affinity self-reactive B cells from negative selection. A similar mechanism has been proposed to exist in individuals with systemic lupus erythematosus (SLE). Similarly, in mouse and human settings of Wiskott–Aldrich syndrome (WAS) deficiency (2), hyper-responsive dual BCR and TLR signaling promotes the positive selection of transitional B cells with BCRs that use a limited subset of genes that encode self-reactive heavy-chain variable (VH) domains. Healthy individuals with the autoimmunity-associated variant $PTPN22^{R620W}$ (3) exhibit altered BCR and CD40 signaling, and have an enrichment of self-reactive BCR specificities within the naive B cell compartment. Although it has not yet been definitively demonstrated, it is likely that enhanced positive selection, rather than relaxed negative selection, predominantly mediates this change. The thickness of the arrows indicates the strength of pathway activation. MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase.

As described earlier, BCR signal strength in response to self-antigen signals serves as a proxy for perceived autoimmune risk, determining the degree and form of negative selection. Thus, while apoptosis is a single, irreversible response towards the most highly self-reactive B cells, the moderate self-reactivity in anergic cells subjects them to less severe forms of negative selection. Various BCR transgenic models of fixed (H and L chain) or semi-fixed (H chain) specificity for self or neo-self antigens have been used to study mechanisms of anergy. From these models, it became clear that 'functional non-responsiveness' can take many forms, including: a shortened lifespan[54,55], reduced ability to compete for survival factors[56], impaired maturation[48,55], segregation from follicles[57], and most importantly, a reduced capacity to signal through the BCR[48,58,59], leading to impaired proliferation[48,60,61] and/or secretion of antibodies[47,48,55]. It has been proposed that the range of anergic phenotypes identified in these models similarly reflects fluctuations in BCR signaling strength, with more severe responses induced in models of higher avidity BCRs[48,49]. In further support of this idea, cells sharing similar markers of anergy were later identified in wild-type mice with fully polyclonal repertoires[37,62,63]. Using the *Nur77*–GFP reporter strain, in which antigen-mediated BCR signaling activates transcription of GFP, Zikherman *et al*. demonstrated that GFP expression correlates with the degree of functional responsiveness[62]. These combined studies provided evidence that self-reactive B cells escaping apoptosis are nevertheless subjected to a degree of negative selection commensurate with their strength of BCR signal and risk for autoreactivity.

Importantly, anergy can be reversed under certain conditions, with important implications for both autoimmunity and pathogenic infection. For instance, the maintenance of anergy requires continuous self-antigen engagement[64], allowing them to become activated during conditions of cross-reactivity to infectious agents or other self-antigens[64,65]. In this context, while anergy is typically thought of as a form of negative selection, it may actually serve the dual purpose of limiting autoimmune risk while simultaneously promoting repertoire diversity.

TLR

Dual BCR and Toll-like receptor (TLR) signaling, facilitated by the trafficking of self-antigen to endosomal TLRs, has an important role in the initial activation of mature naive autoreactive B cells[66]. Although biochemical studies of these pathways in developing B cells have not yet been carried out, mouse and human studies nevertheless support a role for BCR and TLR crosstalk in regulating negative selection, particularly towards nuclear self-antigens. For instance, recent studies clearly demonstrate a role for DNA-specific TLRs (specifically, TLR9) in preventing spontaneous activation of peripheral autoreactive B cells[67]. Consistent with a role for TLR-dependent signaling in removing autoreactive B cells earlier in development, mice lacking the TLR adaptor protein *Myd88* exhibited abnormal central tolerance, and patients who lack *MYD88* or *IRAK4* show similar defects in central and peripheral tolerance[68-70]. Intriguingly, the germinal center-associated protein, activation-induced cytidine deaminase (AID), appears to play some role in inducing apoptosis in immature B cells[71]. Recent *in vitro* studies further implicate dual signals in mediating central tolerance by showing a synergistic impact of BCR and

TLR stimulation on AID expression[68]. (Fig. 1-2A)

Positive Selection of Nonautoreactive B Cells

Tonic BCR

In addition to these negative selection mechanisms, establishment of a safe naïve B cell repertoire requires the concurrent positive selection of nonautoreactive specificities. With the BCR serving as a master regulator, positive selection in this context is most commonly viewed as the survival advantage of a limited number of competing B cells, including those that receive adequate tonic BCR signals[72]. Sustained tonic BCR signaling is in fact required for the survival of both immature BM and mature B cells in the periphery[73] through induction of NFκB-dependent and/or PI3K-dependent pro-survival signaling[74-76]. (Fig. 1-2A)

BAFFR

Systemically-produced BAFF cytokine has prominent roles in B cell survival and homeostasis[77,78]. Although BAFF can bind to three alternative receptors – BAFFR, TACI, and BCMA – BAFFR appears to play the dominant role in peripheral B cell maturation. Transitional B cells predominantly express BAFFR and mice deficient in either BAFF or BAFFR exhibit an identical developmental block at the T2 stage[78-80]. In contrast, TACI or BCMA-deficient animals have relatively normal peripheral B cell numbers[79,81].

In the periphery, BAFFR signaling mediates survival primarily through induction of the

alternative NFκB survival pathway[82], although recent studies suggest additional novel intersections with tonic BCR signaling. For example, BAFFR signaling promotes the phosphorylation of proximal BCR signaling components, including SYK and Igα, and inducible *Syk* deletion results in reduced BAFF- dependent B cell survival despite intact alternative NF-κB signaling[83]. In addition, BAFF seems to co-opt signaling components of the BCR to promote CD19 phosphorylation, resulting in PI3K-dependent B cell survival[84]. Consistent with this, CD19 is required for the survival of SYK-deficient B cells[85]. In addition, BCR signaling has been reported to enhance both BAFFR transcript and alternative NF-κB p100 substrate levels[86,87], which likely modulates NFκB network states[88]. In combination, these studies suggest that a complex interplay between the BCR and BAFFR signaling pathways promotes B cell survival, and that this crosstalk modulates transitional cell selection and the establishment of the naive B cell repertoire.

Lastly, given that immature IgM^+ B cells seem to be normal in *Baff*$^{-/-}$ and *Baffr*$^{-/-}$ mice, BAFF was not thought to affect BM B cell development and selection. However, new studies challenge this idea and may support a model that is similar to that of BAFFR-driven peripheral selection. BAFFR is expressed by a $CD23^+$ immature BM B cell subset, which also expresses B220, IgM and AA4.1. This subset further shares phenotypic and functional characteristics of splenic T2 B cells, including maturation from $CD23^-$ counterparts, dependence on both BAFFR and tonic BCR signaling, and proliferation following T cell help[87,89,90]. BAFFR-deficient 'T2-like' immature cells also exhibit a competitive disadvantage relative to their wild-type counterparts[91]. Collectively, these

data support a revised model of BM B cell tolerance in which BAFFR signaling, in concert with tonic BCR signaling, promotes the differentiation and/or positive selection of certain immature B cells. Additional studies that assess the effects of antigen-mediated BCR signaling are required to better define the potential contribution of this cell population to the establishment of the naive repertoire and/or autoimmune responses. (Fig. 1-2A)

CD40

Emerging data suggest that CD40 signaling, similar to BAFFR, may also modulate transitional B cell selection in concert with the BCR program. CD40 engagement promotes both the alternative and classical NFκB prosurvival pathways[92]. In addition to the well-described upregulation of CD40L following T cell activation, CD40L is also constitutively expressed by naïve CD4 T cells[93]. A range of *in vitro* observations have long implicated CD40 signals in modulating transitional B cell survival including, for example, data demonstrating CD40-dependent rescue of BCR-induced apoptosis in transitional cells[1]. Additionally, CD40 exhibits a compensatory role in the absence of BCR signals, as revealed by the severely reduced transitional B cell compartment size in *Btk* (a proximal BCR signaling component) and *Cd40* doubly-deficient mice[2]. Finally, Schwartz *et al.* recently showed a role for CD40 signaling in promoting B cell positive selection and a broader antigen specificity profile within the mature B compartment, particularly within the MZ B cell repertoire[3]. Thus, although *Cd40*$^{-/-}$ mice have normal B cell numbers[4], CD40 signaling still affects the breadth of specificities in the mature compartment.

Similar to what has recently been described with BAFFR signaling, it is tempting to speculate about an earlier role for CD40 in central tolerance, given the ability of CD23+ immature B cells to respond to CD40 stimulation[5], and the competitive disadvantage of *Cd40*$^{-/-}$ BM B cells observed by Schwartz *et al.*[6,7]. Future mechanistic studies that directly assess the intersecting roles of the CD40 and BCR pathways are likely to provide additional insights into immature and transitional B cell selection. (Fig. 1-2A)

Positive Selection of Autoreactive B Cells

Antigen-mediated BCR

Direct BCR engagement with self-antigens can also promote the positive selection of certain self-reactive B cells, provided it does not surpass a presumed threshold for negative selection[13]. As described earlier, this paradoxical enrichment for self-reactivity is important for maximizing repertoire diversity. In support of this idea, certain V gene families and heavy/light chain pairs are enriched in the mature B cell compartments[14-18]. Recent studies from our laboratory and others have also expanded our understanding of self-antigen-mediated positive selection. For example, our group identified a subset of T2 B cells that enter the cell cycle in response to antigen engagement[19]. Using the M167 VH1 heavy chain transgenic model, in which B cells that express self-reactive phosphorylcholine-specific BCRs are detected by an idiotype-specific antibody, we demonstrated that M167-idiotype^{+} B cells are enriched within this cycling transitional subset and are further enriched in the MZ compartment[22]. Consistent with observations in the M167 model[94] and other transgenic models[8], GFP expression in the *Nur77*-GFP

reporter strain initially increases in T2 B cells, implying that the selection of transitional B cells into FM and MZ compartments is refined by antigen stimulation.

BAFFR

In addition to its effect on B cell survival, BAFF has been implicated in the development of SLE and other autoimmune diseases. For example, BAFF overexpression in *Baff*-transgenic mice recapitulates several cardinal features of human SLE, including polyclonal B cell proliferation, antinuclear antibody production and the development of immune-complex-mediated glomerulonephritis[21]. As mentioned previously, BAFF levels are also increased in a subset of patients with SLE[27], and a BAFF-inhibiting therapeutic antibody demonstrated clinical efficacy in patients with SLE[13], emphasizing the importance of BAFF in the pathogenesis of the disease.

Although the mechanisms by which BAFF promotes autoimmunity have not been completely defined, excess BAFF probably contributes to disease development through effects on B cell selection during development. Specifically, increased BAFF levels rescue low-affinity self-reactive transitional cells, thereby allowing their maturation and entry into 'forbidden' splenic zones[25,26]. In a model in which developing B cells compete for available BAFF, excess BAFF results in relaxed selection[27]. Thus, BAFF-dependent survival signals, presumably downstream of BAFFR, contribute to humoral (autoantibody-mediated) autoimmunity in *Baff*-transgenic mice by increasing the proportion of autoreactive B cells within the mature naive repertoire. (Fig. 1-2B)

CD40

Whether CD40-dependent effects on the pre-immune repertoire also contribute to the risk of autoimmunity has not been addressed. However, individuals who carry the *PTPN22-C1858T* variant do exhibit enhanced self-reactivity at the early emigrant and mature B cell stages, along with enhanced CD40 transcript and surface expression[28]. These combined data suggest a possible contribution for CD40 signaling in promoting the positive selection of self-reactive specificities. Consistent with this idea, although the increased frequency of autoreactive B cells observed in the periphery of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX syndrome) has been attributed to a lack of regulatory T cell-dependent regulation of B cell tolerance[18,29], an alternative interpretation is that the increased levels of CD40L found on $CD4^+$ T cells in these patients instead directly promote the survival and positive selection of autoreactive B cells. (Fig. 1-2B)

TLR

Interestingly, similarly to BCR signaling, TLR activation also seems to have a dichotomous role in promoting both the positive and negative selection of autoreactive B cells. Evidence for TLR signaling promoting transitional B cell positive selection in both humans and mice comes from the primary immunodeficiency Wiskott–Aldrich syndrome (WAS). Mutations in the WAS protein influence actin polymerization and receptor signaling in nearly all haematopoietic cell lineages and, in B cells, WAS mutations result in modestly enhanced signaling downstream of both the BCR and TLRs[30,31]. In this

context, although negative selection is maintained in *Was*$^{-/-}$ mice, late T2 B cells exhibit enhanced proliferation[32-35]. High-throughput B cell repertoire sequencing of *Was*$^{-/-}$ mice and humans with WAS identified the preferential selection of specific heavy-chain variable (VH) gene families (VH10 in mice; VH4-34 in humans) as late transitional B cells become mature naive B cells[32,33,35]. Consistent with the BCR using these VH families to bind to antigenic complexes that also contain TLR ligands, both the increase in transitional B cell cycling and the altered naive repertoire in WAS were MYD88 dependent[36,37]. Thus, in addition to the known effects on the activation of mature autoreactive B cells, altered BCR and TLR signaling is coordinated to enhance the positive selection of self-reactive specificities into the naive B cell repertoire. Therefore, dual BCR and TLR signaling probably affects both negative and positive selection events during B cell development, with the effects depending predominantly on the developmental stage. TLR signaling in immature B cells primarily promotes negative selection, whereas dual signaling in late transitional B cells facilitates positive selection through clonal expansion. (Fig. 1-2B)

In summary, the studies described above support a paradigm in which BCR signaling coordinates with BAFFR, CD40 and TLRs to shape the mature naive B cell repertoire. Although these signaling pathways are frequently considered separately, crosstalk between them seems to be the norm rather than the exception. Depending on the developmental stage and context, this ongoing signal integration can function to modulate either negative or positive selection.

Questions to Address

Emerging data from murine and human studies demonstrate that even modest alterations in B cell-intrinsic signaling programs can be sufficient to promote breaks in B cell tolerance, thus seeding the mature repertoire with harmful autoreactive BCR specificities. These autoreactive B cells then play key roles in initiating autoimmunity through a variety of mechanisms. A better understanding for the molecular pathways regulating B cell tolerance – particularly crosstalk between the BCR and co-receptor signaling pathways – would provide a clearer picture for how signaling dyregulation may potentiate autoimmune risk.

Prior studies in human and murine WAS models have demonstrated a clear role for dual BCR and TLR signaling in driving enhanced positive selection of certain self-reactive BCR specificities, via clonal expansion at the T2 stage. Given evidence of molecular crosstalk between the BCR, BAFFR, and CD40 signaling pathways, similar studies assessing the impact of these combined signals on B cell tolerance under autoimmune conditions is a crucial next step. The *PTN22-C1858T* risk variant emerges as an ideal candidate for study, given its clear relevance to human health and known ability to alter the BCR (and possibly CD40) signaling pathways.

The current study utilized a series of animal models harboring the analogous variant in murine *Ptpn22* for a detailed assessment of how the variant may impact: 1) BCR and co-receptor signaling programs, 2) B cell tolerance checkpoints, and 3) the mature pre-

immune repertoire. Together with limited parallel studies in human risk variant carriers, our results suggest that the *Ptpn22/PTPN22 variant promotes broadly enhanced positive selection throughout B cell development via enhanced BCR, BAFFR, and CD40-mediated signaling programs, leading to an unexpected bias for selection of autoreactive specificities into the FM over MZ compartment.* In providing an alternative interpretation for prior human studies, our model helps resolve some of the long-standing controversies regarding murine versus human variant AgR signaling, while providing novel pathways for future study in designing targeted therapeutics.

CHAPTER 2:

PTPN22 Risk Variant Promotes B Cell Positive Selection.

Introduction

The protein tyrosine phosphatase nonreceptor type 22 gene (*PTPN22)* encodes for the phosphatase, LYP, (or PEP in mice) which functions as a negative regulator of antigen receptor (AgR) signaling through its direct modulation of Src-family kinases[8]. A genetic variant in *PTPN22* (C1858T; encoding LYP-R620W) is a major risk factor for a number of autoimmune disorders including type 1 diabetes (T1D), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Graves' disease, and others[13-16,95]. To model this variant *in vivo*, we previously generated knock-in mice with the analogous risk allele on a mixed 129/Sv and C57BL/6J background. Expression of the *Ptpn22* variant significantly altered lymphocyte function and led to the development of systemic autoimmunity[27].

Although the *PTPN22* risk variant promotes disease via its impact on multiple cell lineages, B cells appear to be particularly important for this process[27,94]. Notably, the disorders associated with *PTPN22* risk variant are characterized by high titers of disease-specific pathogenic autoantibodies[19]. While autoantibodies may result from B and/or T cell-driven processes, our group found that B cell-intrinsic *Ptpn22* variant expression was sufficient to promote autoimmunity[27]. The conclusion that altered B cell tolerance may potentiate similar risks in human subjects arose from the observation that transitional B cells were increased in both human and murine carriers of the risk variant[27,96]. Lending further support to this idea, increased proportions of self-reactive B cells were identified

at two checkpoints during human B cell development based on analyses of cells isolated from the peripheral blood of healthy subjects with the risk allele[97].

Taken together, these data suggest that the *PTPN22* variant plays an important role in shaping the pre-immune B cell repertoire in at-risk individuals and in murine models; however, several key questions remain that warrant further study. First, one major unresolved issue is whether the variant confers a gain- vs. loss-, or alternatively an altered-, functional activity. Indeed, a range of contradictory findings with respect to the impact of the variant on AgR signals have been observed in human and murine studies (reviewed in Rawlings *et al.*[21]). The studies to date have relied upon *in vitro* stimulated cells, thus direct *ex vivo* analysis of AgR signaling is needed. Secondly, other than the BCR signaling pathway, it is unclear whether additional networks are impacted by *PTPN22* variant. Of particular relevance are the BAFFR and CD40 co-receptor pathways, given their importance in regulating B cell tolerance and known crosstalk with the BCR signaling program[72,78,98,99]. Lastly, a more complete understanding of how the *PTPN22* variant shapes the specificities selected into the mature, naïve B cell compartments might help to predict the risk for subsequent aberrant activation of such cells in autoimmune individuals.

In the current study, we use a series of murine models, in association with a rigorous assessment of the naïve repertoire, to track the development and selection of B cells expressing the *Ptpn22* risk variant. Murine studies included mice homozygous for the non-risk allele ($Ptpn22^{CC}$) and heterozygous ($Ptpn22^{CT}$) or homozygous ($Ptpn22^{TT}$) risk

allele animals intercrossed with various selection models. To reduce potential impacts from additional genetic modifiers, we used *Ptpn22* variant and controls backcrossed onto the non-autoimmune C57BL/6J background. In parallel, a flow-based assay tracking a self-reactive heavy chain (HC) was used to monitor peripheral B cell selection in human carriers with the variant. Our combined results suggest the *Ptpn22* variant augments the coordinate BCR, BAFFR, and CD40 programs throughout B cell development, leading to altered tolerance at discrete checkpoints in the bone marrow and periphery. These events promoted enhanced positive selection of transitional B cells, with an unexpected bias for self-reactive specificities into the FM compartment. Healthy human subjects expressing the risk variant exhibited a reduced proportion of transitional B cells utilizing a specific, self-reactive heavy-chain family, findings most consistent with broadly enhanced positive selection for developing B cells with a range of self-reactive specificities. Our collective data add to the understanding of B cell-mediated autoimmunity, suggesting that allelic variants that enhance the BCR and/or key co-receptor pathways preferentially skew self-reactive B cells into the follicular B cell compartment, thereby increasing the probability of subsequent events that trigger autoimmune germinal center responses.

Materials and Methods

Mice

Ptpn22^{CC} (Ly 5.1 and Ly 5.2 lines), *Ptpn22*^{TT}, Nur77-GFP Tg, BAFFR$^{-/-}$, CD40$^{-/-}$, CD40L$^{-/-}$, MD4 Tg, mHEL, sHEL, μMT, and 125 Tg (VH125 and VK125 lines) mice were maintained in the specific pathogen-free animal facility of Seattle Children's Research Institute and handled according to Institutional Animal Care and Use

Committee-approved protocols. *Ptpn22*^{TT} knock in mice were generated as previously described [27] and backcrossed to C57BL/6J for 10 generations before crossing to Nur77-GFP Tg, MD4, 125 Tg (VH125) or 125 Tg (VK125). The experimental mice contained 1 copy of Nur77-GFP, 1 copy of MD4, or 1 copy each of VH125 and VK125 transgenes.

High-throughput BCR sequencing

Murine FM and MZ B cell populations were bulk sorted and genomic DNA was extracted for survey-depth sequencing of the IgH locus (Adaptive Biotechnologies, Seattle WA). Adaptive Biotechnologies' ImmunoSEQ Illumina-based sequencing platform was used to identify productive templates for assignment of IgH V and J genes and to determine CDR3 boundaries (defined as including the first base of the codon for the conserved cysteine in the V gene through the last base of the codon for the conserved residue in the J gene). Average hydrophobicity scores (GRAVY) were calculated using http://www.gravy-calculator.de/. Diversity scores (reciprocal Simpson Index) were calculated as described [100]. The total number of productive templates generated can be found in Fig. 2-S1. Data are representative of two independent experiments.

Human subjects and sample preparation

Frozen PBMCs from age and sex-matched healthy subjects screened for *PTPN22* 1858 were obtained from the Benaroya Research Institute Immune Mediated Disease Registry. Subjects included *PTPN22* C/C (n=35), C/T (n=35) and T/T (n=3). For flow cytometry, single-cell PBMC suspensions were incubated with fluorescently labeled Abs (see below) for 20 min at 4^{o}C in staining buffer, and data collected on an LSRII (BD) and analyzed

using FlowJo software (Tree Star). Subject information can be found in Fig. 2-S2. Data are representative of three independent experiments.

Reagents and Abs

Anti-murine Abs used in these studies include: B220 (RA3-6B2), CD24 (M1/69), CD21 (7E9) and CD23 (B3B4) from Biolegend; IgMa (DS-1) and CD40 (3/23) from BD; Ly 5.1 (A20), Ly 5.2 (104), Gr1 (RB6-8C5), CD11b (M1/70), CD3 (17A2), BAFFR (eBio7H22-E16) and CD93 (AA4.1) from eBiosciences; IgM (1B4B1) and IgD (11-26) from Southern Biotech; recombinant human insulin conjugated to biotin from Fitzgerald Industries; Streptavadin (S-868) from Life Technologies. Anti-human Abs used include: CD19 (HIB19) and IgM (MHM-88) from Biolegend; CD27 (O323) from eBiosciences; CD10 (HI10a), CD24 (ML5), CD38 (HIT2), IgD (IA6-2), and BAFFR (IIC1) from BD; anti–human FITC-conjugated 9G4 antibody[101].

Flow cytometry and cell sorting

Murine single-cell BM and splenocyte suspensions were incubated with fluorescently labeled Abs for 30 min at 4^{o}C in staining buffer, and data collected on an LSRII (BD) and analyzed using FlowJo software (Tree Star). Cell sorting was performed using an Aria II (BD); sort purities were >90% in all experiments.

Single-cell BCR cloning

Murine single FM and MZ cells were FACS sorted into 96-well plates. BCRs were cloned from the cDNA of single cells and used to generate mAbs using methods previously described[99,102]. Data are representative of at least two independent experiments.

ELISA tests

All monoclonal antibody ELISAs were performed with each mAb first normalized to a standard dilution ranging from 10ng/ml to 10ug/ml. Individual FM and MZ mAbs were tested for reactivity with insulin (Fitzgerald Industries International), MDA-LDL (20P-MD L-105; Academy Bio-Medical), dsDNA (Sigma-Aldrich), phosphorylcholine (PC)-12 (Sigma-Aldrich) and smRNP (ATR01-10; Arotech Diagnostics Limited) as previously described[101]. Abs were considered reactive if the observed OD at the highest mAb concentration (10ug/ml) was greater than a threshold value set at 0.5 OD. Anti-HEL Abs in MD4 chimeras were measured by incubating serum on HEL pre-coated plates, followed by detection with anti-IgMa conjugated to biotin (DS-1) and streptavidin-HRP. Data are representative of two independent experiments.

Competitive Chimera BM transplantations

BM was harvested from donor *Ptpn22*^{CC} (Ly 5.1), *Ptpn22*^{TT} (Ly 5.2) and μMT (Ly 5.1/Ly 5.2) mice and single-cell suspensions were mixed at a 10:10:80 ratio for retro-orbital injection of 5 x 10^6 cells into lethally irradiated (900 cGy) *Ptpn22*^{CC} (Ly 5.1/Ly

5.2) recipients. Resulting BM chimeras were sacrificed 12-14 wks post-transplantation. Data are representative of five independent experiments.

HEL BM transplantations

BM was harvested from donor *Ptpn22*^{CC} MD4 or *Ptpn22*^{TT} MD4 mice and made into single-cell suspensions, where 5 x 10^6 cells were retro-orbitally injected into lethally irradiated (900 cGy) mHEL or sHEL recipients. Resulting BM chimeras were sacrificed 8-12 wks post-transplantation. Data are representative of two independent experiments.

Quantitative PCR

RNA was isolated from sorted cells (purity >90% for all samples) using the AllPrep DNA/RNA Micro Kit (QIAGEN) and converted into cDNA by reverse transcription (Maxima Reverse Transcriptase; Thermo Scientific). Real-time PCR was performed using a CFX96 real-time PCR detection system (Bio-Rad) with iTaq universal SYBR Green supermix (Bio-Rad). Ratios were calculated using the comparative C_T method with b2-microglobulin as an endogenous control. Primers used were as follows: β2M FP, 5'-CTTCAGTCGTCAGCATGGCTCG-3'; β2M RP, 5'-GCAGTTCAGTATGTTCGG CTTCCC-3'; BAFFR FP, 5'-CT GAGGCTGCAGAGCTGTC-3'; BAFFR RP, 5'-GGTGAGAAACTGCGTGTCCT-3'; CD40 FP, 5'-CTGCATGGTGTCTTTGCCT-3'; CD40 RP, 5'-GCCATCGTGGAGGTACTGTT-3'; PIM2 FP, 5'-CTTTCGAGGCC GATAACCGA-3'; PIM2 RP, 5'-GATGGCCACCTGACGTCTAT-3'; A1 FP, 5'-CCTGGCTGAGCACTACCTTCA-3'; A1 RP, 5'-CTGCATGCTTGGCTTGGA-3'; Notch2 FP, 5'-TTCGTGTCCCCCAGGCACCC-3'; Notch2 RP, 5'-AATCCGGT

CCACGCACTGGC-3'; Deltex1 FP, 5'-CGGACATTTGAGACCCACTT-3'; Deltex1 RP, 5'-CCACTTTCAAGGAGGGAGAA-3'; Hes1 FP, 5'-GAGAAGAGGCGAA GGGCAAGAAT-3'; Hes1 RP, 5'-GAGGTGACTTCACAGTCA-3'.

In vitro stimulations

Total splenocytes were cultured in RPMI media alone or stimulated with 0.1μg/ml recombinant murine BAFF (R&D) or 0.1μg/ml anti-mouse CD40L (Southern-Biotech) at 37°C for 5-60min in pre-warmed media. Cells were immediately washed post-stimulation with ice-cold PBS in a pre-chilled centrifuge and incubated with fluorescently labeled B220, BAFFR, and CD40 Abs for 30 min on ice in staining buffer containing sodium azide. After washing, cells were immediately fixed in 2% PFA with data collected on an LSRII (BD) and analyzed using FlowJo software (Tree Star). Data are representative of two independent experiments.

Statistical analysis

The p-values were calculated using the two-tailed Student *t* test or Paired *t* test where appropriate (GraphPad software). Differences were considered significant when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Results

Ptpn22 variant increases BCR and co-receptor signaling in developing B cells.

Previous *in vitro* stimulation studies revealed evidence for subtly enhanced BCR signaling in bulk splenic B cells isolated from *Ptpn22* variant mice[27]. This change in

signaling, however, was only evident in cells pre-stimulated with a TLR ligand and correlated with an upregulation in PEP expression. In order to assess the potential impact of BCR signaling within unmanipulated B cells *in vivo*, we utilized the Nur77-GFP Tg reporter strain. In this model, endogenous BCR signals activate a wide spectrum of GFP expression under control of the Nur77 regulatory region, consistent with self-antigen-mediated *in vivo* BCR signaling[103]. For these studies, we assessed *Ptpn22* variant mice with a single copy of the variant crossed to the Nur77-GFP Tg model (*Ptpn22*^{CT} Nur77 Tg), as we were unable to generate pups with two transgene copies (likely due to co-inheritance with the randomly inserted Nur77 transgene). Compared to control animals, B cells from *Ptpn22*^{CT} Nur77 Tg mice exhibited a higher frequency of GFP+ cells as well as a higher GFP MFI beginning at the immature stage in the bone marrow and continuing in all transitional and naïve B cell subsets in the periphery (Fig. 2-1A and Fig. 2-S3). These findings suggest that the *Ptpn22* variant promotes a greater proportion of self-reactive B cells to survive tolerance mechanisms and enter the periphery, consistent with an enhanced BCR signaling program mediating these events.

Recent studies suggest that BCR signals coordinate with both BAFF family receptors and CD40 to shape the mature, naive B cell repertoire[104]. BCR signaling promotes the BAFFR program through modulation of receptor transcript levels[86], as well as providing p100 substrate for induction of the alternative NFκB pathway [87]. We therefore determined whether BCR signaling *in vivo* correlated with surface expression levels of BAFFR and CD40. Using the Nur77-GFP Tg model, we found that higher GFP expression in transitional cells indeed correlates with higher expression of both BAFFR and CD40 (Fig. 2-1B), suggesting a direct and/or indirect role for antigen-mediated BCR signaling in regulating sensitivity to these co-receptor signals.

We also compared BAFFR and CD40 surface levels across bone marrow and splenic subsets in control *Ptpn22*^{CC} mice in order to determine the developmental stages most likely to be impacted by co-receptor signals. Prior reports have identified increased levels of BAFFR on the CD23^{+} subset of immature BM B cells, a population comprising ~20% of all immature IgM^{+} cells (gated as B220^{+} IgM^{+} AA4.1^{+} CD23^{+}) [87,89,91,104,105]. Therefore, we adopted a similar gating strategy in the BM. As shown in Fig. 2-S3B, CD23^{+} immature BM and splenic late transitional (T2) B cells (gated as B220^{+} CD24^{hi} CD21^{mid}) expressed higher levels of BAFFR and CD40 relative to their earlier developmental counterparts. Thus, we focused our subsequent analysis of co-receptor studies on these specific subsets in control and risk variant mice.

Based on the increased BCR signal observed in *Ptpn22*^{CT} Nur77 Tg mice (Fig. 2-1A) and correlation of BCR signal strength with co-receptor expression (Fig. 2-1B), we hypothesized that *Ptpn22*^{TT} animals may exhibit enhanced BAFFR and/or CD40 signaling in CD23+ immature and T2 cells. Consistent with this prediction, T2 cells had greater surface CD40 levels in *Ptpn22*^{TT} mice (Fig. 2-1C). In contrast, surface levels of both BAFFR and CD40 were reduced in *Ptpn22*^{TT} immature CD23^{+} B cells compared to controls (Fig. 2-1C). One potential explanation for the seemingly paradoxical reduction in co-receptor surface levels on *Ptpn22*^{TT} CD23^{+} immature B cells was enhanced receptor internalization. Consistent with this possibility, we first demonstrated that stimulation of control splenic B cells with either BAFF or soluble CD40L is sufficient to reduce surface expression levels of BAFFR or CD40, respectively (Fig. 2-1D). Similar to our findings, BAFFR levels were reported to decline following *ex vivo* BAFF stimulation of human B cells[106]. Therefore, to determine whether reduced levels of these co-receptors on risk variant BM CD23^{+} B cells reflected increased signaling, we assessed transcript levels using quantitative PCR in sort-purified, CD23^{+} and T2 B cells isolated from 8-10wk control and *Ptpn22*^{TT} mice. Consistent with an enhanced co-receptor signaling program, *Ptpn22*^{TT} immature and transitional cells exhibited greater levels of BAFFR and CD40 transcripts, respectively (Fig. 2-1E).

To more directly parse out the interconnected BCR and co-receptor signaling programs, we measured transcript levels of the alternative NFκB-pathway target, Pim2 - responsive to co-receptor signals, and the alternative and classical NFκB-pathway target, A1 – responsive to the BCR and co-receptor signals.[105,107-109]. Pim2 transcripts were

substantially increased at both stages of development in *Ptpn22*^{TT} mice (Fig. 2-1F), while A1 was significantly increased only in the periphery. Collectively, these results demonstrate that the *Ptpn22* variant coordinately increases both antigen-mediated, BCR signaling and the BAFFR and CD40 co-receptor programs. While enhanced BCR signals mediated by the risk variant likely impacts both co-receptor programs (directly and/or indirectly) during this developmental window, our findings suggest that the variant may exert a greater role on BAFFR in the BM and on CD40 in the periphery.

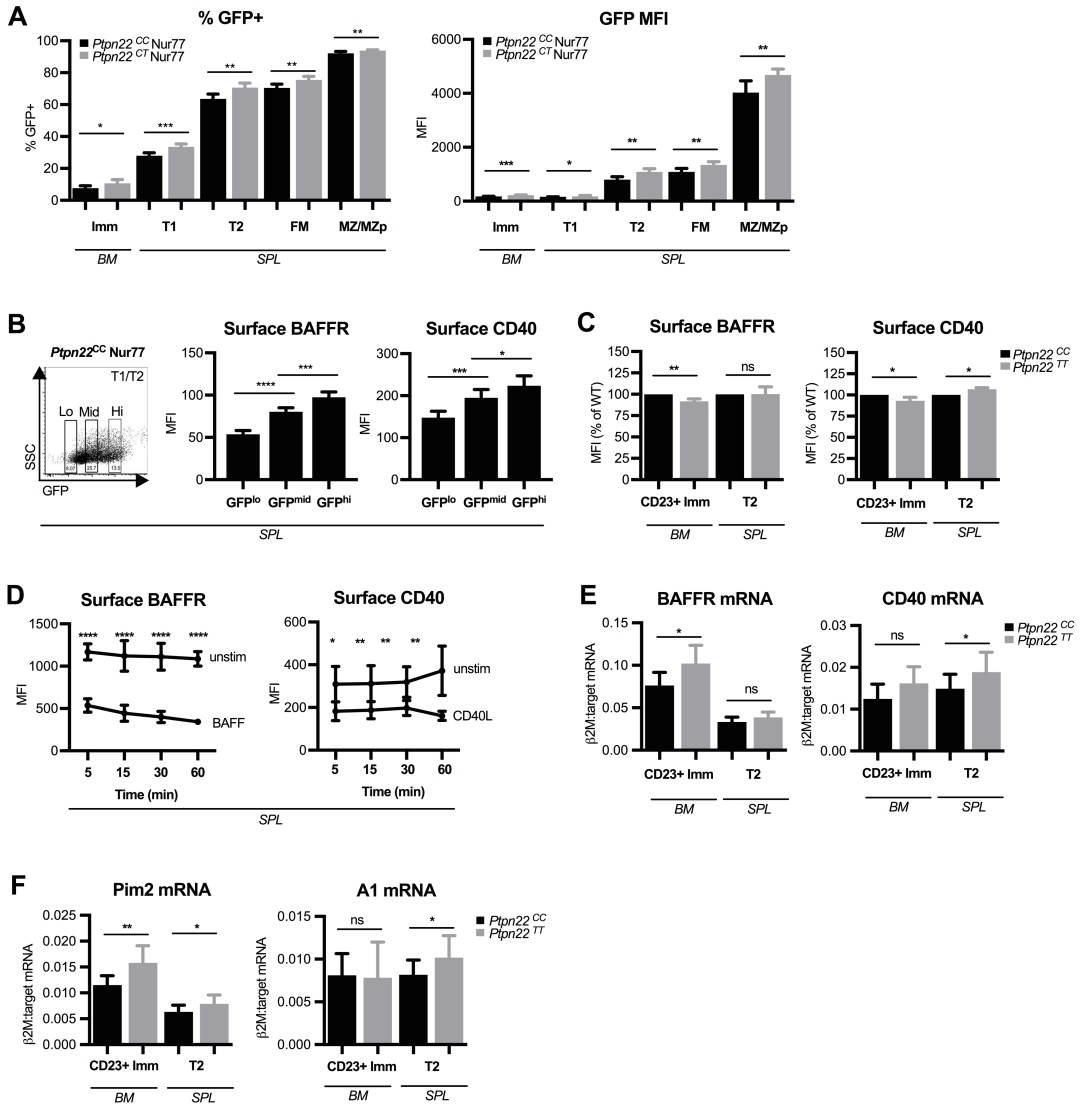

FIGURE 2-1. ***Ptpn22*** **variant B cells exhibit enhanced antigen-mediated BCR, BAFFR and CD40 programs. (A)** The Nur77 GFP Tg model was used to assess *in vivo* antigen-mediated BCR signaling. Frequency of GFP+ cells (left) and mean fluorescent intensity (MFI) of GFP (right) in specific B cell subsets in 12wk *Ptpn22*^{CC} Nur77 (n=6) and *Ptpn22*^{CT} Nur77 (n=6) mice. See Fig. 2-S3A for representative GFP histograms and Fig. 2-S4A-B for details of B cell subset gating. **(B)** Representative gating of GFP^{lo}, GFP^{mid} and GFP^{hi} (left panel); and MFI of BAFFR (middle panel) and CD40 (right panel) on T1/T2 cells (gated B220+ CD24^{hi} CD21$^{lo\text{-}mid}$) across varying GFP intensities in 15wk *Ptpn22*^{CC} Nur77 (n=6) mice. **(C)** MFI of surface BAFFR (left panel) and CD40 (right panel) in bone marrow CD23^{+} immature and splenic T2 B cells in 10-12wk *Ptpn22*^{CC} (n=9) and *Ptpn22*^{TT} (n=9) mice. See Fig. 2-S4B-C for gating. **(D)** Splenocytes from 10wk *Ptpn22*^{CC} (n=6) mice were stimulated with either soluble BAFF (0.1 ug/ml) or CD40L (0.1 ug/ml) for the indicated times and MFI of surface BAFFR (left panel) and CD40 (right panel) of B220^{+} cells were analyzed by FACs. **(E and F)** Quantitative PCR of CD23^{+} immature and T2 cells sorted from 8-10wk *Ptpn22*^{CC} (n=8 samples for BM, 11 samples for SPL) and *Ptpn22*^{TT} (n=6 samples for BM, 11 samples for SPL) mice; mRNA levels of BAFFR (**E**, left panel), CD40 (**E**, right panel), Pim2 (**F**, left panel), and A1 (**F**, right panel) relative to b_2-microglobulin. See Fig. 2-S4B-C for gating. All data are representative of at least two independent experiments. Error bars show SD. Statistical analysis was performed using Student's *t* test: * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

Ptpn22 risk variant expression alters B cell tolerance checkpoints at two stages.

The hen egg lysozyme (HEL) and anti-HEL (MD4) BCR transgenic mouse models have been used extensively to study negative selection of self-reactive B cells[49,110]. In these studies, MD4 B cells express high-affinity BCRs specific to the neo-self antigen, HEL. Upon development in membrane-bound HEL (mHEL) expressing mice, MD4 B cells receive a strong BCR signal and are deleted or undergo receptor editing before entry into the periphery[111,112]. In contrast, exposure to soluble HEL (sHEL) elicits a weaker BCR signal in MD4 B cells that allows them to enter the periphery, where they exhibit features of functional anergy, including down-regulation of surface IgM and an inability to secrete anti-HEL antibodies [48].

To evaluate whether the *Ptpn22* variant modulates either deletion or anergy of self-reactive B cells, we created mixed BM chimeras using $Ptpn22^{CC}$ MD4 Tg or $Ptpn22^{TT}$ MD4 Tg mice as donors for transplantation into lethally irradiated mHEL (Fig. 2-2A) and sHEL recipient mice (Fig. 2-2B). We found that $Ptpn22^{TT}$ MD4 Tg B cells developing in either mHEL or sHEL recipients exhibit comparable deletion (Fig. 2-2C-E, gating as in Fig. 2-S4D) and anergy (Fig. 2-2F-H) as their $Ptpn22^{CC}$ MD4 Tg counterparts.

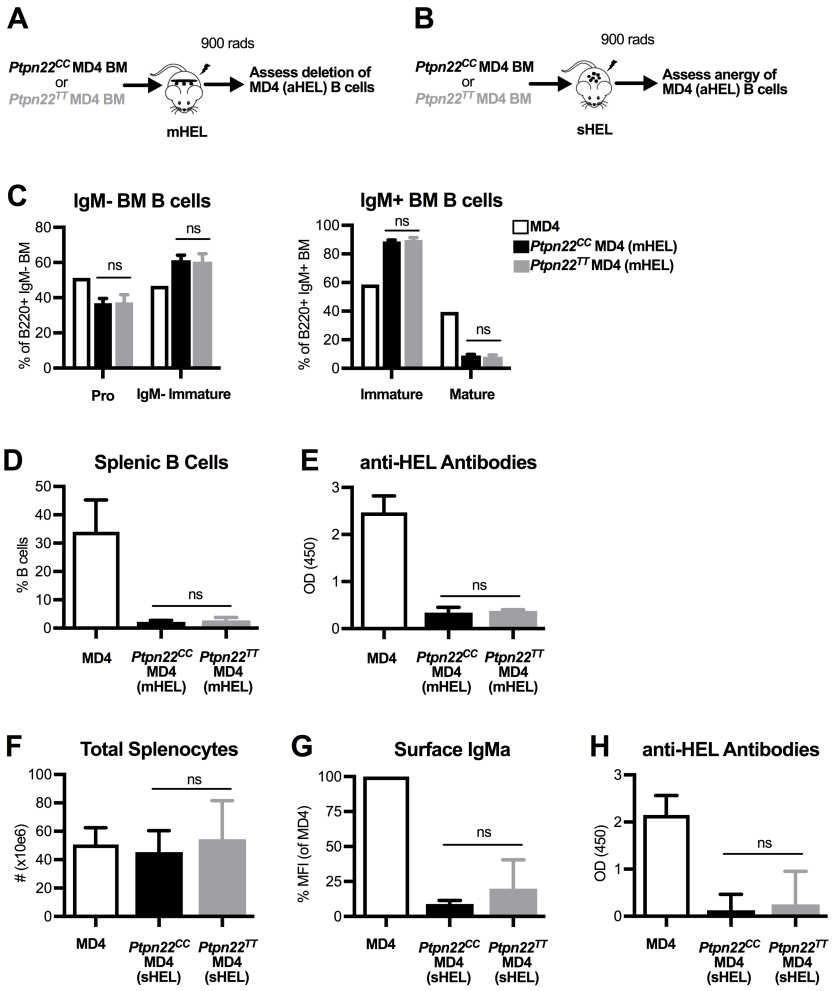

FIGURE 2-2. Intact negative selection of self-reactive *Ptpn22* variant B cells in a high-affinity BCR transgenic model. Mixed BM chimeras utilizing the donor MD4 (anti-HEL Ig transgenic) and either **(A)** membrane-bound (m) or **(B)** soluble (s) HEL recipients were used to assess deletion and anergy, respectively. **(C-E)** mHEL recipients reconstituted with either *Ptpn22*^{CC} MD4 (n=6) or *Ptpn22*^{TT} MD4 (n=6) BM were analyzed 2-3 months after transplant. Reconstitution of donor MD4 B cell subsets in the **(C)** BM and **(D)** SPL of mHEL recipients were analyzed by FACS. **(E)** HEL-specific serum antibody levels from mHEL recipients. **(F-H)** sHEL recipients reconstituted with either *Ptpn22*^{CC} MD4 (n= 12) or *Ptpn22*^{TT} MD4 (n= 12) BM were analyzed 2-3 months after transplant. **(F)** Reconstitution of donor MD4 B cells in the SPL of sHEL recipients. **(G)** MFI of MD4 anti-HEL transgene (IgMa) in sHEL recipients, gated on B220+ splenic B cells. **(H)** HEL-specific serum antibody levels from sHEL recipients. See Fig. 2-S4D. All data represents at least two independent experiments. Error bars show SD. Statistical analysis was performed using Student's *t* test.

While negative selection mechanisms thus appeared intact in *Ptpn22* variant animals, it remained possible that the subtly enhanced BCR signal in $Ptpn22^{TT}$ mice was masked by the high-affinity signal utilized in the HEL models (which express anti-HEL BCRs originally generated from post-germinal center cells). We therefore generated mixed BM chimeras to assess the development of $Ptpn22^{CC}$ versus $Ptpn22^{TT}$ variant B cells within a competitive, polyclonal setting. Congenically marked $Ptpn22^{CC}$ ($Ly^{5.1}$), $Ptpn22^{TT}$ ($Ly^{5.2}$), and B-cell deficient $Ptpn22^{CC}$ (μMT; $Ly^{5.1/5.2}$) donors were mixed at a 10:10:80 ratio for transplantation into lethally irradiated $Ptpn22^{CC}$ ($Ly^{5.1/5.2}$) recipients. The addition of μMT BM allowed us to assess B-intrinsic effects of *Ptpn22* variant expression on B cell selection, since the majority (~90%) of all non-B cells would express wildtype $Ptpn22^{CC}$, whereas B cells were mixed evenly between those expressing $Ptpn22^{CC}$ and $Ptpn22^{TT}$ (Fig. 2-3A).

Chimeras were sacrificed at 3 months post-transplant to evaluate the frequencies of $Ptpn22^{CC}$ ($Ly^{5.1}$) and $Ptpn22^{TT}$ ($Ly^{5.2}$) B cells at each stage of development. Equivalent levels of donor cell engraftment were observed in splenic monocytes based upon congenic marker expression (Fig. 2-3B-C). The Pre+Pro B cell fraction also exhibited equal contributions from each genotype, suggesting that variant expression does not alter early B cell development (Fig. 2-3B-C). In contrast, $Ptpn22^{TT}$ B cells exhibited a competitive advantage at the BM immature and peripheral early transitional (T1) and late transitional (T2) stages (Fig. 2-3B-C). Notably, while this competitive advantage was maintained in mature FM B cells, it was lost in MZ B cells (Fig. 2-3B-C). The enhanced competition at the immature/transitional stage, followed by discrepant competition into

FM vs. MZ B cell compartments, is consistent with a model whereby the *Ptpn22* risk variant alters B cell tolerance at two discrete checkpoints in the bone marrow and periphery.

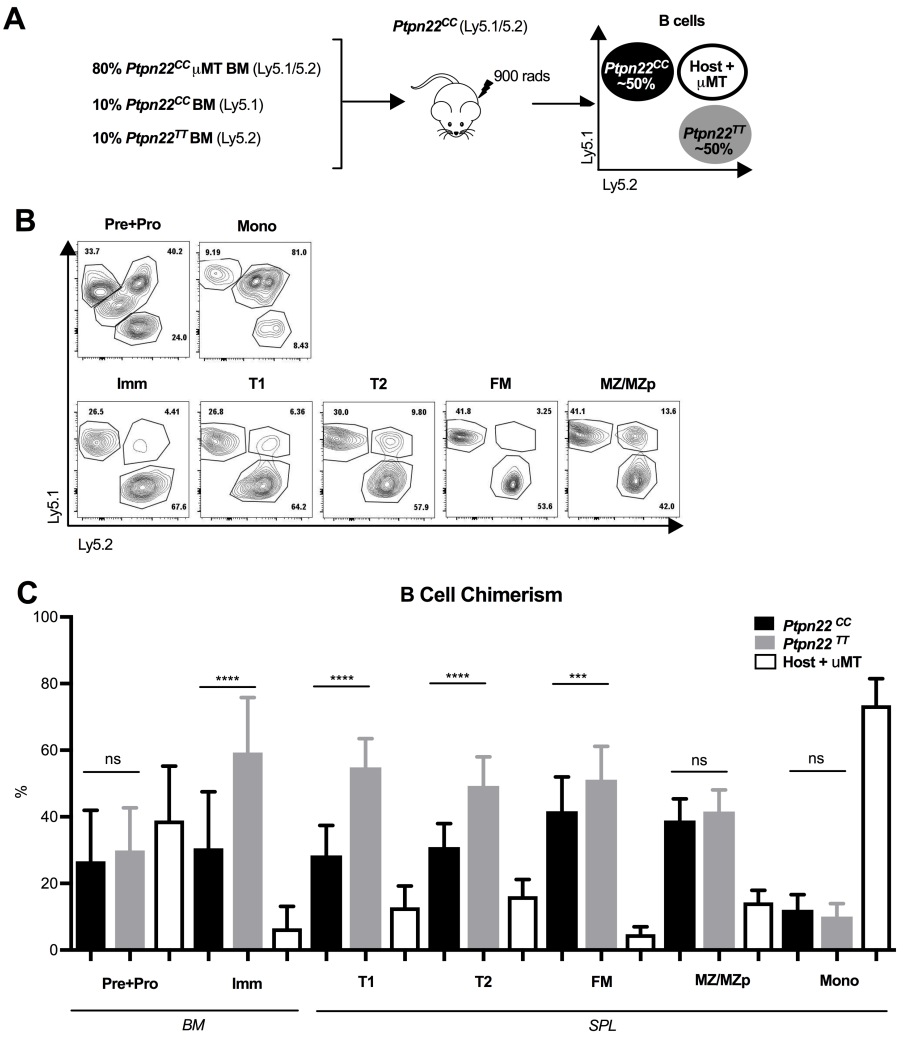

FIGURE 2-3. Competitive advantage of murine *Ptpn22* variant B cells in mixed BM chimeras. **(A)** B cell-intrinsic *Ptpn22*^{CC} and *Ptpn22*^{TT} competitive BM chimeras created by transfer of congenically marked 80% mMT (Ly 5.1/5.2) + 10% *Ptpn22*^{CC} (Ly5.1) + 10% *Ptpn22*^{TT} (Ly5.2) BM into lethally irradiated *Ptpn22*^{CC} mice (Ly5.1/5.2). Chimeras sacrificed three months after transplant (n=28). **(B)** Representative gating of B cell and splenic monocyte (B220^{-} CD3^{-} Gr1^{-} CD11b^{+}) subsets by FACS. **(C)** Frequency of *Ptpn22*^{CC} vs. *Ptpn22*^{TT} vs. host + mMT cells in each subset. Data represents five independent experiments. See Fig. 2-S4A-B for gating. Error bars show SD. Statistical analysis was performed using Student's *t* test: *** p<0.001; **** p<0.0001.

Ptpn22 variant selectively promotes B cells into the FM compartment.

To better understand the differential impact of *Ptpn22* risk variant expression on mature B cell subsets, we decided to test whether FM versus MZ cell fate was altered. Given the role that BCR signal strength has been proposed to play in determining cell fate[113], we predicted that the increased BCR signal in *Ptpn22*^{TT} mice might promote a preferential bias into the FM compartment. Previous studies in *Ptpn22* ^{TT} mice at >12 weeks of age (in a mixed 129/Sv and C57BL/6J background) revealed normal proportions of mature subsets[27], a finding reproduced in the current study in the C57BL/6J background (data not shown). Because the murine MZ B cell compartment is established during the initial ~12 weeks of life, we determined whether earlier assessment of splenic subsets in *Ptpn22*^{TT} animals might reveal subtle differences in FM vs. MZ fate. Indeed, comparing the proportions of splenic peripheral subsets during an 8.5-11 week interval of age, we observed a significant increase in FM and concomitant decrease in the proportion of marginal zone precursor (MZp) and MZ B cells in *Ptpn22*^{TT} mice compared to controls (Fig. 2-4A). Consistent with an alteration in Notch2 dependent signals required for MZ B cell development[114,115], analysis of MZ precursor cells sorted from *Ptpn22*^{CC} vs. *Ptpn22*^{TT} mice at 8.5 weeks of age revealed reduced Notch2 and Notch target gene transcript levels. In addition, a reduction in Hes1 transcripts was also observed in sorted T2 cells (Fig. 2-4B).

While these polyclonal studies suggested widespread selection bias, these data did not assess whether self-reactive B cells in particular were affected. To track the selection of B cells with self-reactivity relevant to an autoimmune disorder associated with the

PTPN22 risk variant, we crossed *Ptpn22*^{TT}mice to the 125 Tg model. In this model, ~95% of peripheral B cells express a fixed H and L chain (identified as IgMa+ by FACS) and are insulin-reactive [47]. Using FACs analysis of splenic B cell subsets, we found that *Ptpn22*^{TT} 125 Tg mice similarly exhibited a marked reduction in the proportion of insulin-reactive MZ B cells, and a subtle (though not statistically significant) increase in FM B cells compared to controls (Fig. 2-4C). Taken together with data demonstrating skewed mature compartments in a polyclonal setting, these findings support the idea that variant protein expression promotes antigen-mediated selection of self-reactive peripheral B cells, biasing their entry into the FM over MZ compartment.

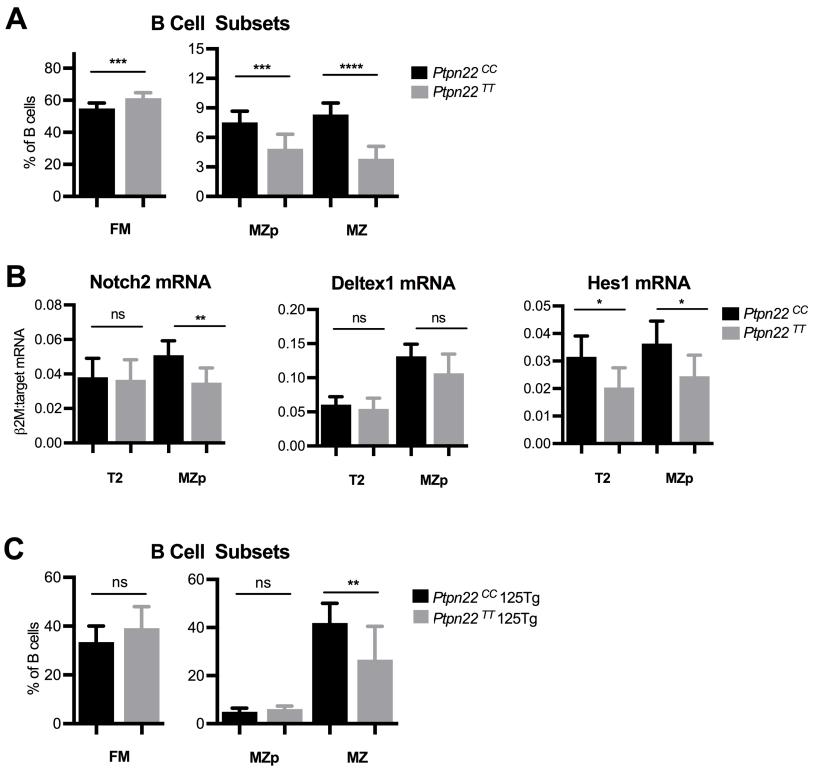

FIGURE 2-4. Preferential selection of murine *Ptpn22* variant B cells into FM compartment. **(A)** 8.5-11wk *Ptpn22*^{CC} (n=9) and *Ptpn22*^{TT} (n=10) mice were analyzed for splenic B cell subsets by FACS. **(B)** Quantitative PCR of B cell subsets sorted from 8.5wk *Ptpn22*^{CC} (n=6) and *Ptpn22*^{TT} (n=6) mice; mRNA levels of Notch2 (left panel), Deltex1 (center panel) and Hes1 (right panel) relative to b_2-microglobulin. **(C)** The 125 Tg (anti-insulin Ig transgenic) model was used to track insulin-specific B cells in the periphery. 9-16wk *Ptpn22*^{CC} 125 Tg (n= 11) and *Ptpn22*^{TT} 125 Tg (n=11) mice were analyzed for total splenic B cell subsets by FACS. All data represents at least two independent experiments. See Fig. 2-S4B for gating. Error bars show SD. Statistical analysis was performed using Student's *t* test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

FM and MZ naïve repertoires are differentially skewed in Ptpn22 variant mice.

Given the enhanced positive selection and differential selection of transitional cells into the FM and MZ compartments in *Ptpn22*^{TT} mice, we hypothesized that these findings might correlate with skewing of the naïve repertoires. We therefore performed a detailed assessment of the naïve repertoire of *Ptpn22*^{TT} mice using combined approaches: high throughput sequencing (HTS) of the BCR H chain, and single cell BCR cloning and assessment of BCR self-reactivity.

We first sought to validate IgH HTS as an appropriate platform for reading out distinct CDR3 profiles between FM and MZ subsets. While useful in many respects, prior sequencing studies have been limited by their restriction to a single VH family, gating strategies that included transitional cells, and/or analysis restricted to the BALB/c background[53,116,117]. To expand on these findings, we bulk sorted FM and MZ B cells from wildtype *Ptpn22*^{CC} mice on the C57BL/6 background for high-throughput sequencing of the IgH locus (obtaining a total of 120,805 – 142,910 productive sequences for each sample; Fig. 2-S1). Our studies revealed broadly altered differences in CDR3 length and composition between mature, naïve FM and MZ subsets (Fig. 2-5). Consistent with published data, we found that MZ B cells had increased usage for JH2 family (Fig. 5A), shorter average CDR3s (Fig. 2-5B), a greater proportion of CDR3s lacking N nucleotides (Fig. 2-5C), and slightly reduced hydrophobicity (Fig. 2-5D) compared to FMs. This high-throughput approach further revealed novel differences, including broadly altered VH family (Fig. 2-5E) and amino acid usage (Fig. 2-5F) between subsets, decreased N2 insertions (Fig. 2-5G), and reduced diversity (Fig. 2-5H) in MZ cells.

Notably, we found no difference in charged amino acid usage between subsets (data not shown), in contrast to prior studies[116,117].

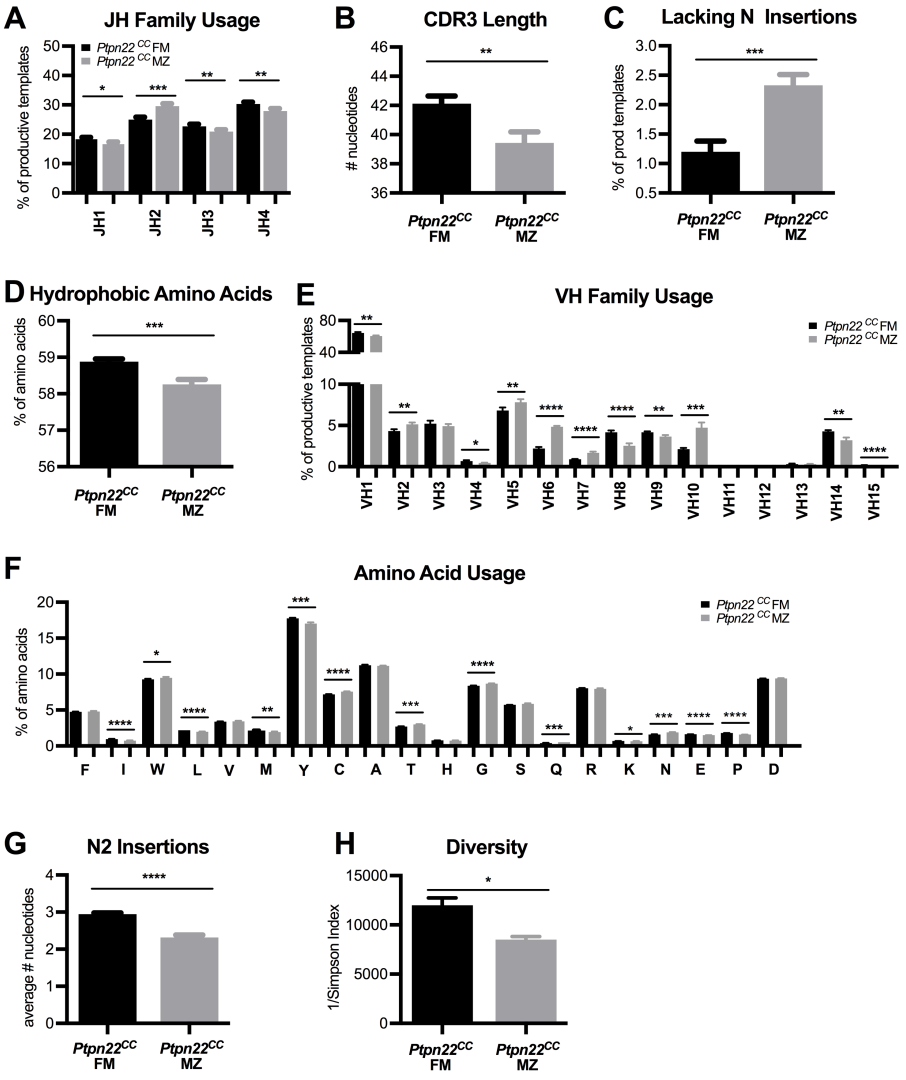

FIGURE 2-5. High-throughput sequencing reveals broadly altered CDR3 characteristics between murine FM and MZ subsets. Follicular (FM) and marginal zone (MZ) cells were sorted from 11-14wk *Ptpn22*^{CC} mice (n=4 per subset) for high throughput BCR IgH chain sequencing. Nonproductive templates were excluded from analysis. **(A)** JH family usage. **(B)** CDR3 length. **(C)** Templates lacking N insertions (between both V-D and D-J junctions). **(D)** Hydrophobic amino acids (includes: F, I, W, L, V, M, Y, C, A). **(E)** VH family usage. **(F)** Amino acid usage. **(G)** N2 insertions (between D-J junction). **(H)** Diversity index. See Fig. 2-S4B for gating. See Fig. 2-S1 for numbers of sequences analyzed. Data represents two independent experiments. Error bars show SD. Statistical analysis was performed using Student's *t* test: * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

Armed with an ability to read out distinct CDR3 profiles between mature, naïve subsets using IgH HTS, we next expanded our sequencing studies to include FM and MZ subsets sorted from *Ptpn22*^{TT} mice (obtaining a total of 119,459 – 142,910 productive sequences for each sample; Fig. 2-S1). Surprisingly, we found that the CDR3 profiles in naïve subsets derived from *Ptpn22* variant mice did not differ markedly from controls. We identified only minor differences in VH usage and N1 insertions in *Ptpn22*^{CC} vs. *Ptpn22*^{TT} MZ B cells (Fig. 2-6). Thus, while an HTS approach was suitable for identifying unique CDR3 characteristics between FM and MZ B cells, it was for the most part unable to detect any impact of the *Ptpn22* variant on the BCR repertoire.

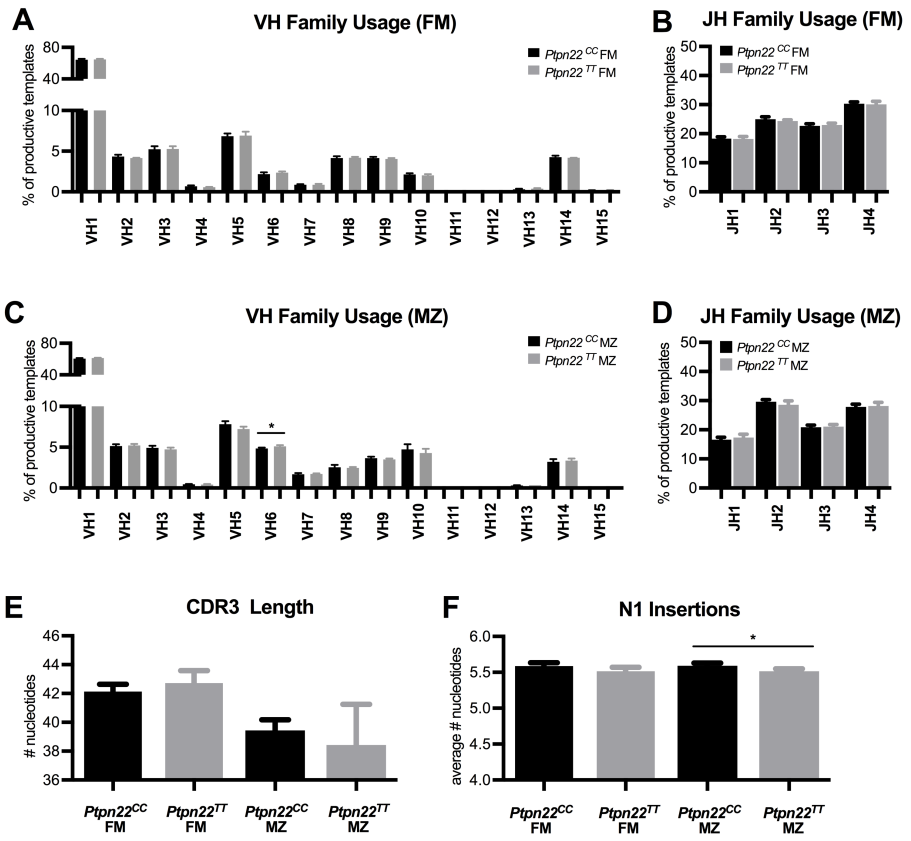

FIGURE 2-6. Control and murine *Ptpn22* variant FM and MZ B cells exhibit indistinguishable IgH CDR3 profiles. Follicular (FM) and marginal zone (MZ) subsets were FACS sorted from 11-14wk *Ptpn22*^{CC} (n=4) and *Ptpn22*^{TT} (n=4) mice for high throughput BCR IgH chain sequencing. Nonproductive templates were excluded from analysis. **(A)** FM VH and **(B)** FM JH family usage. **(C)** MZ VH and **(D)** MZ JH family usage. **(E)** CDR3 length. **(F)** N1 insertions (between V-D junction). See Fig. 2-S4B for gating. See Fig. 2-S1 for numbers of sequences analyzed. Data represents two independent experiments. Error bars show SD. Statistical analysis was performed using Student's *t* test: * $p<0.05$.

One possible explanation, supported by our polyclonal studies (Fig. 2-3 and Fig. 2-4), is that the *Ptpn22* variant impacts a broad range of BCR specificities, leading to a setting where individual CDR3 features might appear largely unchanged. Differences that may exist in a subpopulation, such as in self-reactive cells, could be lost in a high-throughput approach. Therefore, we next turned to single cell BCR cloning for a more targeted assessment of the naïve repertoire. Using established methods[99,102], BCRs were cloned from single FM and MZ cells sorted from $Ptpn22^{CC}$ and $Ptpn22^{TT}$ mice, producing a total of 273 recombinant mAbs. We assessed antigen specificity using ELISAs for a range of self-antigens, including insulin, malondialdehyde (MDA)-LDL, dsDNA, phosphorylcholine (PC-12) and smRNP. Strikingly, for each self-antigen tested, the proportion of $Ptpn22^{TT}$ mAbs considered reactive (reaching an OD threshold greater than 0.5) was approximately doubled in FM cells. In striking contrast, we observed the opposite impact in MZ B cells with the proportion of $Ptpn22^{TT}$ mAbs considered reactive reduced nearly in half compared to control mAbs (Fig. 2-7; ELISA curves in Fig. 2-S3C-D). The proportions of polyreactive clones exhibited similar differential skewing, with higher and lower proportions found in the FM and MZ compartments, respectively, of variant mice (Fig. 2-7). As predicted by our tolerance studies, we conclude that *Ptpn22* variant mice exhibit a subset-specific skewing of the naïve repertoire, with a preferential bias for self-reactive BCRs within the FM over MZ compartment.

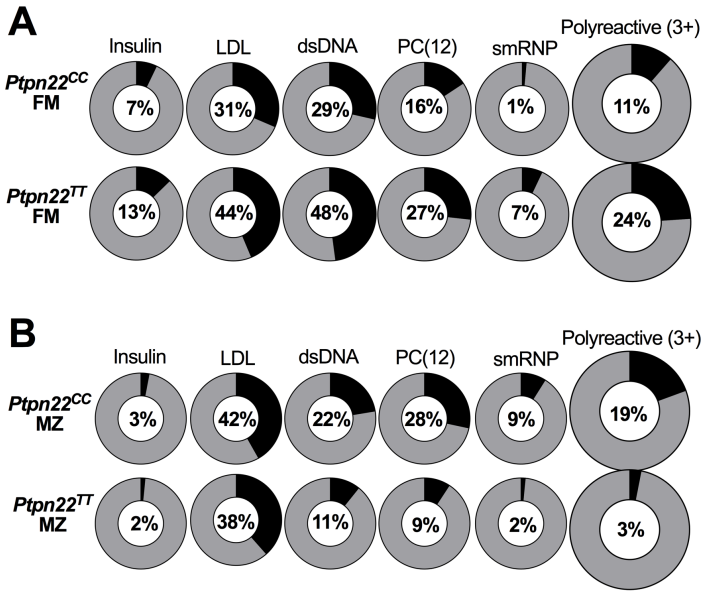

FIGURE 2-7. *Ptpn22* variant mice exhibit an increased proportion of self-reactive FM B cells but fewer self-reactive MZ B cells. Follicular (FM) and marginal zone (MZ) cells were FACS sorted from 8-12wk *Ptpn22*^{CC} (n=5) and *Ptpn22*^{TT} (n=5) mice for single cell BCR cloning of mAbs (n=70 *Ptpn22*^{CC} FM, n=71 *Ptpn22*^{TT} FM, n=67 *Ptpn22*^{CC} MZ, n=65 *Ptpn22*^{TT} MZ mAbs). Proportions of **(A)** FM and **(B)** MZ mAbs reactive to self-antigens (insulin, MDA-LDL, dsDNA, phosphorylcholine [PC-12], smRNP) or polyreactive (reactive to three or more of above self-antigens) by ELISA assay. See Fig. 2-S4B for gating and Fig. 2-S3C-D for ELISA curves. Data represents two independent experiments.

Carriers of the PTPN22 variant exhibit broadly enhanced positive selection.

The increased self-reactivity of the FM compartment in *Ptpn22*^{TT} mice closely mirrors the increase in self-reactivity observed in circulating naïve B cells of healthy human subjects with the *PTPN22* risk variant[97]. In an effort to better understand the signaling events that drive the selection of autoreactive B cells in *PTPN22* variant carriers, we turned to a flow based assay based on tracking of a self-reactive heavy chain (HC). We chose the VH4-34 family as a candidate for study due to its well-documented polyreactivity towards a range of self-antigens including B cells, RBCs, and dsDNA[118-120], as well as its relatively high frequency within the transitional and naïve mature B cell compartments in healthy subjects. In addition, previous work from our laboratory and others have documented enrichment of VH4-34 family expressing transitional and mature B cells in autoimmune settings, thought to be driven through modulations in dual BCR and TLR signals[101,121]. Based on these previous studies, we anticipated that healthy *PTPN22* risk variant carriers might exhibit an increased proportion of VH4-34^{+} B cells (as detected by the anti-idiotypic mAb, 9G4), as would be consistent with a naïve repertoire skewed towards autoreactivity prior to disease development. Surprisingly, in contrast to this prediction, we found that healthy *PTPN22* risk subjects had fewer 9G4^{+} B cells across all peripheral blood compartments compared to healthy non-risk controls, including most notably within the transitional subset (Fig. 2-8A-B; cells gated as in Fig. 2-S4E).

One possible explanation for the reduced frequency of 9G4+ B cells in *PTPN22* risk variant subjects, supported by our murine studies, is that globally enhanced positive

selection across multiple specificities might reduce the relative proportion of BCRs utilizing this specific HC family. In partial support of this idea, CD40 levels and signaling activity were previously reported to be increased in *PTPN22* risk variant subjects[97]. To test whether BAFFR levels were similarly increased, and might reflect events similar to our observations in the murine model, we next compared surface BAFFR levels in *PTPN22* non-risk vs. risk subjects. Strikingly, the MFI of BAFFR expression was increased in all peripheral B cell subsets in risk subjects (Fig. 2-8C). The elevated BAFFR levels in risk subjects were unlikely to reflect differences in available cytokine, as we have previously reported that non-risk and risk subjects exhibit similar serum levels of BAFF[96] However, it remained possible that the reduced frequency of self-reactive 9G4+ B cells in risk subjects might reflect an impaired ability compete for available BAFF. To test for this possibility, we compared surface BAFFR levels among $9G4^+$ (representing predominantly self-reactive BCRs) or $9G4^-$ peripheral B cell subsets (representing a more heterogeneous population) in both non-risk and risk subjects. Strikingly, relative to non-risk subjects, $PTPN22^{CT}$ subjects exhibited a global increase (or trend for increase) in BAFFR levels across all populations, including $9G4^-$ and $9G4^+$ cells within the transitional, naive and IgM memory B cell compartments (Fig. 2-8D). Overall, these data imply that in risk subjects, BCR and/or other coordinate signals promote increased BAFFR expression, thereby permitting such cells to compete more effectively for BAFF family survival and differentiation signals.

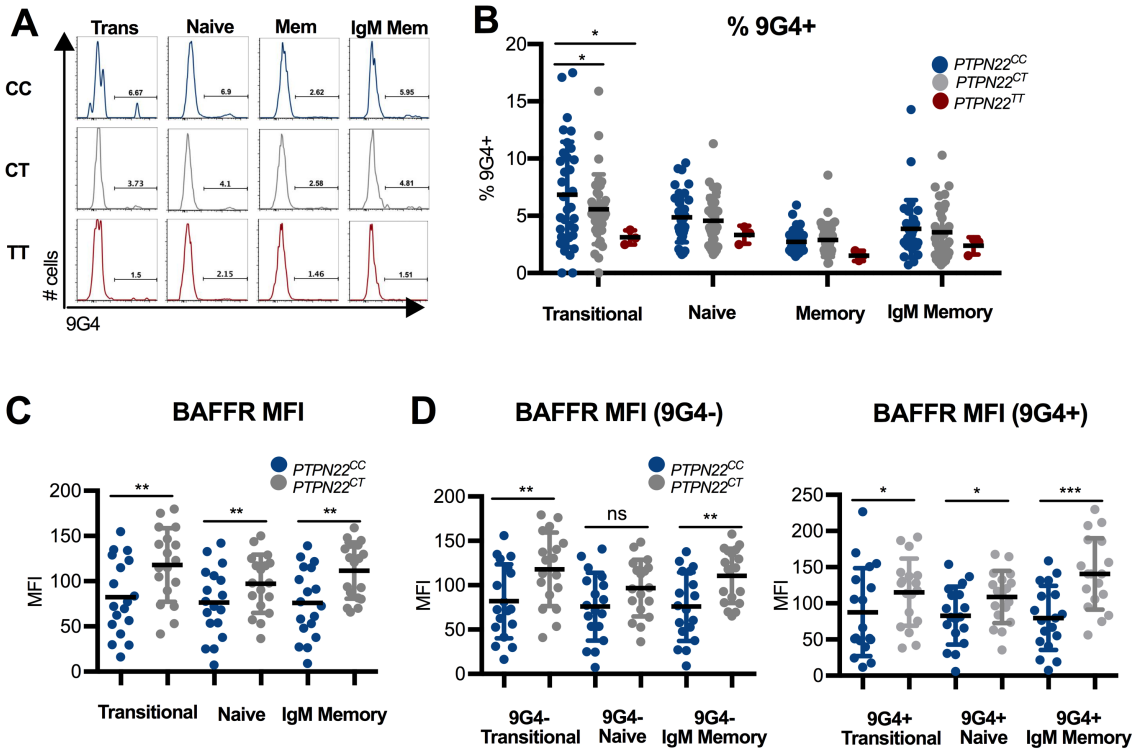

FIGURE 2-8. Healthy subjects with the *PTPN22* risk variant exhibit broadly enhanced positive selection. (A-B) PBMCs from age and sex-matched healthy subjects screened for *PTPN22* 1858 C/C (n=34), C/T (n=34), and T/T (n=3) genotypes were analyzed by FACS for VH4-34 family (9G4+) B cells. **(A)** Representative histograms quantifying 9G4+ B cells. **(B)** Percentage of 9G4+ cells across peripheral B cell subsets. **(C-D)** PBMCs from age and sex-matched healthy subjects screened for *PTPN22* 1858 C/C (n=18) and C/T (n=18) genotypes were analyzed by FACS for surface BAFFR MFI of total B cells **(C)** and 9G4- and 9G4+ gated B cells **(D).** See Fig. 2-S4E for gating. See Fig. 2-S2 for subject information. All data represents at least two independent experiments. Error bars show SD. Statistical analysis was performed using Student's *t* test (B) or paired *t* test (C): * $p<0.05$; ** $p<0.01$.

Discussion

Our findings in the *Ptpn22*^{TT} murine models indicate that while negative selection mechanisms are intact, enhanced BCR and/or co-receptor signaling programs promote greater positive selection of self-reactive, follicular mature B cells. Moreover, the counter selection of VH4-34+ expressing cells in human carriers, coupled with global increases in BAFFR and CD40 co-receptor expression described here and in a previous study[97], respectively, support a similar role for the risk variant in human B cell selection. Our combined datasets imply that the risk variant facilitates a subtle yet widespread increase in positive selection signaling programs throughout BM and transitional B cell development. These combined events ultimately allows for a *broader* range of autoreactive B cells to compete for survival into the mature, naïve B cell compartment. While additional work is required to definitively assess the specific biochemical impact(s) of *PTPN22-C1858T* variant on BCR and/or co-receptor signaling pathways in developing B cells, our findings strongly suggest that similar mechanisms alter central and peripheral B cell tolerance in both murine and human immune development (Fig. 2-9).

FIGURE 2-9. Summary of human and murine B cell phenotypes in *PTPN22* risk variant settings.

Phenotype	Human *PTPN22* risk variant carriers	Murine *Ptpn22* risk variant models
Signs of autoimmunity	- Increased risk for autoimmune diseases	- Splenomegaly (129/BL6) - Increased tissue pathology (129/BL6) - Spontaneous GCs (129/BL6) - Increased auto-antibodies (129/BL6)
Evidence of a role for B cells in disease	- Presence of pathogenic auto-antibodies	- B-restricted varient expression recapitulates many autoimmune phenotypes (129/BL6)
Evidence of altered B cell tolerance	- Increased proportion of early emigrant B cells - Increased frequency of self-reactive B cells at early emigrant and mature naïve stages - Unknown reactivity of splenic MZ cells	- Increased proportion of transitional B cells (129/BL6) - Competitive advantage of developing B cells at BM and peripheral checkpoints (BL6) - Increased frequency of self-reactive B cells at FM stage; decreased frequency at MZ stage (BL6)
Evidence of altered selection into mature compartments	- Unknown	- Increased proportion of FM cells; decreased proportion of MZ cells (BL6)
BCR signals	- Hypo-responsive yet with paradoxical upregulation of BCR target genes	- Hyper-responsive (129/BL6; BL6)
CD40 signals	- Enhanced signaling program	- Enhanced signaling program (BL6)
BAFFR signals	- Enhanced surface BAFFR expression; signaling program unknown	- Enhanced signaling program (BL6)
Current working model	***Enhanced BCR and co-receptor signals in PTPN22/Ptpn22 variant B cells leads to broadly enhanced positive selection and an enrichment of autoreactive specificities into the FM compartment***	

A summary of the evidence for altered B cell tolerance in human carriers of the *PTPN22* variant (ref 13-16, 19, 27, 95, 97) and in a murine *Ptpn22* variant KI model in the mixed 129/BL6 background (ref 96). Both human and murine results from the current study (BL6 background) are also depicted.

As described earlier, multiple studies in murine models and human subjects support the idea that *PTPN22* variant alters B cell tolerance mechanisms[27,96,97]. However, it remained unknown whether this defect was B cell-intrinsic. To eliminate the potential impact of disrupted T cell tolerance and homeostasis present in *Ptpn22* variant mice and human subjects[21,27,122], our BM chimera studies restricted variant expression predominantly to B cells. Our finding that *Ptpn22*^{TT} expressing B cells exhibit a competitive advantage at key developmental checkpoints in the bone marrow and periphery highlight the importance of B cell-intrinsic signaling pathways in regulating these events. Consistent with this, we found an identical competitive advantage for variant expressing B cells in competitive chimeras depleted of CD4 T cells (data not shown).

To better address the key question regarding how the *Ptpn22* risk variant alters BCR signaling in murine B cells, we utilized the newly described Nur77-GFP model[103]. This model has the advantage of permitting assessment of BCR signaling directly *ex vivo* at discrete stages of B cell development. Our results indicate that risk variant expression promotes a greater proportion of B cells to survive tolerance mechanisms throughout development, mediated in part through stronger BCR signals. Taken as a whole, these data provide compelling evidence that the *Ptpn22* variant modulates key survival programs dependent upon BCR signals *in vivo*, and that engagement with self-antigen in the periphery can promote these events.

Although BCR signaling serves as a master regulator of B cell tolerance, its synergy with key co-receptor pathways, predominantly BAFFR and CD40, ultimately determines the

developmental fate of a given B cell[104,123]. Each of these co-receptor pathways, in turn, play an important role in promoting positive selection in the periphery[72,78,98,99]. In addition, a growing body of work, including our own, suggests they serve a similar function in the bone marrow[87,91,99,124]. Our finding that *Ptpn22*^{TT} mice exhibit slightly augmented BCR and co-receptor signals is consistent with subtle signaling changes whereby the variant promotes greater positive selection throughout immature and naïve B cell development.

While the complexity of receptor crosstalk[104] prevented us from fully dissecting the relative contributions of the BCR versus co-receptor signals in mediating selection, our studies demonstrate how subtle fluctuations in these signaling networks can influence the mature repertoire, and may do so in distinct ways. For instance, enhanced positive selection observed in *Ptpn22* risk variant mice differs from other autoimmune settings. Unlike models in which a transgenic self-reactive BCR[125] or a specific, self-reactive BCR family[101] facilitates positive selection at the T2 stage via clonal expansion, the *Ptpn22* risk variant instead impacts a broad range of BCR specificities during BM and splenic transitional B cell development, allowing multiple self-reactive B cells to compete for BAFF and CD40L signals. Thus, while highly specific VH family skewing is evident in both mice and humans with defects in the Wiskott-Aldrich syndrome (*WAS*) gene – an autoimmune setting of augmented BCR and TLR signals –[101], we did not observe major differences in either IgH CDR3 profiles or VH family use in *Ptpn22*^{TT} mature B cells. We further propose that the broad impact of the *Ptpn22* variant limited the relative expansion of individual (including self-reactive) VH families. Although the

implication of these subtle distinctions in dual BCR/TLR vs. BCR/BAFFR/CD40-mediated positive selection in distinct models remains to be seen (including implications likely beyond autoimmunity), they nevertheless illustrate the usefulness of our integrated approach in assessing the naïve repertoire.

This integrated approach also helped us identify an unexpected bias for selection of self-reactive cells specifically within the FM compartment of *Ptpn22*^{TT} mice. Several lines of evidence led to this idea: First, a larger proportion of FM relative to MZ cells was observed in both polyclonal and insulin-specific *Ptpn22*^{TT} murine models, and marginal zone precursors exhibited a significant decrease in Notch2 expression and in Notch target gene transcripts. Secondly, and consistent with a preferential bias for selection into the FM compartment, our single cell BCR cloning studies revealed an increased proportion of self-reactive BCRs within the FM compartment. In parallel, we observed the opposite finding in the MZ with decreases in both self-reactive and polyreactive specificities in individual MZ B cells. These observations support the conclusion that the *Ptpn22* risk variant skews the naïve FM B cell compartment towards self-reactivity, while revealing an additional novel role for restricting MZ development and/or fate. While the mechanistic basis for these surprising observations with respect to MZ B cell development remain unclear, these findings align with recent studies in which BCR and Notch2 signaling exhibit crosstalk that critically regulates MZ lineage commitment[126].

Notably, while we were unable to directly study human splenic B cell subsets, we made progress in translating our murine findings to human subjects. Our observations provide

an alternative interpretation of the prevailing model for how the *PTPN22* variant impacts human B cell development. As noted above, previous human studies suggest that *PTPN22-C1858T* carriers exhibit an attenuated BCR signal, promoting relaxed negative selection and subsequent enrichment of self-reactive B cells within the naïve compartment[24,96,97,127]. Interestingly, these previous studies have proposed that hypo-responsive BCR signals in risk subjects leads to *both* increased numbers of self-reactive, new-emigrant (transitional) B cells[96,127], and a seemingly paradoxical aberrant activation of these cells, as demonstrated by elevated BCR target genes involved in cell activation, proliferation, and survival[97]. Most notably, previous work described higher levels of both CD40 transcripts and expression and increased CD40 signaling in risk variant new emigrant B cells[97]. Thus, while the interpretation differs from our conclusions, these earlier findings indicate that healthy subjects with the risk allele exhibit an increase in the CD40 co-receptor program in transitional B cells. Consistent with these observations, our murine studies revealed intact negative selection and evidence for augmented BCR and/or BAFFR and CD40 co-receptor signals in *Ptpn22* variant mice, leading to broadly enhanced positive selection and preferential skewing for self-reactivity within the FM compartment. Thus, while the end result appears the same, explanations for *how* a greater proportion of self-reactive B cells enter the naïve repertoire differ with respect to previous human and our current murine studies.

As a means to begin to test whether our findings of broadly enhanced positive selection in murine models also applied to human carriers, we utilized a flow-based assay to track the selection of a polyreactive VH family in peripheral blood (VH4-34; identified as

9G4+)[121]. Our observation that healthy *PTPN22* variant subjects had fewer $9G4^+$ B cells, yet exhibited increased BAFFR levels in all peripheral B subsets, including both $9G4^+$ and $9G4^-$ cell populations, is most consistent with a model whereby positive selection is globally enhanced, thereby reducing the relative contribution of this single family VH family. Finally, an additional (but not mutually exclusive) interpretation for the reduced proportion of $9G4^+$ B cells in *PTPN22* carriers, is that this may reflect impaired MZ fate/development – an untested yet intriguing possibility given our murine findings of impaired Notch2 signaling, as well as the presumed enrichment of 9G4+ B cells into an MZ-like B cell subset within human peripheral blood[101,121].

FIGURE 2-S1. High-throughput sequence data.

Genotype	Subset	# Productive Sequences
Ptpn22^{CC}	FM	142910
Ptpn22^{CC}	MZ	120805
Ptpn22^{TT}	FM	142757
Ptpn22^{TT}	MZ	119459

Total productive IgH sequences obtained from murine bulk sorted splenic FM (B220+ CD24mid CD21mid) and MZ (B220+ CD24hi CD21hi CD23-) subsets from *Ptpn22*^{CC} and *Ptpn22*^{TT} mice.

FIGURE 2-S2. *PTPN22* screen controls information.

Subject	Age	Sex	% T	% N	% M	% IgM M
CC 1	23	F	5.81	92.4	5.65	31.7
CC 2	23	F	4.56	81.6	14.6	72.1
CC 3	25	M	3.49	83.2	12.7	32.6
CC 4	26	M	2.7	84.7	9.84	41.7
CC 5	27	M	2.74	82.5	13.3	32.1
CC 6	28	F	8.44	85	12.6	67.8
CC 7	28	M	2.99	78.8	17	50.8
CC 8	30	F	2.04	79.5	16.3	41.2
CC 9	31	F	6.26	86.8	11.2	49.9
CC 10	32	F	1.15	91.9	5.9	40.7
CC 11	32	F	0.37	85.9	9.4	31.9
CC 12	32	F	3.38	88.8	8.11	20.6
CC 13	33	M	2.36	71.4	24.7	36.6
CC 14	34	F	3.77	62.3	32.4	31.1
CC 15	34	M	6.61	68.6	27.8	42.2
CC 16	35	F	6.06	69.6	27.6	70.4
CC 17	36	M	6.1	65.1	31.7	55.1
CC 18	36	F	4.3	59.9	35.8	34.9
CC 19	36	M	5.35	66.6	29.6	24.1
CC 20	37	F	5.42	81	16.2	52.5
CC 21	38	M	3	73.1	22.3	57.5
CC 22	38	F	3.92	68.3	28.5	39.4
CC 23	39	F	1.18	54.2	39.2	74.7
CC 24	40	F	2.64	61.2	34.9	56.6
CC 25	42	F	2.63	61.6	31.8	70.7
CC 26	42	F	4.01	78.2	18.1	52.7
CC 27	44	F	4.7	78.2	17.9	58.3
CC 28	50	F	9.17	92	6.66	42
CC 29	54	F	3.34	72.9	23.7	44.3
CC 30	55	F	2.19	53.5	38.3	44.1
CC 31	56	M	8.58	87.7	11	42.2
CC 32	57	M	0.81	59.6	32.9	82.2
CC 33	63	F	9.11	86.8	11.4	29.4
CC 34	63	F	18.9	68.4	27.1	38.8
CC 35	67	M	9.76	62.4	33.9	44.3
Average	*39.03*		*4.80*	*74.96*	*21.14*	*46.78*
SEM	*2.59*		*0.59*	*1.94*	*1.76*	*2.58*

Subject	Age	Sex	% T	% N	% M	% IgM M
CT 1	19	F	0.55	89	7.18	49.1
CT 2	21	F	3.77	94.8	3.13	30.1
CT 3	25	M	6.21	81.7	13.6	54.4
CT 4	28	M	4.89	87.9	8.62	87.6
CT 5	27	M	1.88	63.2	31.3	25.6
CT 6	23	F	2.62	72.9	22.5	72.6
CT 7	30	M	0.82	76.5	17.9	58.6
CT 8	29	F	2.48	83.3	12.7	43.1
CT 9	30	F	1.28	77.2	19.5	37.5
CT 10	30	F	8.35	81.8	14	54
CT 11	37	F	1.31	75.8	18	33
CT 12	32	F	1.71	74.4	21.2	35.3
CT 13	32	M	4.69	85.4	12.1	30.4
CT 14	34	F	3.2	71.8	24	29.4
CT 15	34	M	4.73	79.1	17.2	65.7
CT 16	35	F	4.46	78.8	16.8	60.9
CT 17	33	M	4.83	84.9	12	68.3
CT 18	36	F	3.44	70.7	24.7	55.7
CT 19	36	M	4.09	60.2	36	29.8
CT 20	36	F	6.59	81.4	15.8	75
CT 21	38	M	2.07	76.8	18.5	58.9
CT 22	39	F	2.13	79.6	15.4	71.4
CT 23	39	F	16.7	83.3	14	71.2
CT 24	39	F	2.92	62.7	32.6	50.1
CT 25	42	F	7.86	83.4	12.5	36.2
CT 26	42	F	2.89	74.1	21.9	37.4
CT 27	46	F	6.34	62.5	31.9	53.5
CT 28	49	F	7.08	78.6	16.7	47
CT 29	53	F	6.74	93.2	5.61	38.7
CT 30	56	F	4.18	88.7	9.82	55.4
CT 31	55	M	2.39	77.6	16.1	58.2
CT 32	56	M	1.14	66	29	24.7
CT 33	63	F	7.55	75.9	21.2	25
CT 34	63	F	5.27	82.2	14.8	57
CT 35	68	M	4.97	74	20.8	39.4
Average	*38.71*		*4.35*	*77.98*	*17.97*	*49.15*
SEM	*3.1*		*0.51*	*1.42*	*1.28*	*2.74*

Subject	Age	Sex	% T	% N	% M	% IgM M
TT 1	31	F	2.9	69.8	14.1	11.0
TT 2	45	F	5.0	61.2	24.4	23.5
TT 3	35	M	2.1	56.6	20.4	9.1
Average	*37.00*		*3.33*	*62.53*	*19.63*	*14.54*
SEM	*4.16*		*0.88*	*3.87*	*3.00*	*4.51*

Human peripheral blood subsets defined as Transitional (% CD24+ CD38+ of CD19+ B cells); Naive (% CD27- of CD19+ B cells); Memory (% CD27+ of CD19+ B cells); IgM Memory (% IgM+ of CD19+ CD27+ cells)

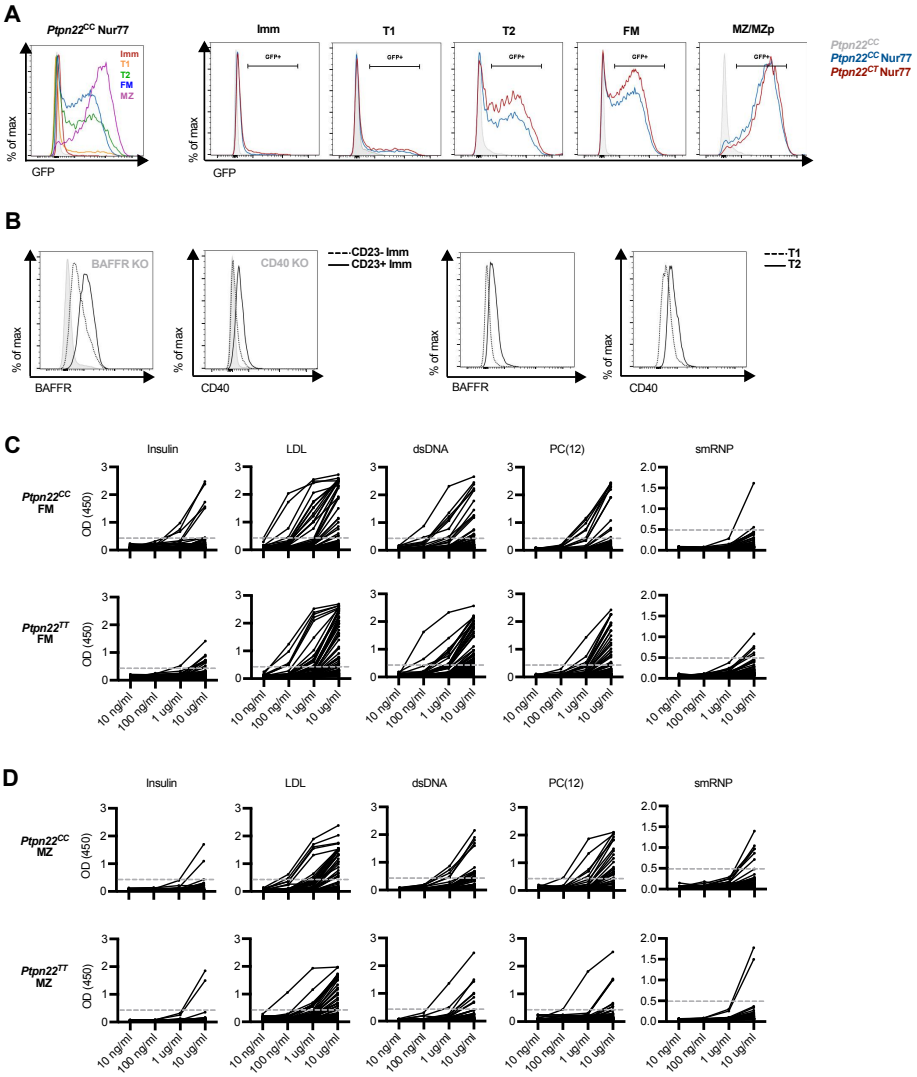

FIGURE 2-S3. BCR and co-receptor pathways during development and self-reactivity of naïve compartment. (A) Representative GFP expression of BM and SPL B cell subsets in *Ptpn22*^{CC} Nur77 and *Ptpn22*^{CT} Nur77 mice as described in Fig. 2-1A. See Fig. 2-S4A-B for gating. **(B)** Representative surface BAFFR and CD40 expression of BM (left panel) and SPL (right panel) B cells in *Ptpn22*^{CC} mice as described in Fig. 2-1. BAFFR and CD40 knockout mice used as negative staining controls and are depicted by filled gray histogram. Dotted lines represent CD23^{-} immature and T1 cells. Solid lines represent CD23^{+} immature and T2 cells. See Fig. 2-S4B-C for gating. **(C-D)** OD (450) values of self-antigen ELISA assays run using FM **(C)** and MZ **(D)** mAbs cloned from *Ptpn22*^{CC} and *Ptpn22*^{TT} mice as described in Fig. 2-7.

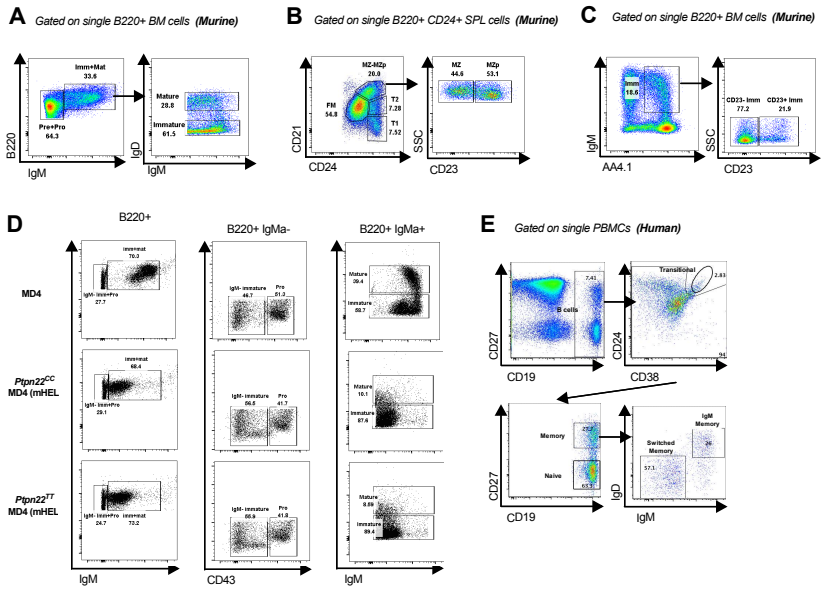

FIGURE 2-S4. Representative gating used for analysis and/or cell sorting of murine and human B cell subsets. (A) Murine bone marrow B cell gating used in Fig. 2-1(A), Fig. 2-2, Fig. 2-3, and Fig. 2-S3A. **(B)** Murine splenic B cell gating used in Fig. 2-1(A, C, E, F), Fig. 2-3, Fig. 2-4, Fig. 2-5, Fig. 2-6, Fig. 2-7, and Fig. 2-S3A-B. **(C)** Murine bone marrow B cell gating used in Fig. 2-1(C, E, F) and Fig. 2-S3B. **(D)** Representative murine BM B cell gating of MD4/mHEL chimeras as described in Fig. 2-2C. **(E)** Human PBMC B cell gating used in Fig. 2-8.

CHAPTER 3:

Concluding Remarks

Our collective murine and human data provides an alternative model for how the *PTPN22-C1858T* variant promotes self-reactivity into the naïve B cell repertoire, via broadly enhanced positive selection throughout development. The further unexpected bias for selection of self-reactivity in the FM compartment consequently increases the probability of triggering germinal center autoimmune B cell responses in at-risk individuals. These studies highlight the importance of synergistic BCR and co-receptor signaling pathways in regulating these events, and in doing so, identifies novel pathways for future study.

Outside of autoimmunity, our results also provide additional clues into how *PTPN22-C1858T* variant may interface with infectious diseases. In addition to its well-established association with autoimmunity, several studies link variant expression to *Mycobacterium tuberculosis* resistance[128]. Since IFNs provide anti-inflammatory effects during *M. tuberculosis* infection[129,130], it has been proposed that the blunted type I interferon (IFN) response of *PTPN22* variant-expressing myeloid cells may be the reason for this enhanced protection [22]. However, given the established importance of T and B functions in controlling *M. tuberculosis* infection[131,132], it is also possible that *PTPN22* variant functions in lymphocytes to improve the adaptive immune response. Interestingly, our findings that *Ptpn22* variant mice have augmented BCR signals and a larger proportion of follicular B cells is consistent with the idea that variant expression may enhance B:T

interactions, leading to possibly higher affinity antibodies and/or (T) cell-mediated immunity towards *M. tuberculosis*. Future studies assessing the breadth of antigen specificities (outside of self-reactivity) in *Ptpn22* variant mice should help us better understand the role of B-intrinsic variant expression on controlling this and other infections.

Similarly, our novel MZ findings provide a new context for understanding the surprising linkage between *PTPN22* variant expression and susceptibility towards *Streptococcus pneumoniae* infection[133]. Innate-like MZ B cells are thought to play a crucial role in providing protection against encapsulated bacteria such as *S. pneumoniae*[134,135]. Our evidence for impaired MZ B cell development in murine *Ptpn22* variant models would then predict defective innate-like immune responses. Further consistent with this idea, is the reduced frequency of VH4-34 cells we observed in human *PTPN22* variant subjects - notably, these cells were recently linked to control of pathogenic commensals[136]. Future mechanistic studies exploring BCR and Notch signaling in our murine studies, combined with parallel studies of the MZ in human splenic samples, would help us better understand the molecular underpinnings of this crosstalk and its implications for infectious disease.

In summary, while the primary focus of these studies has been to better understand the autoimmune-associated functions of *PTPN22-C1858T* variant for the purpose of designing more targeted therapies, we believe that a full understanding of its widespread and confounding persistence in the global population will require an integrated approach

assessing its dual role in controlling infectious diseases. The intriguing breadth of disease associations, widespread cellular expression, and certainly tmany pertinent human and animal models available, makes this (admittedly biased) researcher think there is likely no variant more pertinent for addressing the deceptively simple question: why do autoreactive cells exist?

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