The Function of *PTPN22* and the Autoimmune Risk Variant LypW in Immune Responses to Vaccination

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Dedication

I dedicate my thesis to my husband, Jose Gomez Garcia, who was an incredible source of support throughout my Ph.D. I would also like to dedicate this body of work to my Creater and Lord, Jesus Christ, who gave me a curiosity for uncovering the mysteries and the workings of biology, and who strengthened and kept me during this arduous but joyful journey.

Table of Contents

List of Tables

List of Figures

List of Figures (cont.)

Chapter 1: Introduction

PTPN22 and the autoimmune-associated SNP variant C1858T

PTPN22, or Protein Tyrosine Phosphatase Non-receptor 22, is a gene expressed exclusively in cells of the hematopoietic lineage (1). Early expression studies noted a high level of expression in the thymus and the spleen, (2) prompting studies of the role of the gene in T cell biology. Its structure is characterized by a phosphatase domain at the N-terminus and four C-terminal PEST domains (defined as proline, glutamic/aspartic acid, serine, threonine) named P1-P4 (1). These PEST domains mediate protein-protein binding interactions. Early studies of PTPN22 found that the P1 domain interacts with the SH3 domain of CSK, a major negative regulator of T cell antigen receptor signaling (3,4). Studies in T cells determined substrates for the catalytic phosphatase domain of *PTPN22* were important mediators of T cell receptor (TCR) signaling. Those include LCK, CD3ε, CD3ζ, ZAP-70, Fyn, VAV and the ATPase VCP (4,5). The dephosphorylation of these TCR signaling mediators led to the conclusion that *PTPN22* acts as a repressor of TCR signal strength. Studies using genetic knockdown and phosphatase inhibition of *PTPN22* confirmed this finding, showing increased phosphorylation of CD3ζ, ZAP-70 and SLP76 in stimulated T cells after *PTPN22* depletion or inhibition (6,7). Generation of *Ptpn22* knockout (KO) mice and studies of TCR signaling revealed increased phosphorylation of LCK on tyrosine 394, as well as increased [Ca^{2+}]

flux after CD3/CD28 stimulation (8–10). These data confirmed the conclusion that *PTPN22* acts to repress TCR signaling.

At the same time that the function of *PTPN22* as a TCR signaling repressor was reported in *Ptpn22* KO animals, the discovery and association was made between a single nucleotide polymorphism (SNP) in exon 14 of *PTPN22* in humans and increased risk of autoimmune disorders. The C1858T SNP in *PTPN22* is a missense mutation, resulting in the substitution of a tryptophan for an arginine in the amino acid sequence (termed R620W) (11–13). The frequency of this SNP ranges widely, with Caucasians of Scandinavian and Northern European descent representing the highest frequency (11-15%), Western Europeans having a slightly lower frequency (7-8%) and a much diminished frequency of the allele in Southern and Mediterranean Europe (2-2.5%) (14). The R620W variant has been associated with increased risk of bacterial infections of Gram-positive cocci (15), while studies of Tuberculosis (TB) patients associate the SNP variant with conferring protection against *Mycobacterium tuberculosis* infection (16,17). These studies suggest that the role of *PTPN22* in protection against infection is complex, and disease outcomes will likely depend on the specific pathogen. It is also interesting to speculate that the geographic stratification of the frequency of the R620W variant in Europe could be due to some selective pressure, such as TB infection itself. While there is no data to confirm this hypothesis directly, a study of the TB infection rate in England and Wales during the 19th century attributed the decline of TB in part due to natural

selection (18). Thus it is possible that TB could have served as a selective pressure to enrich the carriage rate of the R620W variant in Northern and Western Europe, although such a hypothesis is purely speculative at this time.

In addition to infectious disease, *PTPN22* has important roles in the development of autoimmune diseases. The R620W variant was shown by three separate groups to associate with Type 1 diabetes (T1D) (11), Rheumatoid Arthritis (RA) (12) and Systemic Lupus Erythematosus (SLE) (13). Genome Wide Association studies (GWAS) confirmed the link between the *PTPN22* R620W allele and increased risk of T1D, RA (19) and SLE (20). These disease-associations led to investigations into how the R620W mutation altered the function of *PTPN22* to promote autoimmunity. The missense mutation of R620W localizes to the P1 domain of *PTPN22*, and the variant protein is unable to bind to Csk (11). One group showed a more complex regulatory relationship between Csk and *PTPN22*, whereby association of *PTPN22* with Csk allowed proximal localization to LCK, whereby LCK could phosphorylate *PTPN22* on its inhibitory Y536 residue, dampening the catalytic activity of the phosphatase domain of *PTPN22*. The R620W variant's inability to bind Csk prevents this inhibitory phosphorylation by LCK, and leads to prolonged phosphatase activity on proximal TCR signaling substrates (21). Another group expounded on this complex relationship between Csk and *PTPN22*, showing that reduced association with Csk allows *PTPN22* to localize to lipid rafts, increasing its proximity to TCR signaling elements (7). Vang and colleagues went on to show

that the R620W variant localized to lipid rafts at a rate 3 times that of canonical *PTPN22*, and correlated with lower TCR signal strength (7). The Buckner group looked at TCR signal strength in primary T cells from health *PTPN22* R620W carriers and found that CD3/CD28 stimulation in the variants had significantly decreased [Ca^{2+}] flux compared to carriers of the major allele, specifically in cells of the memory compartment (22). These observations accorded with the conclusion that the R620W variant of *PTPN22* served as a gain-of-function mutation, whereby R620W carriage resulted in further decreased TCR signal strength. Conflicting evidence came from a study of the phospho-proteome of primary T cells from healthy human R620W carriers and major allele controls after *in vitro* stimulation with CD3/CD28. Memory CD4 T cells from R620W carriers exhibited increased and prolonged phosphorylation of several TCR signaling proteins including Akt, ERK, SLP76 and Ikkα (23). This led to conflicting evidence as to whether the R620W variant was a gain-of-function or a loss-of-function variant with regard to T cell receptor signaling repression.

PTPN22 **and R620W effects on T cell responses**

The initial study of *Ptpn22* KO mice by Hasegawa et al. showed that effector *Ptpn22* KO T cells exhibited increased proliferation *in vitro* to CD3/CD28 stimulation and *in vivo* using DO11.10 TCR transgenic *Ptpn22* KO T cells responding to their cognate antigen OVA (8). Critically, they noted no augmentation of naïve T cell proliferation or [Ca^{2+}] flux in response to TCR

stimulus (8). In an approach to study the function of the R620W variant on T cell responses, two separate groups generated knockin mice expressing variant *Ptpn22* R619W, a murine orthologue to *PTPN22* R620W (24,25). Studies with the R619W variant closely mimicked results of the *Ptpn22* KO mice, as T cells exhibited increased proliferation in effector/memory subsets and had increased TCR signal strength as measured by [Ca^{2+}] flux (24). These observations supported the model of a loss of function in repressing TCR signaling, contrary to the results reported by Rieck et al. in human R620W T cells (22). Thus there became a divide in the field as to whether R620W was a gain-of-function or a loss-of-function variant with respect to TCR signaling repression.

Investigations into the effect of *PTPN22* and the R619W or R620W variant on T cell cytokine production were similarly conflicting. Stimulation of *Ptpn22* KO T cells *in vitro* with CD3/CD28 yielded higher production of T_{H1} cytokines IFNγ and IL-2 and T_{H2} cytokines IL-4 and IL-5, consistent with loss of TCR repression (8). Studies of *in vivo* T cell responses to antigen in Complete Freund's Adjuvant (CFA) in *Ptpn22* KO mice noted higher expression of T_{FH} cytokine IL-21 and T_{H2} cytokine IL-4 (26), while immunization with the self-antigen MOG in CFA resulted in decreased production of T_{H17} cytokine IL-17 and T_{H1} cytokine IFNγ (10). The latter result is complicated by the control of Regulatory T cells (T_{Reg}) which in *Ptpn22* KO mice had a higher T_{Reg}:$T_{Effector}$ ratio (10). Studies of the R619W orthologue during *in vitro* stimulation of effector T cells revealed higher IL-2 production after CD3/CD28 stimulation (24), consistent with the *Ptpn22* KO

results and a variant that represents a loss of TCR repressive function. Studies of primary human T cells with the R620W variant differed as well. Two separate groups performed *in vitro* stimulation of T cells with CD3/CD28 with one showing no difference in production of IFNγ, IL-2, or IL-4, but a significant decrease in IL-10 production by the R620W variant (22), while the other group noted significantly increased IFNγ, TNFα and IL-2 production and significantly decreased IL-17 production by the R620W variant (23). These differing results have led groups to conclude that *PTPN22* and the R620W variant mediates preferential polarization to specific T_{Helper} effector subsets, depending on the prevalence of the cytokines produced in response to TCR stimulus. It remains unclear whether and how *PTPN22* modulates T cell cytokine responses to antigen receptor stimulus. Differences between the mouse orthologue R619W and the human variant R620W of *PTPN22* may be explained by the low frequency of sequence conservation (52% sequence identity and 65% sequence conservation), which could have important effects on their function (27). With regard to CD4 effector T cell polarization, it remains untested whether *PTPN22* modulates the formation of different T_{Helper} subsets via the measure of expression of the master transcriptional regulators Tbet, GATA3, RORγt, Bcl6 and Foxp3. Future studies of the role of *PTPN22* in CD4 T cell effector polarization should use this approach, rather than relying solely on cytokine profiles. Additionally, the data to date on R620W rely on *in vitro* studies, looking at general TCR stimulation of bulk T cells. The role of antigen-specific stimulus *in vivo* has not

been described for the R620W variant.

PTPN22 variants and Thymic Selection

T cell receptor signal strength tightly controls the thymic selection of developing thymocytes (28). The evidence that *PTPN22* negatively regulates TCR signal strength, and that the R619W and R620W genetic variants of *PTPN22* promote altered TCR signal strength, led to the hypothesis that the *PTPN22* variants altered thymic positive and negative selection. Changes in thymic selection can lead to increased auto-reactive T cells reaching the periphery, or could impact peripheral tolerance by way of altering T_{reg} development. A breakdown in tolerance to self-antigen results in autoimmunity, and genetic variants of *PTPN22* are associated with increased risk of multiple autoimmune diseases, therefore investigators interrogated the role of *PTPN22* in thymic selection.

Hasegawa et al. provided the first evidence supporting a role for *Ptpn22* in regulating T cell thymic selection by using the H-Y antigen to model positive and negative selection in *Ptpn22* KO mice. They found that female mice had increased numbers of $CD8^+$ thymocytes with TCRs reactive against the H-Y antigen, indicating an increase in positive selection of those TCRs (8). A similar increase in positive selection was shown for $CD4^+$ thymocytes using the DO11.10 model in *Ptpn22* KO mice (8). While *Ptpn22* KO mice exhibit increased positive selection for both $CD8^+$ and $CD4^+$ thymocytes, there was no evidence of altered negative selection (8). Another group investigated the role of *Ptpn22* in

development of T_{regs}. Strength of TCR signal guides T_{reg} development in the thymus, with T_{regs} receiving stronger TCR stimulus than conventional T cells (29). Maine et al. showed that *Ptpn22* KO mice have higher numbers of T_{reg} precursors and T_{regs} in the thymus, and higher numbers of T_{regs} in the periphery (10). Together, these data indicated that *Ptpn22* was important for thymic selection of conventional and regulatory T cells.

These findings of altered thymic selection in *Ptpn22* KO mice motivated investigations into whether genetic variants of *Ptpn22* also impart altered thymic selection. Utilizing the *Ptpn22-R619W* knockin mice, Dai et al. showed enhanced numbers of DO11.10 and H-Y TCR in female mice, indicating enhanced positive selection, similar to the *Ptpn22* KO studies (24). However unlike the *Ptpn22* KO mice, the R619W knockin mice displayed enhanced negative selection of the H-Y TCR in male mice (24). Another group evaluated the effect of the human R620W variant on thymic selection. Wu et al. utilized two different mouse lines, one that fused the LCK proximal promoter to the LypW gene to drive overexpression in the thymus, and one using a BAC transgene with the native promoter of LypW. While the overexpression of LypW did result in decreased phospho-ERK activation in stimulated thymocytes, consistent with LypW working as a gain-of-function variant, no effects on positive or negative selection of H-Y TCR thymocytes was detected (30). Overexpression of LypW did not result in any alterations in the number of Foxp3+ Tregs in the thymus, nor any alteration of the Vβ chain repertoire in SKG mice (30). Similarly, analysis of the effect of LypW

using mice bearing the native promoter showed no difference in positive or negative selection of H-Y TCR thymocytes (30). The conflicting results between the effect of the R619W variant and the human R620W variant on thymic selection could be explained by the nature of their effect on TCR signal strength. The R619W variant acts as a loss-of-function with regard to suppression of TCR signal strength, resulting in stronger TCR signal strength (24). Whereas the R620W variant, in overexpression studies, was shown to exert a gain-of-function effect, resulting in weaker TCR signaling (30). The majority (85%) of developing thymocytes express TCR clones which are of low affinity, imparting weak TCR signal strength, while only 15% of TCR clones are of a high enough affinity to undergo selection (31). Thus any effect that strengthens TCR signaling would be easier to detect, as it effects a smaller population of cells. In order to detect a change in the population of cells that generate low TCR signal strength, a much larger N would be required to detect an effect. It is important to note that the experiments done by Wu et al. represent a low N, and thus may be substantially underpowered to detect small differences in thymic selection of a variant that imparts weaker TCR signaling.

PTPN22 and B cells

In addition to the important function of *PTPN22* in regulation of T cell antigen receptor signaling, much work has been done to characterize its function in B cell antigen receptor signaling responses. The B cell malignancy Chronic

Lymphocytic Leukemia (CLL) provided early insights to the function of *PTPN22* in B Cell Receptor (BCR) signaling, as *PTPN22* was found to be overexpressed in CLL which resulted in blocked BCR signaling (32). Thus, like the TCR, *PTPN22* functions as a negative regulator of BCR signaling. *In vitro* studies of human B cells from R620W carriers revealed that the risk-associated variant conferred decreased BCR signaling as measured by decreased [Ca^{2+}] flux and decreased phosphorylation of Syk and PLCγ, important proximal BCR signaling proteins (33). Stimulation of B cells with α-IgM also revealed decreased proliferation (34) and an increased resistance to apoptosis by higher upregulation of Bcl2 (33). In addition to modulating BCR signaling, R620W B cells showed higher surface expression levels of CD40 as well as stronger CD40 signaling (35) indicating a complex relationship between *PTPN22*-mediated regulation of B cell activation and antibody class switching responses.

The function of *PTPN22* in regulating BCR signaling has important effects on the development of the B cell compartment. *Ptpn22* KO mice have increased follicular B cells and exhibit spontaneous development of large, mature germinal centers (8,26). This correlated with increased serum IgG and IgE, however there was no increase in the development of autoantibodies in these mice (8). Analysis of the R619W orthologue revealed increased frequencies of autoreactive Marginal Zone B cells, as well as increased frequencies of transitional B cells, germinal center B cells and Age-associated B cells (ABCs) in the spleens of aged mice (≥ 6months) (24). Unlike the PTPN22 KO, the aged

R619W mice did produce increased autoantibodies, including elevated levels of anti-dsDNA and anti-Insulin antibodies (24). Studies of human B cells in R620W carriers revealed similar changes in the B cell compartment, with one group reporting increased frequencies of transitional and anergic B cells in the peripheral blood (33) and another reporting an elevated frequency of autoreactive naïve mature B cells (35). Thus the regulation of BCR signaling by *PTPN22* is an important regulatory function in maintaining B cell tolerance, and the increased repression of BCR signaling by the R620W variant can lead to breaking B cell tolerance to self-antigen. What remains to be understood is whether factors extrinsic of B cell signaling contribute to the loss of B cell tolerance.

PTPN22 **in Myeloid cell responses**

PTPN22 is expressed in cells of the hematopoietic system (1), which includes its expression in cells of the myeloid lineage including macrophages and dendritic cells. Since myeloid cells do not possess antigen receptor signaling complexes, it was unknown what function *PTPN22* served in these cell subsets. Recent work by two separate groups have identified that *PTPN22* serves as a signaling regulator of cytokine responses downstream of innate immune surface and cytosolic pattern recognition receptor (PRR) signaling and response pathways. A role was first described for *PTPN22* in macrophages and dendritic cell PRR signaling that resulted in transcriptional activation of Type 1 Interferons

(T1 IFN). Wang and colleagues demonstrated that *Ptpn22* KO mice exhibited deficient production of T1 IFN after Toll-Like Receptor stimulation (36), a finding which was independently confirmed by another group (37). Molecular and biochemical analyses showed that *Ptpn22* binds to TRAF3 downstream of TLR engagement, and potentiates the activating K63-linked polyubiquitination of TRAF3 to promote downstream signaling to activate the transcription factor IRF3, a function that is independent of the phosphatase domain of *Ptpn22* (36). Studies of the response to TLR stimulation *in vivo* revealed that *Ptpn22* KO CD8α dendritic cells (DCs) were not only deficient in T1 IFN production, but displayed decreased activation and lower surface levels of costimulatory molecules CD80 and CD86, as well as CD40 (36). While T1 IFN production was decreased in *Ptpn22* KO myeloid cells, there was no effect on transcriptional regulation of TNFα, IL-6 and IL-1β, indicating that *Ptpn22* does not regulate NFκB responses downstream of TLR ligation (36). The defect in T1 IFN production and reduced DC maturation and costimulation in *Ptpn22* KO mice extended to defects in priming of CD8 T cells, as WT OT-I transgenic T cells adoptively transferred into *Ptpn22* KO recipient mice displayed decreased proliferation in response to OVA and Poly(I:C) TLR3 adjuvant (36). Thus it has become clear that understanding the function of *Ptpn22* in T cells also requires understanding of how it modulates other aspects of the immune response extrinsic to the T cell receptor.

To determine whether the R620W variant of *PTPN22* displayed altered function with regard to TLR-derived T1 IFN production, BAC transgenic mice

were generated on a *Ptpn22* KO background that expressed either the major allele variant R620 (termed LypR) or the minor allele variant W620 (termed LypW) (36). Using these transgenic "humanized" mice, as well as primary human cells derived from Peripheral Blood Mononuclear Cells (PBMC), the investigators determined that the LypW variant displayed the same decrease in T1 IFN production after TLR stimulus. Biochemical analysis revealed that the LypW variant had reduced ability to potentiate the K63-linked polyubiquitination, which accounted for the decreased T1 IFN production (36). Thus the R620W variant behaves as a loss of function with respect to TLR signaling and T1 IFN production in macrophages and DCs. Importantly, the loss of T1 IFN production in these myeloid subsets in *Ptpn22* KO and LypW transgenic mice resulted in worse outcomes in two models of autoinflammatory disorders, including the KBxN serum transfer arthritis model and the DSS colitis model (36). Therefore the role of *PTPN22* in the regulation and control of inflammation provides key insights into how the R620W variant provides increased risk for autoinflammatory and autoimmune disorders.

Another group recently described a role for *Ptpn22* in regulation of the NLRP3 inflammasome. The inflammasome is a multi-protein complex that catalyzes the proteolytic cleavage of pro-inflammatory cytokines IL-1β and IL-18 into their bioactive secreted forms (38). Work by Spalinger et al. showed that bone marrow-derived macrophages and DCs from *Ptpn22* KO mice exhibited decreased levels of IL-1β after treatment with TLR4 agonist LPS and NLRP3

agonist SiO_2. Similar experiments using the R619W mouse orthologue revealed an increase in secreted IL-1β compared to WT. This indicated that *Ptpn22* normally functions to activate the inflammasome, and that the R619W orthologue is a gain of function. Further analysis revealed that NLRP3 is phosphorylated on Y861, and that this phosphorylation inhibits formation of the inflammasome complex. *Ptpn22* dephosphorylates the Y861 residue of NLRP3, serving to positively regulate inflammasome formation (39). Analysis of the sera from Crohn's Disease patients that carry the R620W variant allele revealed a significantly higher level of IL-1β than patients without the variant allele or healthy controls, indicating that R620W behaves as a gain-of-function variant similar to the mouse R619W orthologue (39). These studies of TLR and NLR signaling pathways in innate immune cells give insight into the role of *PTPN22 i*n the regulation of inflammation in response to microbial and danger-associated molecular patterns. They also bring key insights into understanding the mechanisms by which the autoimmune risk variant R620W works to promote inflammation and the development of autoimmunity. It is also necessary to realize that the effects of *PTPN22* on the innate immune system can have important effects on the adaptive immune response of T and B lymphocytes, and further characterization of this interplay will be necessary.

PTPN22 **and Immunization Responses**

Immunization of genetically engineered mice provide a great tool for

studying the function of *PTPN22* in a complex immune response *in vivo*, allowing dissection of the various cellular and molecular mediators of immunity to a neo-antigen. The majority of the work on *PTPN22* in immunization responses has relied upon a vaccine comprised of neo-antigen emulsified in Complete Freund's Adjuvant (CFA). Studies of *Ptpn22* KO transgenic T cells specific for ovalbumin (OVA) in CFA revealed increased proliferation compared to WT transgenic T cells (8). Immunization of *Ptpn22* KO mice with the T-dependent antigen NP-KLH in CFA yielded higher frequency and number of T follicular helper cells (T_{FH}), and correspondingly higher Germinal Center B cell numbers and α-NP antibody titers than that of WT mice (26). These results fit well with a loss of TCR signal repression leading to increased proliferation, and suggested that *Ptpn22* may regulate CD4 T cell effector polarization.

Investigation of the immunization response in the mouse R619W orthologue revealed increased production of IL-2 compared to WT T cells after OVA and CFA immunization, however no analysis of the proliferation or CD4 T cell effector polarization profiles was reported (24). Antibody responses to immunization were increased in the R619W for both T-dependent antigen NP-KLH and T-independent antigen TNP-Ficoll (24), indicating that R619W exerted intrinsic effects within B cells and CD4 T cells during immunization. These data are consistent with R619W exhibiting a loss of function as a lymphocyte antigen receptor repressor. It remains to be shown whether the R620W variant exerts similar effect on T cell and B cell responses during immunization, or whether

carriers of the *PTPN22* R620W variant would have defective immunization responses. Additionally, an important consideration when investigating the effect of *PTPN22* variants on immunization responses is the T and B cell extrinsic effects due to altered myeloid cell cytokine production and antigen presenting cell maturation (36,39). Immunization studies in healthy human R620W carriers or humanized LypW mice will provide critical insight to how the R620W variant alters the function of innate and adaptive immunity in response to antigen stimulation.

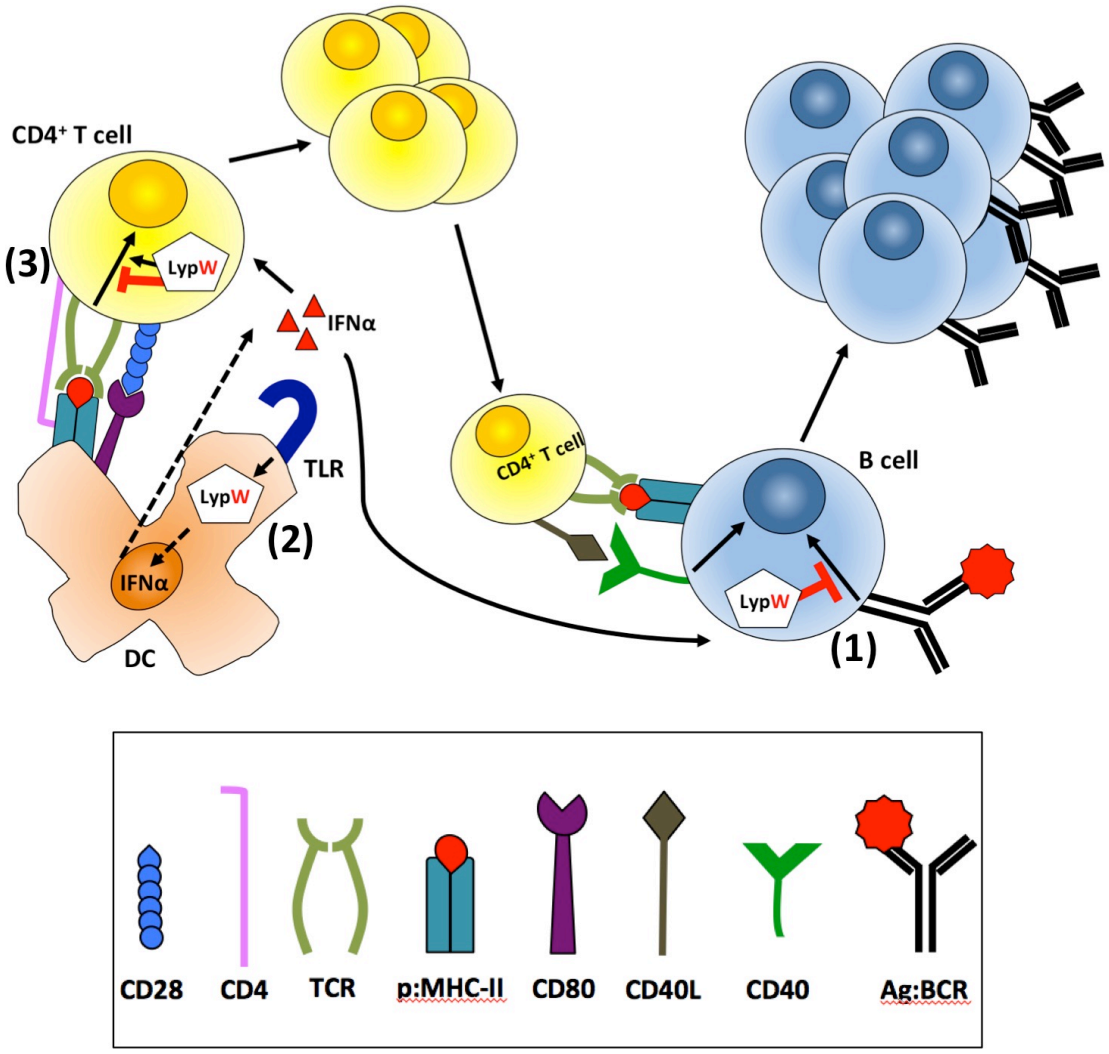

Figure 1-1: Effect of the *PTPN22* variant LypW on cellular immune responses. The LypW variant of *PTPN22* is implicated in (1) decreased B cell receptor signaling in B cells, and (2) decreased Toll-Like Receptor signaling in Dendritic cells and other Antigen-Presenting cells. (3) Conflicting evidence exists for the role of LypW in T cell receptor signaling in T cells, with some reports of a gain-of-function in TCR signal repression, and other reports of a loss-of-function in TCR signal repression.

Chapter 2: Autoimmune variant *PTPN22* C1858T is associated with impaired responses to influenza virus vaccination

Abstract

High-affinity antibody production, T cell activation, and Interferon upregulation all contribute to protective immunity that occurs in humans following influenza immunization. Hematopoietic cell-specific *PTPN22* encodes Lymphoid Phosphatase (Lyp), which regulates lymphocyte antigen receptor and Pattern Recognition Receptor (PRR) signaling. A *PTPN22* variant R620W (LypW) predisposes to autoimmune and infectious disease, and confers altered signaling through antigen receptors and PRRs. We tested the hypothesis that LypW-bearing humans would have diminished immune response to trivalent influenza vaccine (TIV). LypW carriers exhibited decreased induction of influenza-specific CD4 T cells expressing effector cytokines, and failed to increase antibody affinity following TIV. No differences between LypW carriers and non-carriers were observed in virus-specific CD8 T cell responses, early interferon transcriptional responses, or myeloid APC costimulatory molecule upregulation. LypW association with defects in TIV-induced CD4 T cell expansion and antibody affinity maturation suggests that LypW may predispose to diminished capacity to generate protective immunity against influenza.

Introduction

Influenza virus infection poses a substantial public health burden, resulting in approximately 200,000 hospitalizations and between 7,505 to 37,102 deaths annually in the U.S. (40,41). Annual influenza vaccination can provide protection against circulating seasonal strains, provided there is good matching between viral vaccine strains and circulating strains (42–44).

Protective immunity to influenza virus results from combined humoral and cellular immune responses. Neutralizing antibodies can provide sterilizing immunity to homologous viral strains by preventing viral attachment and entry to host epithelial cells (45). Antibody neutralizing capacity is heavily dependent upon specificity and epitope binding affinity (46). However, due to high mutation rates in influenza genes encoding hemagglutinin (HA) and neuraminidase (NA) proteins, most neutralizing antibodies elicited by vaccination are strain-specific and often do not provide heterosubtypic immunity (47,48).

T cells are important cellular effectors of antiviral immunity. CD8 T cells mediate direct lysis of infected epithelial cells (49), and high levels of virus-specific CD8 T cells correlate with lower viral shedding and lower disease severity in humans (50,51). CD4 T cells contribute to anti-viral immunity by providing help to B cells for efficient isotype-switching (52) and affinity maturation (53) needed in the

generation of virus-specific antibodies (54), and by providing help to CD8 T cells (55). CD4 T cells can also secrete IFNγ in the lungs (56), and can directly lyse virus-infected, MHC-II-expressing bronchial epithelial cells in a perforin-dependent manner (57). Both CD8 and CD4 T cells are strongly implicated in heterosubtypic immunity to influenza, due to their recognition of viral proteins that are largely conserved across strains (50,57–59).

In addition to stimulating adaptive responses, influenza infection or vaccination can induce transcription of numerous interferon (IFN)-regulated genes (60,61). An early IFN signature correlates with B cell responsiveness to vaccination and the development of neutralizing antibodies, suggesting that IFN signaling plays a role in anti-influenza antibody production (62). Indeed, in animals, type I IFN are critical for generation of both protective antibody and CD4 T cell responses to influenza vaccination (63). The requirements for type I IFN and innate immunity in vaccine-induced protective immunity among humans remain incompletely defined.

Protein Tyrosine Phosphatase Non-receptor 22 (*PTPN22*) encodes Lymphoid Phosphatase (Lyp), a hematopoietic-specific intracellular protein. Lyp functions as a negative regulator of TCR signaling (27), and positively modulates interferogenic Toll-like Receptor (TLR) signaling in macrophages and dendritic cells (36,64). A human *PTPN22* coding polymorphism, C1858T, associates with

significantly increased risk of numerous autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes, and with altered susceptibility to selected Gram-positive and mycobacterial infections (65,16,15). C1858T is carried by 6-9% of persons of European ancestry (66). C1858T encodes a single amino acid substitution-bearing (R620W) protein variant, termed LypW, that exhibits altered function in TCR signaling and in TLR-driven type I IFN production.

To date, studies testing LypW function in primary human lymphocyte and innate immune cell responses have utilized in vitro or ex vivo approaches (8,11,23,33–35,67). Information concerning the role of human LypW during immunization responses has not been reported. Since *PTPN22* regulates both lymphocyte and innate immune cell signaling and activation, we tested the hypothesis that R620W carriers would mount diminished or defective innate, cellular and humoral responses to influenza vaccination.

Methods:

Subject recruitment and sample collection

Healthy adult volunteers submitted DNA for *PTPN22* rs2476601 (C1858T) genotyping. *PTPN22* LypW carriers (heterozygote n=17; homozygote n=1) and age and sex-matched non-carriers (LypR, n=17) received intramuscular Fluzone (2013-2014 trivalent inactivated influenza vaccine (TIV); Sanofi Pasteur).

Subjects submitted blood pre-vaccine, and at days 1, 14-15, and 25-28 following vaccination. IRB approval was obtained (University of Minnesota, protocol #1210M21901), and all subjects provided informed written consent.

Genotyping

DNA was extracted from participant blood using the DNeasy Blood & Tissue kit (Qiagen), and subjects were genotoyped for the *PTPN22*-C1858T SNP by Taqman assay (Applied Biosystems). HLA-A genotype was determined by BLAST analysis of sequenced HLA-A PCR amplicons (68) using the IMGT/HLA database (https://www.ebi.ac.uk/ipd/imgt/hla/).

Hemagglutination inhibition assay

Serum was mixed with Aprotinin (Sigma) before freezing. Hemagglutination inhibition assays (HAI) were performed using the WHO 2013-2014 Influenza Reagent Kit and Turkey RBCs in Alsevers (Colorado Serum) according to manufacturer's instructions.

Biolayer Interferometry Assay of Human Serum Samples

Real time binding assays of antibodies in human sera and purified hemagglutinin proteins were performed using biolayer interferometry with an Octet RED system (ForteBio, Inc.). Human sera were heated at 56° for 30min before the assay. Biotinylated (EZ-link Micro NHS-PEG4-Biotinylation Kit, Thermo Scientific)

A/California/04/09 hemagglutinin proteins (20 μg/ml) were immobilized onto streptavidin-coated biosensors (ForteBio) for 5min. After measuring baseline signal in kinetics buffer (1X PBS 0.01% BSA 0.002% Tween-20) for 3min, biosensor tips were immersed into wells containing human sera (starting dilution of 1:10 and serially diluted 3-fold) for 5min to measure association. Biosensors were later immersed into kinetics buffer to measure dissociation. Binding kinetics were calculated using the Octet RED software package (Data Acquisition 8.2), to fit the observed binding curves in a 1:1 binding model to calculate the association and dissociation rate constants. Equilibrium dissociation constants were calculated as the kinetic dissociation rate constant divided by the kinetic association rate constant.

CD4 T cell assessments

PBMC were isolated from whole blood, and stored at -80 °C until batched analysis. Thawed PBMCs were washed with complete pre-warmed RPMI 1640. Cells were rested before stimulating 6 hours in the presence of Brefeldin A (eBioscience) with either 2013-2014 Fluzone (10 μL), PMA (50ng/mL) and ionomycin (1μg/mL; Sigma), or medium alone. Cells were stained with fixable viability dye and surface antibodies before permeabilization and intracellular staining. Data were acquired on an LSRFortessa (BD), and were analyzed using FlowJo software (Tree Star). The influenza-specific CD4 T cell fraction was calculated by subtracting numbers of cytokine-positive events observed in no-

stimulation control from total cytokine-producing events observed in Fluzone stimulated conditions.

Isotype ELISA assays

96-well PRO-BIND plates (Falcon) were coated with rHA from strain A/California/07/2009 H1N1 (BEI Resources) at 2ug/mL in carbonate coating buffer (Sigma) overnight at 4°. Plates were washed and blocked with 2% BSA. Serum was diluted in PBS at the indicated dilutions and incubated at 37°. HRP-conjugated antibodies against human IgM, total IgG or IgA (Abcam) were used for detection of the different anti-HA isotypes. Plates were incubated with TMB substrate, the reaction was stopped with 1N H_2SO_4 and plates were read on a SPECTRAmax Plus 384 (Molecular Devices).

CD8 T cell assessments

Influenza-specific CD8 T cells were identified in selected vaccinated subjects who were HLA-A*02 carriers. PBMCs were stained with HLA-A*0201 M1 peptide GILFVFTL tetramer (Beckman Coulter) and surface antibodies. After permeabilization and intracellular antibody staining, cells were analyzed by FACS.

Transcriptional Profiling

RNA was extracted from whole blood using PAXgene Blood RNA Kit

(PreAnalytix). cDNA was synthesized using iScript cDNA synthesis kit (BioRad). Transcriptional response was assessed using Human Interferon Signaling and Response 96 StellARay (Bar Harbor Biotechnology) and Fast Start Universal Sybr Green Master Mix (Roche). Q-PCR reactions were run on a 7900HT (ABI Prism).

Costimulatory molecule upregulation

Thawed PBMCs were plated in complete RPMI 1640 and rested before stimulation with Fluzone (10uL), LPS (100 ng/mL) or medium for 18 hours at 37° C. Cells were incubated with 2 mM EDTA for 15 minutes before washing with cold PBS + 2% FBS. Cells were stained with fixable viability dye and surface antibodies, and analyzed by FACS.

Statistical Analysis

Chi-square test was used to compare categorical variables of antibody seroconversion and seroprotection. The Student's T-test was used to compare antibody titers, isotype and affinity; CD4 and CD8 T cell frequency; and costimulatory molecule MFIs between groups. Within-group baseline and post-vaccination values were compared using a paired T-test. The Mann-Whitney test was also performed as an alternative non-parametric test. For transcriptional analyses, Mann-Whitney test was performed for each gene to compare the expression levels between groups, followed by a gene-set enrichment

association test (GSEA) (69) with 10,000 permutations. Bonferroni correction was used for multiple testing. Statistical analysis was performed using Stata (version 13.1, StataCorp) and GraphPad Prism 5 software.

Results:

LypW carriage associates with poor antibody affinity maturation after TIV

To examine the role of *PTPN22* R620W variant (LypW) in human in vivo responses to influenza vaccination, we immunized healthy LypW carriers (n=18) or non-carriers (n=17; Table 2-1 shows subject characteristics) with TIV.

Table 2-1: Subjects

	LypW[a]	LypR	Total
N	18	17	35
Median Age (Range)	21 (18-55)	21 (18-51)	21 (18-55)
Sex:			
Female (%)	13/18 (72)	12/17 (71)	25/35 (71)
Ethnicity:			
White (%)	18/18 (100)	17/17 (100)	35/35 (100)
American Indian (%)	1/18 (5.5)	0/17 (0)	1/35 (2.9)
Previous Influenza Immunization (%)	17/18 (94)	16/17 (94)	33/35 (94)

[a] n=17 *PTPN22*-R620W heterozygous (R/W); n=1 homozygous (W/W)

First, we compared trivalent inactivated influenza vaccination (TIV)-induced antibody levels in LypW carriers and non-carriers. We found that pre-vaccine seroprotection, defined as hemagglutination inhibition (HAI) titer ≥1:40, was present in 82-100% of subjects (Table 2-2). These results suggested high rates of previous vaccination or infection. 25 days after vaccination, the majority of subjects exhibited significant increases in mean neutralizing antibody titers for

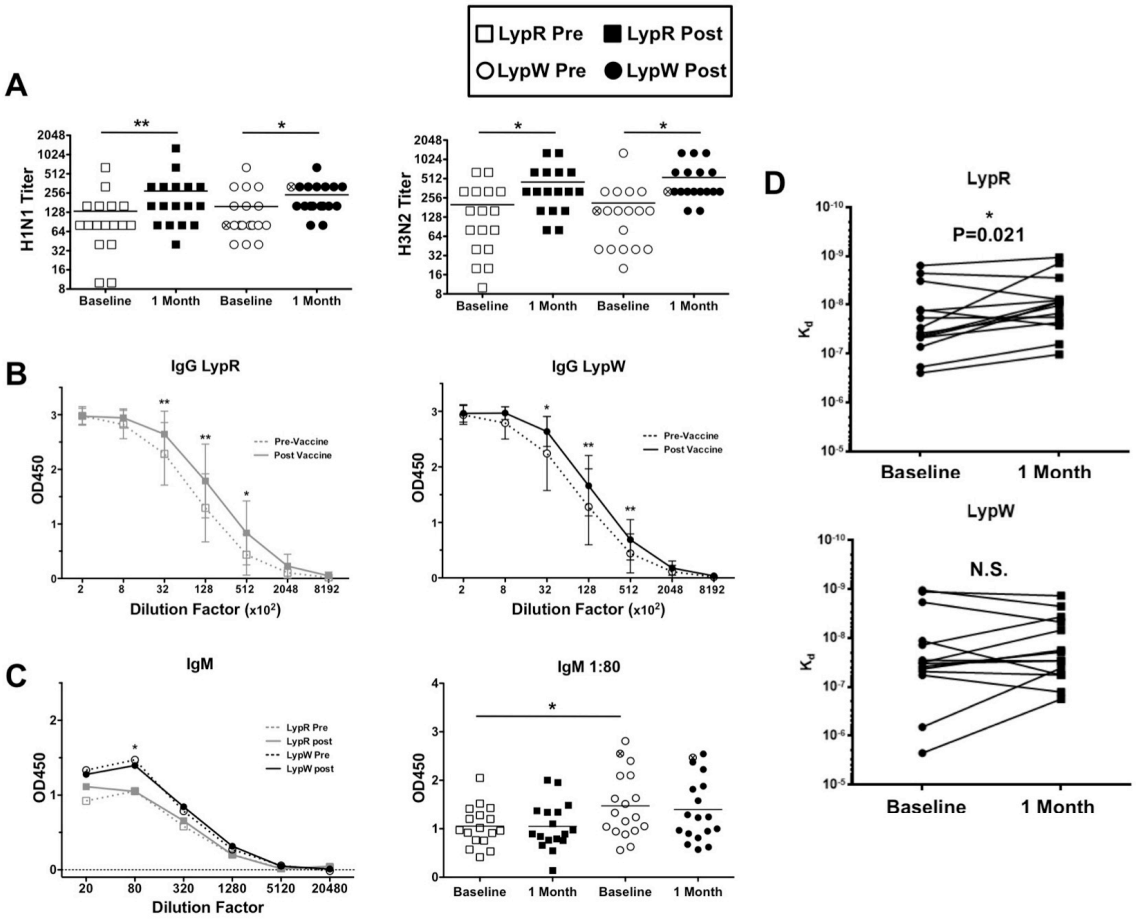

Figure 2-1: LypW Carriers have reduced affinity maturation after TIV
(A) Hemagglutinin-inhibition assays were performed on sera from subjects to determine H1N1 and H3N2 titers. (B-C) Ig Isotype ELISA for H1N1 HA-specific antibodies for IgG and IgM. (B) Anti-HA IgG binding curves for LypR and LypW subjects from serial dilutions of the serum at baseline and 1 month after vaccine. Bars represent S.D. (C) IgM curves and scatterplot for LypR and LypW subjects from serial dilutions of the serum at baseline and 1 month after vaccine. (D) Dissociation constants from biolayer interferometry analysis of H1N1 HA-specific antibodies in the sera of LypR (N=14) and LypW (N=14) subjects at baseline and 1 month after vaccine. Circle with x denotes LypW homozygote. (A, B, D) paired t-test (C) unpaired t-test *p<0.05 **p<0.01

H1N1 and H3N2 (Fig. 2-1A). However, we noted no statistically significant differences between LypW carriers and non-carriers (LypR) in average absolute HAI titers, in rates of seroconversion (defined as fold-increase in neutralizing titer ≥4), or in rates of seroprotection (100%) after TIV vaccination (Table 2-2).

Table 2-2: HAI seroconversion and seroprotection

	Strain	LypR	LypW	p[a]
Seroconversion[b]	H1N1	23.5%	27.7%	0.774
	H3N2	35.3%	44.4%	0.581
Seroprotection[c] (Baseline)	H1N1	88%	100%	0.134
	H3N2	82%	94%	0.261
Seroprotection (1 month)	H1N1	100%	100%	N.A.
	H3N2	100%	100%	N.A.

[a] Chi-Square test
[b] Seroconversion is defined as a fold-increase in titer ≥ 4.

Antibody isotype switching and affinity maturation are important for development of a neutralizing anti-influenza antibody repertoire (46). To assess immunization-induced isotype switching, we measured serum levels of IgM, IgG and IgA specific for H1N1 HA. LypW carriers and non-carriers showed equivalent enhancement of IgG and IgA anti-HA in response to vaccination (Fig 2-1B, Fig. 2-2). However, mean anti-HA IgM levels in LypW carriers were significantly higher than levels in LypR subjects at baseline (Fig. 2- 1C). We also assessed the role of LypW in antibody affinity maturation during the influenza response. Biolayer interferometry was used to measure the dissociation constants (affinity) of serum antibodies against H1N1 HA. While serum from LypR non-carrier subjects displayed a significant increase in anti-HA binding affinity (mean 2.6-

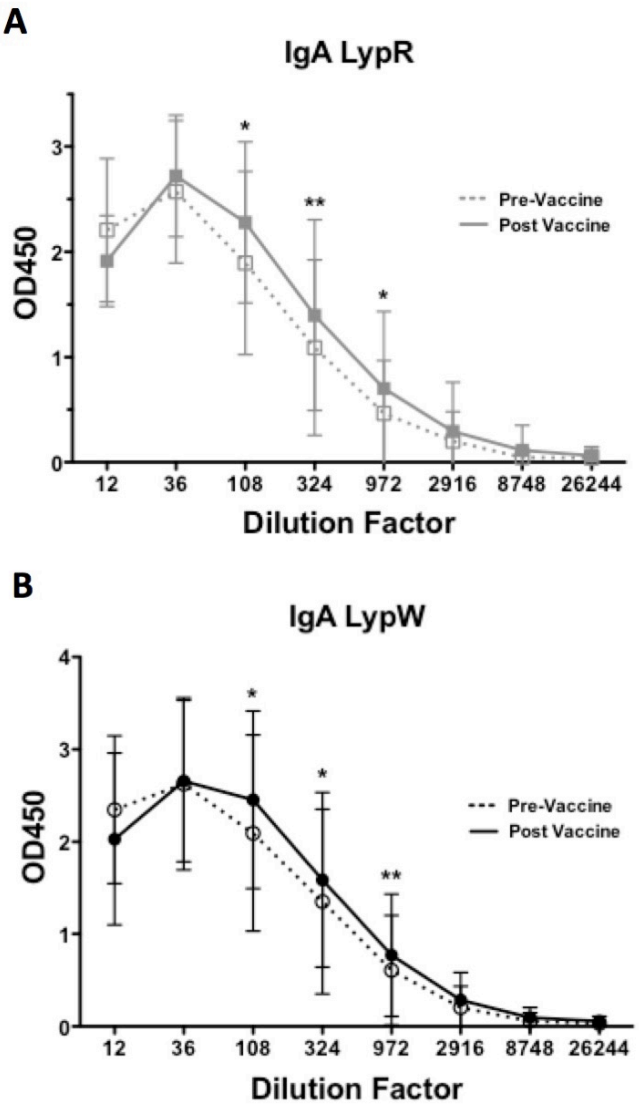

Figure 2-2: IgA production is significantly induced by vaccination
IgA isotype ELISA for H1N1-specific HA antibodies. Curves represent IgA levels measured by serial dilutions of serum from LypR (A) and LypW (B) Subjects at baseline and 1 month post vaccination. Bars represent S.D. Paired t-test, *p≤0.05 **p≤0.01.

fold) after vaccination, LypW carrier serum did not (Fig. 2-1D, Table 2-3). Together, these results suggested that LypW carriage associates with increased IgM anti-HA antibodies, and with altered capacity to form high-affinity antibody to TIV.

Table 2-3: HA-specific antibody affinity

	Baseline K_d	1 Month K_d
	Mean (95% C.I.)	Mean (95% C.I.)
LypR	5.43e-8 (1.22e-9)	2.12e-8 (4.76e-10)
LypW	2.31e-7 (1.02e-8)	4.11e-8 (8.69e-10)

LypW carriers show diminished CD4 T cell responses to TIV

CD4 T cell help is critical for optimal isotype switching and affinity maturation (52,53). To study the role of LypW in CD4 T cell immunization responses, we exposed PBMC from immunized subjects to TIV in vitro. Vaccine-stimulated CD4 T cell production of any of three cytokines−IFNγ, TNFα, or IL-2−was used as an indicator of influenza specificity (70) (Fig. 2-3A). Unstimulated and PMA/Ionomycin stimulated CD4 T cells were used as negative and positive controls for cytokine production, respectively, and no difference between LypR and LypW subjects was detected in either of these stimulation conditions (Table 2-4).

Table 2-4: Frequency of CD4 T cells positive for Cytokines IFNy, TNFa or IL-2

	LypR		LypW		
	Mean	S.D.	Mean	S.D.	P[a]
Baseline:					
Unstimulated	0.0400	0.0284	0.0416	0.0338	0.8800
PMA/Ionomycin	37.63	13.42	35.18	16.01	0.6279
2 Weeks:					
Unstimulated	0.0221	0.0232	0.0258	0.0202	0.6172
PMA/Ionomycin	39.69	10.82	33.47	15.35	0.1776

a: unpaired student's t-test

Frequency of influenza-specific CD4 T cells among PBMC increased in LypR (non-carrier) subjects 2 weeks post-vaccination (Fig. 2-3B,C). However, LypW carriers showed no vaccination-induced increase in the frequency of influenza-specific CD4 T cells, either by analysis of pooled cytokine-producers (Fig. 2-3C), or of CD4 T cells producing individual cytokines (Fig. 2-4).

A similar pattern was seen with enumeration of absolute numbers of TIV-specific CD4 T cells (Fig. 2-5). Geometric mean fold-change increase in TIV-induced influenza-specific CD4 T cells among non-carriers was 2.6 (95% C.I. 2.019-3.426), compared to 1.3 among LypW carriers (95% C.I. 0.926-2.018)(Fig. 2-3D). The percentage of TIV-induced influenza-specific CD4 T cells showing evidence of prior activation ($CD45RA^{-}CD27^{+}$) increased significantly in non-carrier LypR subjects, but not in LypW carriers. These data suggested that LypW carrier CD4 T cells might be subject to impaired TCR-dependent priming (Fig. 2-6A). We observed no alteration in expression of PD1, a marker for dampened T cell

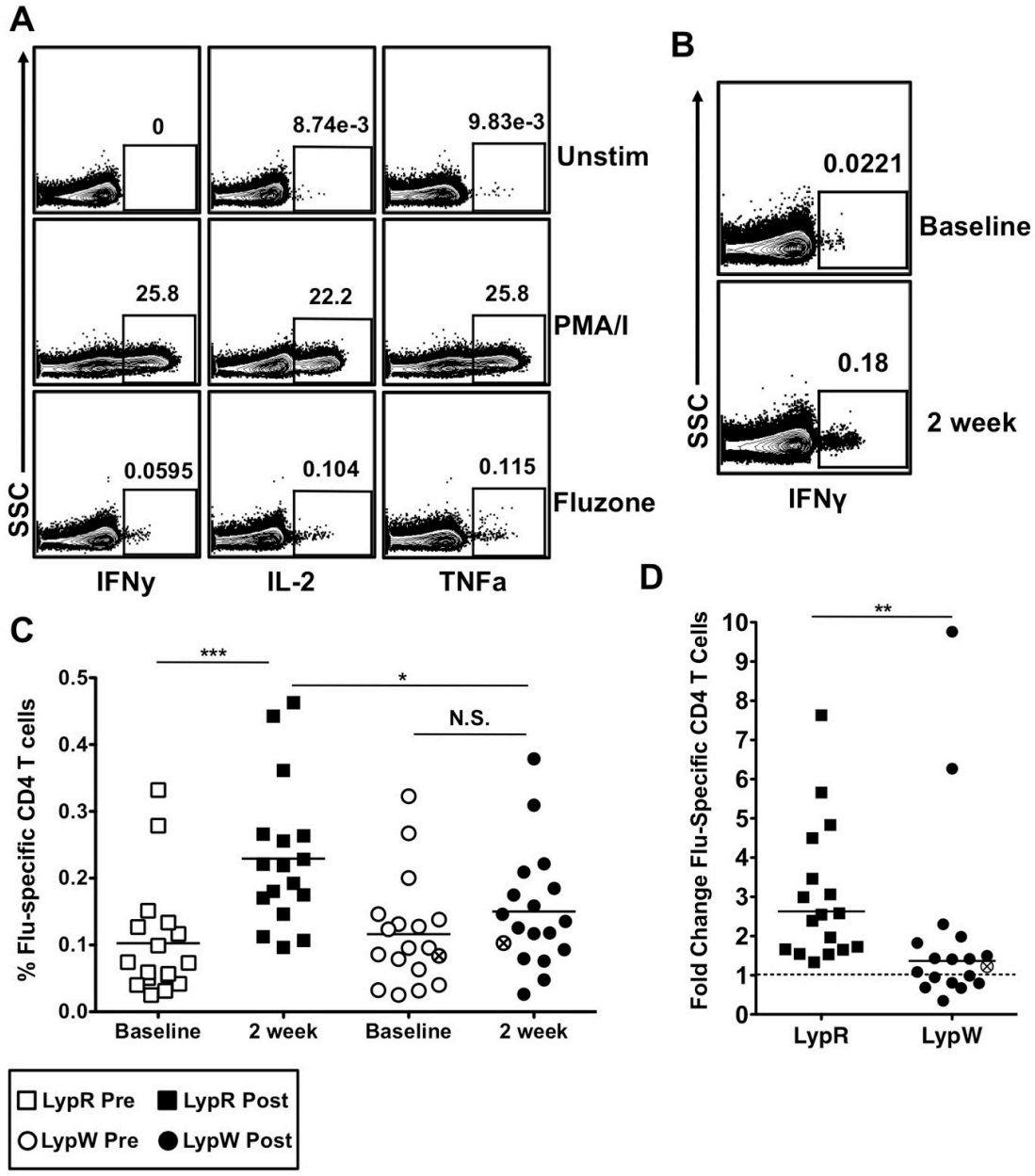

Figure 2-3: LypW Carriers exhibit reduced CD4 T cell response to influenza vaccine

Subject PBMC were stimulated in vitro with the 2013-2014 inactivated influenza vaccine Fluzone and assayed for cytokine production to denote flu-specific CD4 T cells. (A) Representative cytokine staining for CD4 T cells with indicated stimulus. (B) Representative IFNγ staining of CD4 T cells stimulated with Fluzone before and 2 weeks after immunization. (C) Total frequency of flu-specific CD4 T cells. (D) Fold change of flu-specific CD4 T cells in response to vaccination. Circle with x denotes LypW homozygote. (C) Paired and unpaired t-test (D) Mann-Whitney N.S.= not significant *p<0.05 **p<0.01 ***p<0.0001

responsiveness, in either basal or vaccine-induced influenza-specific CD4 T cells between LypR and LypW carriers (Fig 2-6B). We observed no differences in responder T cell polyfunctionality (71) between non-carriers and LypW carriers, as the fractions of influenza-specific CD4 T cells expressing single or multiple cytokines were unchanged by vaccination or genotype (Fig. 2-6C). Together, these findings suggest that LypW carriage confers reduced capacity for TIV-induced expansion and activation of CD4 T cells, but that restricted T cell expansion is not a result of increased PD1 expression in LypW carrier T cells.

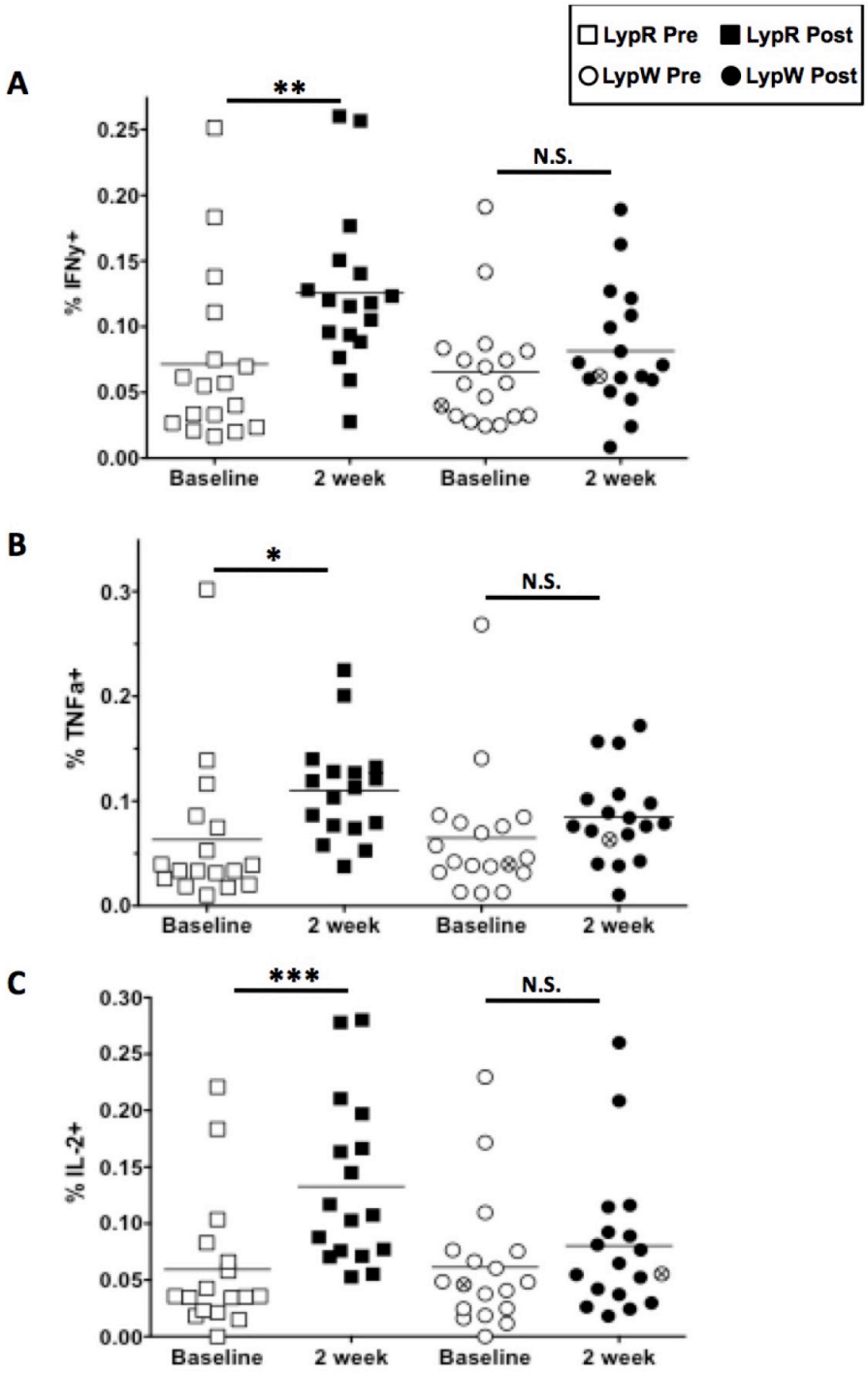

Figure 2-4: Individual cytokine production of TIV-specific CD4 T cells Frequency of TIV-specific CD4 T cells in LypR (blue) and LypW (red) subject PBMC producing A) IFNγ B) TNFα or C) IL-2 cytokines. Circle with x denotes LypW homozygote. Paired t-test N.S. not significant *p≤0.05 **p≤0.01 ***p≤0.0001

Table 2-5: CD86 gMFI

	Unstimulated					LPS				
	LypR		LypW			LypR		LypW		
	Mean	S.D.	Mean	S.D.	P[a]	Mean	S.D.	Mean	S.D.	P
cDC	881	132	842	234	0.5768	614	273	668	325	0.6187
$CD123^+$ DC	7638	4618	7189	4057	0.7715	9512	7068	8930	6373	0.8112
pDC	36	17	57	44	0.0930	590	646	449	351	0.4415
Mono	2112	935	1807	860	0.3446	446	168	355	275	0.2716

a: unpaired student's t-test

Table 2-6: CD40 gMFI

	Unstimulated					LPS				
	LypR		LypW			LypR		LypW		
	Mean	S.D.	Mean	S.D.	P[a]	Mean	S.D.	Mean	S.D.	P
cDC	801	425	968	487	0.3149	862	403	904	584	0.8169
$CD123^+$ DC	2419	2556	3125	1722	0.3622	10042	6693	9941	6291	0.9658
pDC	278	227	427	267	0.1015	1498	863	1215	716	0.3191
Mono	4843	1658	4921	1192	0.8786	3595	827	3120	1455	0.2739

a: unpaired student's t-test

LypW carriage does not affect TIV induction of costimulatory molecules

Antigen presenting cell (APC) activation and interferon signaling represent T cell-extrinsic factors that enhance CD4 responses to immunization (36,63). *PTPN22* variants differentially modulate these processes in animals (36,72); therefore, we investigated potential roles for LypW in human T cell-extrinsic immunization responses. Optimal T cell activation and clonal expansion depend upon

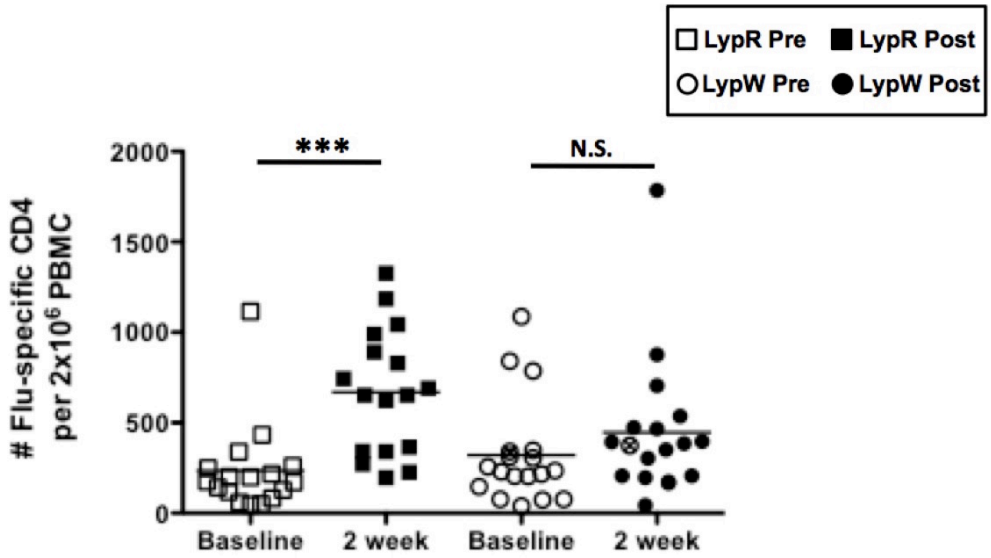

Figure 2-5: Total number of TIV-specific CD4 T cells Number of CD4 T cells per $2x10^6$ PBMC expressing IFNγ, TNFα or IL-2 in response to TIV stimulation. Circle with x denotes LypW homozygote. Paired t-test N.S. not significant ***p=0.0002.

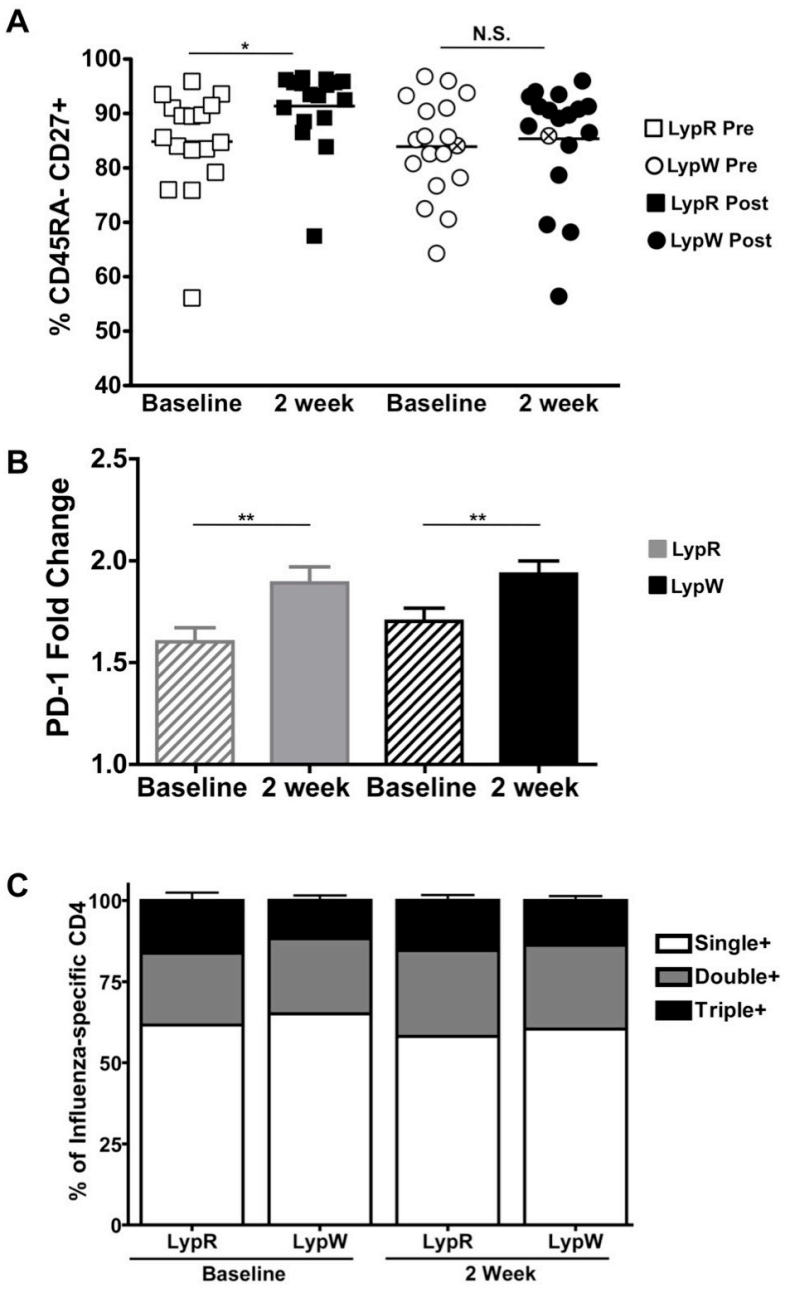

Figure 2-6: LypW carrier Influenza-specific CD4 T cells exhibit decreased activation, but equal polyfunctionality and PD-1 expression. (A) Frequency of influenza-specific CD4 T cells that are $CD45RA^{-}CD27^{+}$ in LypW and LypR subjects. Circle with x denotes LypW homozygote. (B) Surface PD-1 expression depicted as fold change of influenza-specific CD4 T cells in LypW (black) and LypR (grey) subjects before and after immunization. (C) Frequency of influenza-specific CD4 T cells that produce 1, 2 or 3 cytokines. Error bars represent standard deviation. N.S. = not significant *$p<0.05$.

costimulatory signals delivered through CD86 and CD40 expressed on APC (73,74). In animals, *Ptpn22* deficiency results in blunted CD80/CD86 and CD40 upregulation on myeloid APC after viral infection, and carriage of the LypW allele results in reduced CD80 and CD40 upregulation on macrophages after TLR stimulation (36). To assess LypW carrier capacity for upregulation of costimulatory molecules, we stimulated PBMCs with Fluzone, medium alone (unstimulated) or LPS. We monitored CD86 and CD40 expression on myeloid subsets, including monocytes (HLA-DR$^+$CD14$^+$), conventional dendritic cells (cDC; HLA-DR$^+$CD11c$^+$), CD123$^+$ CD11c$^+$ DCs, and plasmacytoid dendritic cells (HLA-DR$^+$CD11c$^-$CD123$^+$BDCA-2$^+$) (21). We found that TIV induced significant upregulation of CD40 expression on monocytes, CD123$^+$CD11c$^+$ DCs, and pDCs (Fig. 2-7A), and drove upregulation of CD86 on pDC (Fig. 2-7B). However, LypW carriers and non-carriers showed no difference in co-stimulatory molecule upregulation. Additionally, myeloid cell subsets from LypR and LypW carrier subjects showed no difference in upregulation of CD86 (Table 2-5) or CD40 (Table 2-6) in response to LPS. Taken together, these results suggest that LypW carriage does not alter capacity for APC to become activated by TIV.

LypW carriage does not affect interferon transcriptional response to TIV

An early transcriptional response dominated by interferon-stimulated genes has been linked with development of adaptive immune responses after TIV (60–62). LypW carrier myeloid cells show defects in interferon-stimulated gene upregulation after TLR stimuli (36,64). We therefore measured transcriptional

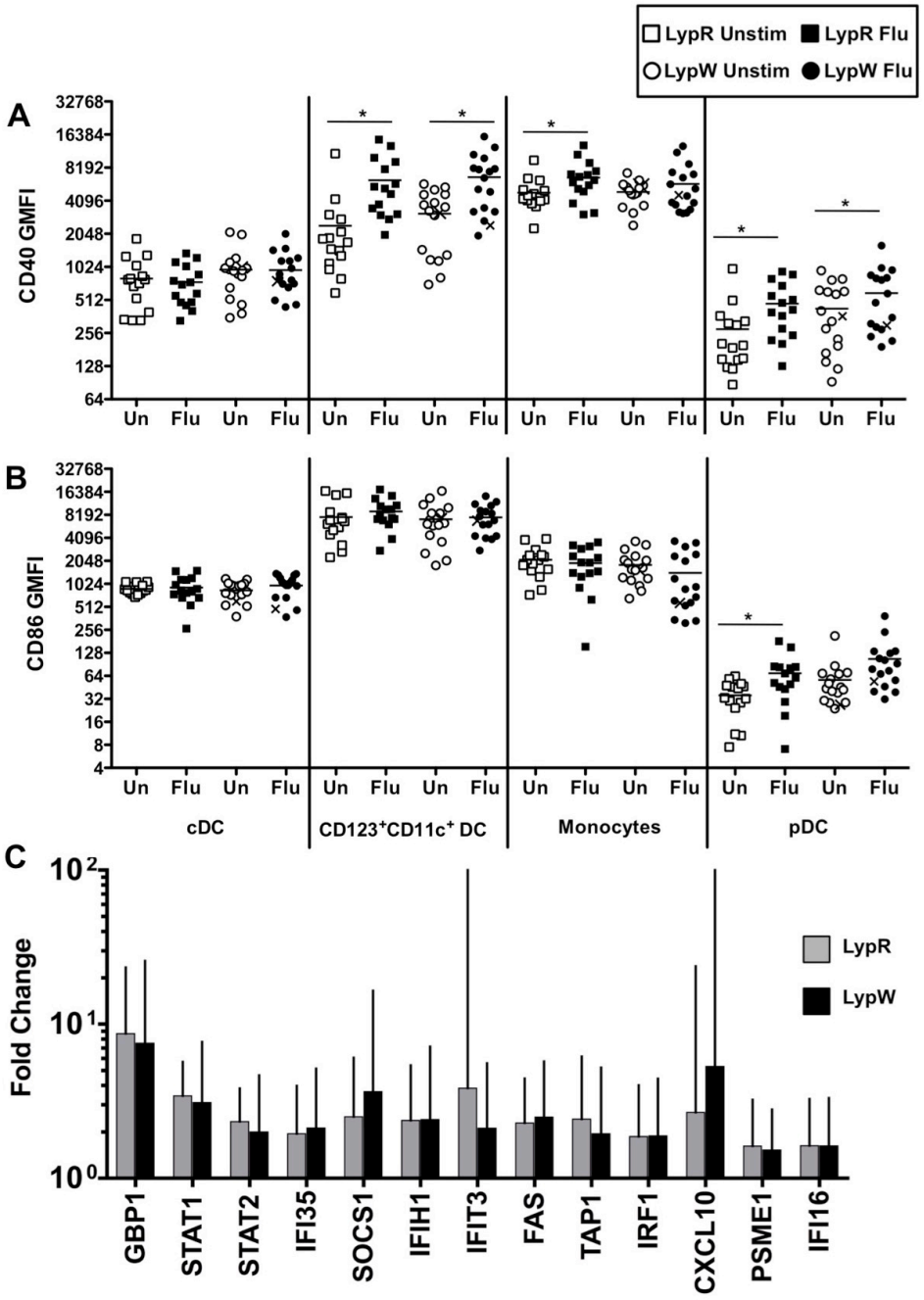

Figure 2-7: LypW carriers show no defect in innate response to influenza vaccine

GMFI of (A) CD40 and (B) CD86 expressed on indicated PBMC cell subsets from LypR and LypW subjects stimulated with Fluzone vaccine in vitro for 18 hours. Un = Unstimulated, Flu = Fluzone. X denotes LypW homozygote. (C) Fold change of IFN signaling and response genes significantly induced by TIV at 1 day for LypR (gray) and LypW (black) subjects. Error bars represent standard deviation. (A-B) *p<0.05.

responses 1 day after TIV immunization using a limited array enriched for interferon signaling and response genes. 13 of 96 analytes were significantly induced 1 day after TIV immunization (Fig. 2-7C). LypW carriers and non-carriers did not differ in either upregulation of individual gene transcripts or in grouped global responses, as assessed by gene-set enrichment association testing (GSEA) (69). These data suggest that TIV-induced in vivo interferon-induced transcriptional responses are not affected by LypW carriage. Together with vaccine-induced APC activation results (Fig 2-7A), these findings are consistent with the hypothesis that diminished LypW-associated CD4 T cell immunization responses stem from T cell-intrinsic mechanisms of LypW action.

TIV does not impact peripheral blood CD8 T cell numbers or function

In animals, *Ptpn22* deficiency is associated with decreased expansion of virus-specific CD8 T cells after RNA viral infection (36). We assessed the role of LypW carriage in expansion of human antigen-specific CD8 T cells after TIV immunization. We used tetramers to determine frequency of influenza-specific CD8 T cells among PBMC in those vaccine recipients found to harbor MHC Class I HLA-A*02 (LypR n=9; LypW n=8). We observed no vaccine-inducible change in the frequency of influenza-specific CD8 T cells two weeks after TIV in either LypR or LypW subjects (Fig. 2-8A).

Moreover, neither group showed evidence of vaccine-inducible increases in influenza-specific CD8 T cell proliferation, activation state, or development of

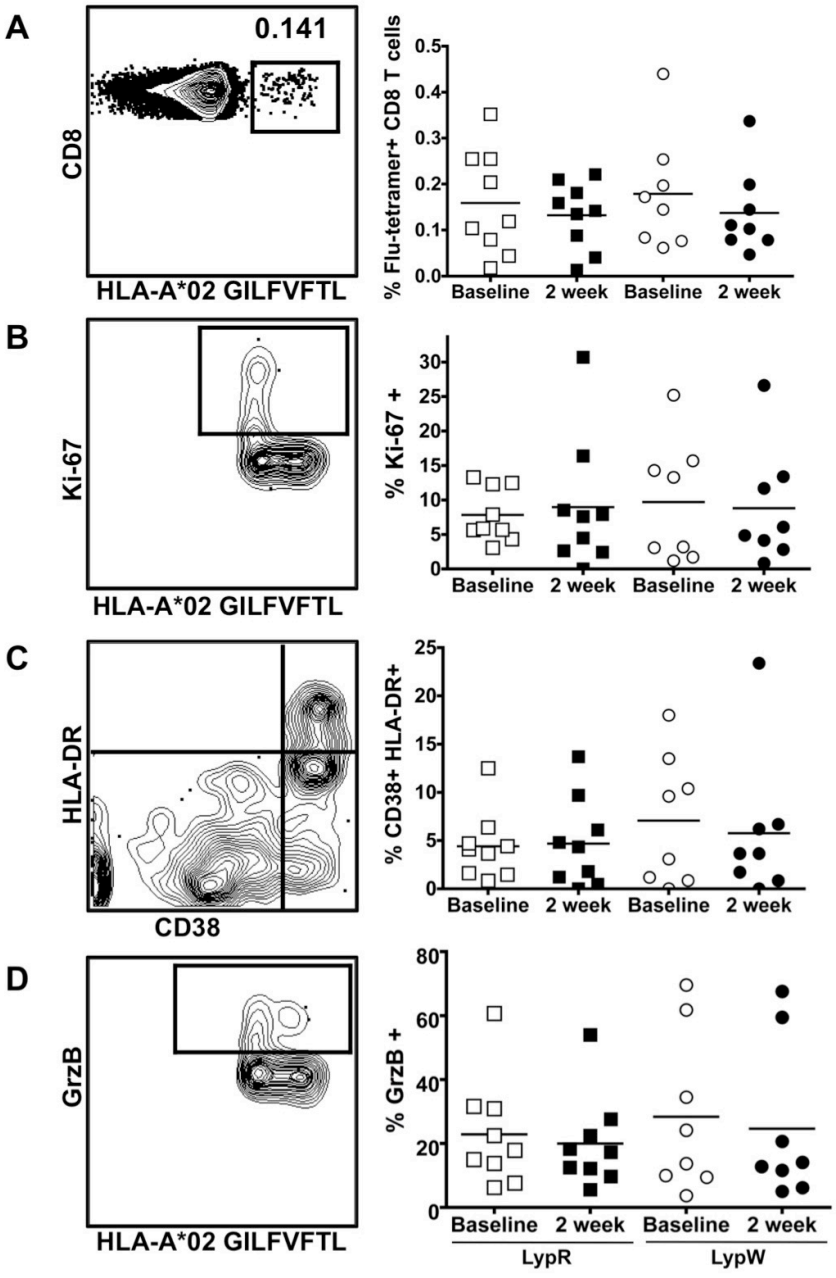

Figure 2-8: Inactivated influenza vaccine does not induce peripheral CD8 T cell response

PBMCs were stained ex vivo with influenza peptide-MHC class I tetramers to identify virus-specific CD8 T cells at baseline and at 2 weeks after immunization. Representative staining and total frequency of (A) tetramer binding CD8 T cells, (B) Ki-67$^+$ flu-specific CD8 T cells, (C) CD38$^+$HLA-DR$^+$ flu-specific CD8 T cells, and (D) GrzB$^+$ flu-specific CD8 T cells.

effector function, as detected by upregulation of Ki-67 (Fig. 2-8B), by change in frequency of $CD38^{+}HLA\text{-}DR^{+}$ (Fig. 2-8C), or by expression of Granzyme B (Fig. 2-8D), respectively. These data strongly suggest that seasonal TIV does not induce detectable expansion or activation of peripheral blood influenza-specific CD8 T cells at two weeks following TIV. The data do not permit conclusions about a potential role for LypW in human CD8 responses to vaccine.

Discussion:

This study addresses *PTPN22* variant LypW effects on human immune responses induced in vivo. After TIV vaccination, we found that LypW carriers exhibit markedly impaired influenza-specific CD4 T cell expansion, and show no significant increase in anti-influenza antibody affinity, compared to LypR (non-carrier) subjects. While both LypR and LypW subjects show equal induction of isotype-switched antibodies against HA, LypW carriers harbor significantly higher HA-specific IgM titers at baseline and after vaccination. The immunological implication of our findings is that human LypW carriage results in multiple alterations in adaptive immune responses to anti-viral immunization. Our results also have potential clinical significance. Since numbers of influenza-specific CD4 T cells correlate with protection against influenza infection (57,75), and since high affinity anti-influenza antibodies associate with reduced viral titers after infection (46), the reduced CD4 T cell expansion and impaired affinity maturation observed in LypW carriers could translate to increased vulnerability to influenza

infection following vaccination (57,75). One important caveat to our findings is the small sample size of this study. Nevertheless, our data suggest LypW should be studied further in larger cohorts as a candidate genetic modifier of protective responses to immunization against influenza.

Impaired augmentation of antibody affinity for HA in immunized LypW carriers might stem from altered function in either T or B cell compartments. Antigen-specific CD4 T cell help to B cells is a limiting factor in generating germinal centers and flu-specific antibodies during influenza infection (54). Such CD4 T cell help promotes somatic hypermutation that results in affinity maturation (76). Thus, reduced CD4 T cell proliferation in LypW carriers could translate into less help to B cells for initiating immunization-induced germinal center responses. A second possible contributor to blocked immunization-induced anti-HA affinity maturation could be the significantly higher baseline anti-HA IgM levels we observed in LypW carriers. Pre-existing anti-HA antibodies can impair vaccine antibody responses (77). Accordingly, higher pre-vaccine HA-specific IgM levels in LypW carriers could hamper efficient affinity maturation. Additionally, overall serum HA affinity represents a summation of all affinity-matured immunoglobulin, including IgM, which is less subject to hypermutation-mediated affinity boosting than IgG or IgA. Even if immunization-induced affinity maturation is occurring among IgG subtype antibodies in LypW carriers, affinity modulation may not be detectable because greater amounts of less mutable anti-influenza IgM

overwhelm the signal arising from hypermutation-matured IgG.

The underlying cause of elevated HA-specific IgM levels in immunized LypW carriers is not clear. Studies of B cell function suggested a role for LypW in altering BCR and CD40 signaling (33–35), both of which are involved in isotype switching (77). Further quantifying frequency and phenotype of IgM+ B cells generated in LypW carriers after TIV will likely provide insight into how LypW contributes to elevated IgM levels in carriers.

Animal studies have explored roles of *PTPN22* variants in immunization responses. Early observations in *Ptpn22*-deficient mice suggested a T cell-intrinsic effect of *Ptpn22* in restraining T cell expansion to peptide immunization (8). Two groups have studied animals bearing knockins for Ptpn22-R619W, a murine LypW orthologue (24,25). R619W-expressing T cells produce more antigen-stimulated IL-2 following OVA/adjuvant immunization (24). Further, R619W knockin mice show increased antibody titers against T-dependent antigens after adjuvanted peptide immunization (24,25). Together, these data argued that in mice, *Ptpn22* functions to repress immunization-induced T cell expansion/activation and that R619W is a loss of function variant in T cell vaccination responses.

Multiple factors could contribute to apparent discordance between the above-

described animal study results and our finding of diminished CD4 expansion in TIV-exposed human LypW carriers. First, T cell responses in naïve, syngeneic mice may differ from those elicited in antigen-experienced, outbred humans. Second, the present study documents human responses to a non-adjuvanted "split" viral protein preparation, whereas animal immunizations were conducted with adjuvanted neo-antigen. Third, functional differences between murine 619W and human 620W in T cell signaling could contribute to divergent outcomes of immunization observed in human and animal systems.

Controversy exists regarding the net effect of LypW on TCR signaling in human cells. Some in vitro studies suggest that LypW carriage confers increased TCR signal strength and increased cytokine responsiveness among both naïve and memory human CD4 T cells (23,25). These data suggest that LypW acts as a loss-of-function negative regulator. In contrast, other studies suggest that in antigen-experienced human T cells, LypW exerts "gain-of-function" as a TCR signaling suppressor, perhaps due to higher phosphatase enzymatic activity (11,21,22). Such gain-of-function behaviors, which would be consistent with the T cell expansion defects described here, have not been reported for murine 619W. In the face of uncertainty about T cell-intrinsic function of LypW, our findings of diminished CD4 T cell expansion in immunized LypW carriers will require validation in larger studies of genotyped humans. Further studies should also address possibilities that LypW carriage could modulate secretion capacity for

cytokines such as IL-10 (22) and IL-2 (78) by immunization-induced CD4 T cells. Discrepancy in data concerning in vivo LypW function might also be approached through study of immunization responses by LypW-expressing memory T cells in murine models, and studies of adjuvanted neo-antigen immunizations in human LypW carriers. Finally, previous studies raise the possibility that effector T cell-extrinsic mechanisms such as altered regulatory T cell (Treg) frequency or function could contribute to the decreased CD4 response to TIV in LypW carriers (10,23,24). Whether the size or function of the human Treg compartment is altered by LypW carriage after vaccination requires further investigation.

Reports that LypW acts as a loss-of-function variant in myeloid cell pattern recognition receptor signaling (36) lead us to hypothesize that LypW could also differentially regulate vaccine-induced CD4 expansion by altering innate immune activation. Vaccine-induced, IFN-driven transcriptional responses and costimulatory molecule upregulation on APC subsets have been linked to pattern recognition receptor signaling (63). Therefore we were surprised that we detected no LypW-dependent alterations in APC activation after TIV exposure in the present study (Fig. 4). However, interpretation of these results is complicated by the fact that LypW-bearing influenza-specific T cells present in PBMC could be modulating APC responses via cytokine secretion or contact-dependent processes. In addition, O'Gorman *et al* found that TIV could engage human innate immune cells through Fc receptors, independently of TLR (79). In the

current study, we observed no effect of LypW carriage on innate immune cell-produced cytokines (e.g. CXCL10, data not shown) or transcriptional responses to IFNγ, both of which are reportedly induced by TIV-immune complex stimulation of Fc receptors (79). Thus, the degree to which diminished CD4 T cell expansion to TIV in LypW carriers reflects differential modulation of innate immune responses remains to be determined.

Chapter 3: *Ptpn22* modulates memory CD4 T cell responses to antigen-stimulation

Abstract

Studies of human LypW carriers' immune response to influenza immunization revealed a defect in antigen-specific CD4 T cell expansion. An absence of evidence for T cell-extrinsic effects of LypW on CD4 T cell responses suggested that the expansion defect was due to intrinsic TCR signaling effects of LypW. We use *Ptpn22* KO mice and humanized LypR and LypW transgenic mice to determine whether *Ptpn22* deficiency and genetic variants of *PTPN22* exert repression of antigen-specific T cell proliferation after TCR stimulation. Using multiple immunization strategies we show that naïve *Ptpn22* KO CD4 T cells do not have a defective proliferation response to antigen priming. Memory *Ptpn22* KO and LypW CD4 T cells, however, do exhibit defects in fold proliferation after antigen re-challenge. These studies provide a useful animal model for studying the mechanisms by which genetic variants of *PTPN22* alter memory CD4 T cell responses to immunization.

Introduction

Our previous study of the responses of healthy human carriers of the disease-associated *PTPN22* R620W variant to Trivalent Inactivated Influenza Vaccine (TIV) revealed a loss of function with respect to antigen-specific CD4 T cell expansion (80). The lack of defect in the innate immune response to TIV immunization suggests that this is a result of T cell-intrinsic function of *PTPN22* R620W (termed LypW). To study the mechanism by which *PTPN22* and genetic variants of *PTPN22* modulate CD4 T cell expansion during immunization, it is necessary to develop an animal model that replicates the CD4 T cell expansion defect displayed by human LypW carriers.

Current knowledge about *Ptpn22* function in immunization responses has relied upon characterizing bulk (non antigen-specific) CD4 T cells (24,26), or by using TCR transgenic models (8). Conclusions from these studies showed that *Ptpn22* functions to modulate CD4 T cell effector polarization responses, yielding higher frequencies of T follicular helper cells (T_{FH}) (26), and that loss of *Ptpn22* function results in increased CD4 T cell proliferation responses to immunization (8).

When studying the effect of *Ptpn22* on CD4 T cell responses to immunization, it is important to consider that the phenotypes observed are directly altered by the strength of T cell antigen receptor signaling capacity. Thus it is imperative to identify and isolate the T cells responding to antigen from the T cells that remain unstimulated. Studying bulk CD4 T cell responses to antigen

stimulation may therefore complicate observed phenotypes of *PTPN22* variants and T cell antigen receptor stimulus. The use of a TCR transgenic system allows identification of antigen-specific T cell responses, and thus is a better system for elucidating *PTPN22*-mediated effects on T cell immunization responses. However one important caveat to these studies is effect of naïve T cell precursor frequency on proliferation responses (81,82). A high frequency of transferred TCR transgenic T cells has been shown to substantially alter proliferative expansion and also modifies the inflammatory environment (81). The early experiments investigating immunization responses in *Ptpn22* KO TCR transgenics utilized a high transfer model of OT-1 T cells, transferring $1x10^6$ cells per mouse (8). This high transfer rate can have profound effects on T cell proliferation, and make conclusions about *Ptpn22* effects on proliferation difficult.

A clearer picture of antigen-specific T cell responses to TCR stimulation can be observed by the use of peptide:MHC class II tetramers (83). Using p:MHCII tetramer reagents allows us to identify and track all of the T cells with receptors specific for a given peptide antigen in the context of its MHC (83). Using this p:MHCII tetramer method, we tracked antigen-specific CD4 T cell responses to immunization in *Ptpn22* KO mice and in humanized LypR and LypW transgenic mice.

Methods

Mice

C57Bl6 (B6) wild type (WT) and B6 CD45.1 recipient mice were purchased from the Jackson Laboratories (Bar Harbor, ME). *Ptpn22* KO mice were crossed >10 times to the B6 strain, and have been described (8). LypR and LypW transgenic mice were generated as previously described (36). All mice were maintained under specific pathogen free conditions and all studies were conducted in accordance with Institutional Animal Care and Use Committee-approved protocols at the University of Minnesota (Minneapolis, MN).

Flow Cytometry and Reagents

Cell surface staining for flow cytometry was performed with ice cold PBS supplemented with 2% fetal bovine serum (FACS buffer). Single cell suspensions were washed with FACS buffer, stained with surface antibodies, then washed twice before fixing with 4% paraformaldehyde. After fixing, cells were washed once and resuspended in FACS buffer for multiparameter flow cytometric acquisition on a BD LSR Fortessa (Becton Dickinson, San Jose, CA). For intracellular transcription factor staining, after surface staining and washing, cells were permeabilized using the Transcription Factor Staining Buffer Kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. Cells were then stained with intracellular antibodies at 4°C overnight. Cells were then washed twice with Permeabilization buffer and resuspended in FACS buffer for flow cytometric acquisition. Directly conjugated fluorescent antibodies used included α-CD3ε, α-CD4, α-CD8, α-CD11c, α-CD11b, α-F4/80, α-NK1.1, α-Ly6G,

α-CD19, α-PD1, α-CXCR5, α-CD69, α-Tbet, α-Bcl6, α-RORγt, α-Ki-67.

Immunizations and Infections

Influenza virus H1N1 strain A/PuertoRico/8/193 (PR8) was provided by Ryan Langlois at the University of Minnesota (Minneapolis, MN). *Listeria monocytogenes* expressing the influenza NP311 CD4 epitope (Lm-NP311) was provided by Thomas Griffith from the University of Minnesota (Minneapolis, MN). 2W1s peptide sequence EAWGALANWAVDSA was purchased from GenScript (Piscatawway, NJ). For immunization, peptide was emulsified in Complete Freund's Adjuvant (Sigma), and injected i.p. into mice. Whole inactivated influenza virus was prepared as previously described (84). Mice were anesthetized with isoflurane and immunized via intranasal instillation of 25μL of flu vaccine (50μg/mouse). Live influenza virus infections were also administered via the intranasal route under isoflurane anesthesia, with 40 pfu per mouse in 25μL. For Lm-NP311 infections, mice were injected i.v. in the tail vein with $1x10^7$ cfu in 100μL PBS.

Tetramer Reagents and Cell Isolations

2W1s:I-A^b-APC conjugated tetramers were provided by Marc Jenkins at the University of Minnesota (Minneapolis, MN). NP311:I-A^b-APC conjugated tetramers were provided by Thomas Griffith at the University of Minnesota

(Minneapolis, MN). For tetramer-specific CD4 T cell isolations, 1 µL of tetramer reagent was added per 99 µL single cell suspension and cells were incubated in the dark at room temperature for 1 hour. The StemCell EasySep™ Mouse APC Positive Selection kit was used according to manufacturer's instructions to isolate tetramer-bound cells (StemCell Technologies, Vancouver BC, Canada). For generating single cell suspensions of lung lymphocytes lungs were minced into small pieces with scissors, and treated with Type I collagenase in complete RPMI for 30 minutes in a 37°C rotating incubator. Lung pieces were washed in complete RPMI and then incubated with 1µM EDTA in complete RPMI for 30 minutes in a 37°C rotating incubator. Cells were filtered and lymphocytes purified by Percoll gradient. The buffy layer was isolated and cells were washed with complete RPMI before proceeding to surface antibody staining. For memory CD4 T cell isolations, single cell suspensions were selected with the EasySep™ Mouse Memory $CD4^+$ T cell isolation kit from StemCell Technologies according to the manufacturer's instructions. Post negative selection, an aliquot of purified cells was stained with α-CD4 and NP311:I-A^b tetramer to determine purity and frequency of tetramer-positive CD4 T cells. Cells were washed and resuspended in PBS, and 80,000 tetramer-positive CD4 T cells were injected per mouse i.v. in the tail vein.

Bone Marrow Chimeras

Mixed bone marrow chimeras were generated by mixing T cell-depleted bone

marrow preparations from Thy1.1^{+} (WT) and CD45.2^{+} (*Ptpn22* KO) mice at a 1:1 ratio and injecting 10x10^{6} cells into lethally irradiated (1100 rads) CD45.1^{+}/Thy1.2^{+} recipient animals (85).

Statistics

All statistical tests were performed using Prism GraphPad software version 6. The data were unpaired and non-parametric, therefore we employed the Mann-Whitney statistical test to compare data sets.

Results

No difference in *Ptpn22* KO and WT antigen-specific CD4 T cell expansion during primary immunization

In order to further test T cell-intrinsic mechanisms of *Ptpn22* action that would prevent normal expansion of CD4 T cells to immunization, we utilized mouse models of *Ptpn22* deficiency to study immunization responses. Using peptide:MHC-class II tetramer reagents we can track antigen-specific CD4 T cells responding to immunization. We immunized C57BL/6 wild-type mice (WT) and *Ptpn22* KO mice with a whole-inactivated influenza virus vaccine, or with 2W1s peptide emulsified in CFA and measured the antigen-specific CD4 T cell response at peak of expansion. Surprisingly, we did not detect a difference in the number of tetramer-positive CD4 T cells in *Ptpn22* KO animals after flu or 2W1s/CFA immunizations (Figure 3-1 A,C). Although we did not detect

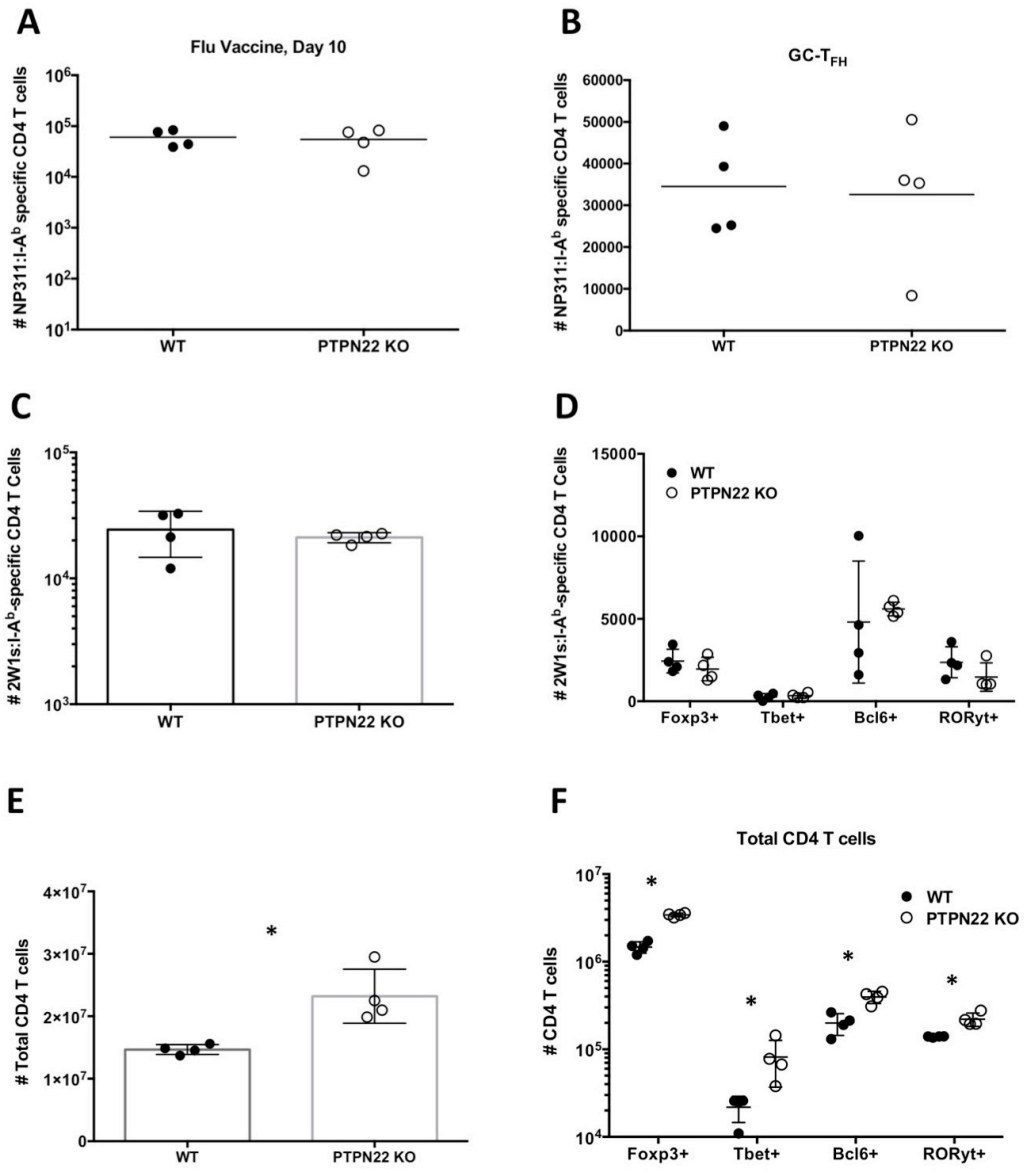

Figure 3-1: PTPN22 does not alter CD4 T cell proliferation or T_H effector polarization

(A-B) WT and PTPN22 KO mice were immunized i.n. with whole-inactivated influenza virus vaccine, and NP311:I-A^b-specific CD4 T cell responses were measured at Day 10 (A). (B) Number of NP311:I-A^b-specific CD4 T cells that express GC-T_{FH} markers $PD1^{hi}CXCR5^+$. (C-F) WT and PTPN22 KO mice were immunized with 2W1s peptide in CFA i.p. and 2W1s:I-A^b-specific CD4 T cells were enumerated on Day 7 (C). (D) Number of 2W1s:I-A^b-specific CD4 T cells positive for the transcription factors Foxp3, Tbet, Bcl6 or RORγt. (E) Number of total CD4 T cells in WT and PTPN22 KO mice. (F) Number of total CD4 T cells in WT or PTPN22 KO mice expressing Foxp3, Tbet, Bcl6 or RORγt. * P<0.05, Mann-Whitney. Mean ±S.D.

differences in absolute number of antigen-specific CD4 T cells, it has been shown that human LypW carriers and *Ptpn22* KO mice have altered CD4 T cell differentiation profiles in response to TCR stimulation (23,26). We therefore asked whether immunization would induce altered T_{Helper} cell differentiation in *Ptpn22* KO mice. After flu immunization we saw equal frequencies of Germinal Center-T_{FH} cells, as measured by co-expression of the chemokine receptor CXCR5 and high levels of PD1 (Figure 3-1 B). In the 2W1s/CFA immunization we saw equal frequencies of antigen-specific CD4 T cells expressing Tbet, Foxp3, Bcl6 and RORγt in WT and *Ptpn22* KO mice (Figure 3-1 D). This indicates that for both immunization strategies, PTPN22 deficiency does not alter the differentiation of CD4 T cells. When the bulk non-antigen-specific CD4 T cell population was analyzed for T cell differentiation after 2W1s/CFA immunization, there was an increased frequency and number of *Ptpn22* KO T cells expressing Tbet, Foxp3 or Bcl6 (Figure 3-1 F), a finding consistent with results published by Maine et al. (26). However this is likely due to the increased total number of CD4 T cells in the *Ptpn22* KO mice (Figure 3-1 E) and not an alteration of CD4 T cell polarization due to *Ptpn22* deficiency. Thus in a primary immunization we saw no difference in antigen-specific CD4 T cell expansion or differentiation.

Ptpn22 KO and WT mice have no difference in precursor frequency of antigen-specific CD4 T cells

No difference in peak expansion or differentiation of antigen-specific CD4 T cells

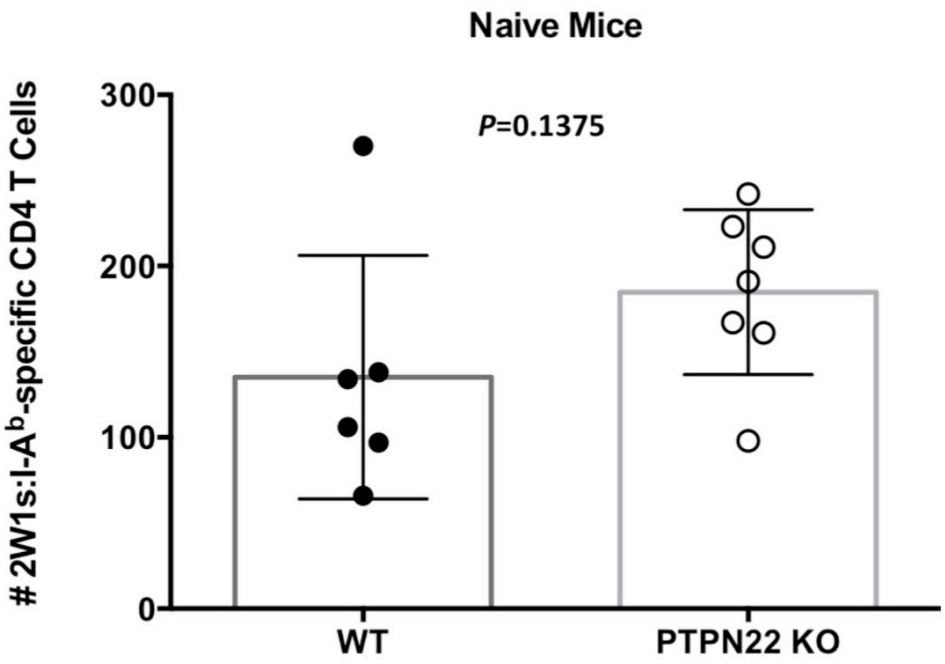

Figure 3-2: PTPN22 KO mice do not have altered naïve T cell precursor frequency
Number of 2W1s:I-A^b-specific CD4 T cells in naïve WT and PTPN22 KO mice. Mann-Whitney, Mean ± S.D.

was detected in immunized *Ptpn22* KO mice. However if *Ptpn22* deficiency results in an altered T cell repertoire, there could be differences in the precursor frequency of a given antigen-specificity compared to WT animals. If this were true, then there would be a difference in the fold-expansion of *Ptpn22* KO CD4 T cells compared to WT. To test this hypothesis we measured the number of 2W1s:I-A^b-specific CD4 T cells in naïve WT and *Ptpn22* KO mice. We found no difference in the precursor frequency of 2W1s:I-A^b-specific CD4 T cells in WT and *Ptpn22* KO animals (Figure 3-2). The equal precursor frequency of 2W1s:I-A^b-specific CD4 T cells and equal numbers of 2W1s:I-A^b-specific CD4 T cells after immunization between WT and *Ptpn22* KO mice (Figure 3-1 C) indicate that there is no difference in fold expansion in *Ptpn22* KO CD4 T cells. These data confirm that during primary immunization responses, there is no defect in CD4 T cell proliferation of *Ptpn22* KO mice.

T cell-extrinsic effects of *Ptpn22* KO do not alter CD4 T cell expansion during immunization

Although our data show a lack of difference in T cell expansion or differentiation during priming of antigen-specific CD4 T cells during immunization, it is unclear whether *Ptpn22* KO has no effect on T cell-intrinsic signaling, or whether any TCR signaling effects related to *Ptpn22* deficiency are masked by effects of this deficiency to other cellular components that are important for effective T cell priming. It has been described by our group and others (36,37,39) that *Ptpn22*

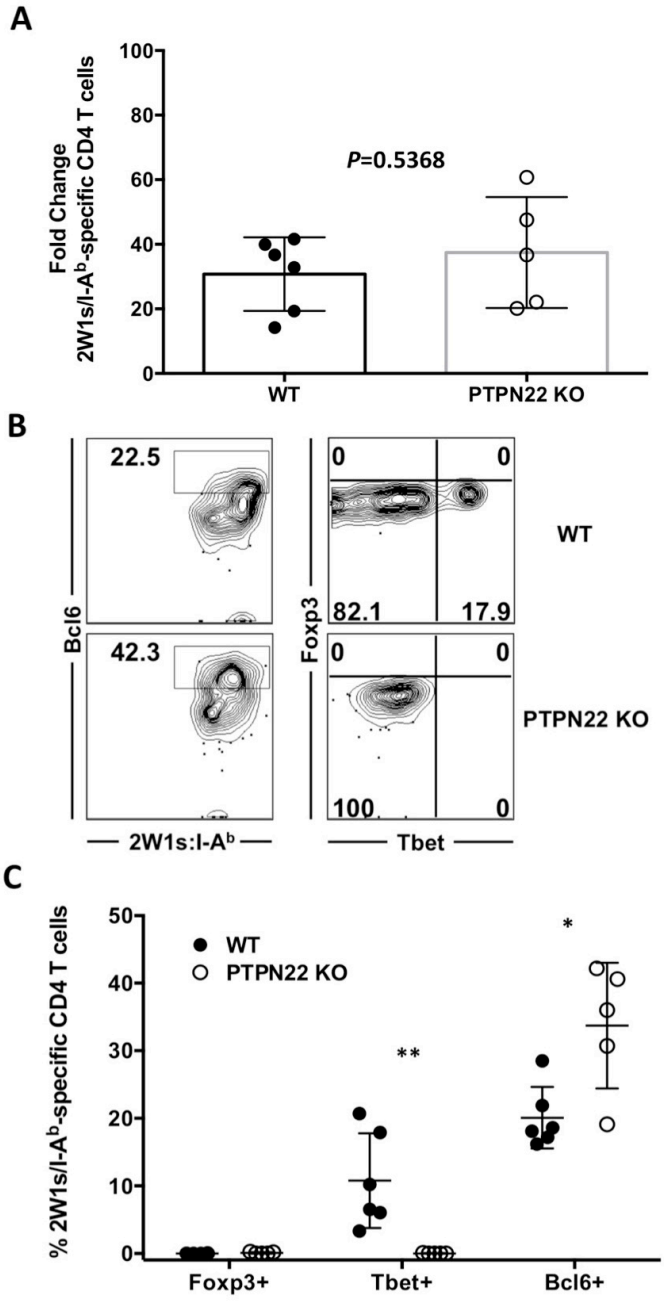

Figure 3-3: T cell-intrinsic effects of PTPN22 do not alter primary CD4 T cell expansion

Mixed bone marrow chimeric mice were generated using 1:1 ratio of WT and PTPN22 KO bone marrow. Fully reconstituted animals were then immunized with 2W1s peptide in CFA i.p. and analyzed for CD4 T cell responses at Day 7. (A) Fold expansion of 2W1s:I-A^b-specific WT and PTPN22 KO CD4 T cells. (B) Representative intracellular staining and (C) frequency of 2W1s:I-A^b-specific CD4 T cells expressing Foxp3, Tbet or Bcl6 in WT and PTPN22 KO mice. *P<0.05, ** P<0.01 Mann-Whitney, Mean ± S.D.

has important regulation functions in TLR and inflammasome signaling pathways in myeloid cell subsets, which can affect their maturation and the cytokine milieu generated. These defects in myeloid cells, including dendritic cells, can alter the efficiency of T cell priming (36). To isolate the effect of *Ptpn22* deficiency on T cell-intrinsic function we generated 1:1 mixed bone marrow chimeras with WT and *Ptpn22* KO bone marrow. The resulting chimeric mice have equal fractions of WT and *Ptpn22* KO myeloid cells important for priming, allowing effects of *Ptpn22* on T cell-intrinsic responses to be measured. We immunized chimeric mice with 2W1s/CFA and measured their antigen-specific CD4 T cell response. The fold-expansion of WT and *Ptpn22* KO CD4 T cells specific to 2W1s:I-A^b were not significantly different at day 7-post immunization (Figure 3-3 A), indicating that *Ptpn22* deficiency in T cells does not alter the proliferation capacity of naïve CD4 T cells during their initial exposure to antigen.

To determine whether T cell-intrinsic function of *Ptpn22* modulates CD4 T cell differentiation during priming, we also assessed the frequency of T_{reg}, T_{H1} and T_{FH} polarization in the chimeric mice. *Ptpn22* KO 2W1s:I-A^b specific CD4 T cells exhibited a statistically significant increase in the transcription factor Bcl6, and a statistically significant decrease in the transcription factor Tbet, compared to WT antigen-specific CD4 T cells (Figure 3-3 B,C). This suggests that the loss of *Ptpn22* function during priming modulates CD4 T cell polarization away from the T_{H1} lineage in favor of the T_{FH} lineage. Neither the WT nor *Ptpn22* KO 2W1s:I-A^b-

specific CD4 T cells had measurable expression of Foxp3, indicating that there were little to no generation of antigen-specific regulatory T cells (Figure 3-3 B,C). Thus *Ptpn22* is not required for normal proliferation of naïve CD4 T cells during initial antigen-encounter, but is likely important for modulating CD4 T cell effector differentiation.

Antigen-experienced CD4 T cells have decreased expansion to secondary challenge in *Ptpn22 KO* mice

Previous experiments showing no difference in *Ptpn22* KO CD4 T cell expansion were conducted in naïve mice, encountering antigen for the first time. While there seems to be no defect in *Ptpn22* KO T cell priming, it has been shown that effects of *Ptpn22* on TCR signaling are most pronounced in antigen-experienced T cells (22). We therefore asked whether *Ptpn22* KO CD4 T cells would have altered expansion during a recall response to antigen. To test this question we immunized WT and *Ptpn22* KO mice with whole-inactivated influenza virus vaccine, waited 35 days and then challenged mice with *Listeria monocytogenes* expressing the influenza CD4 epitope NP311 (Lm-NP311). This heterologous challenge avoids the effect of neutralizing antibody on limiting availability of antigen for CD4 T cell recall responses to the NP311 epitope. Similar to previous experiments, antigen-specific CD4 T cell expansion in *Ptpn22* KO mice was equal to WT at day 9-post immunization (Figure 3-4 A). At day 35-post immunization, *Ptpn22* KO mice showed no significant difference in NP311:I-A^b-

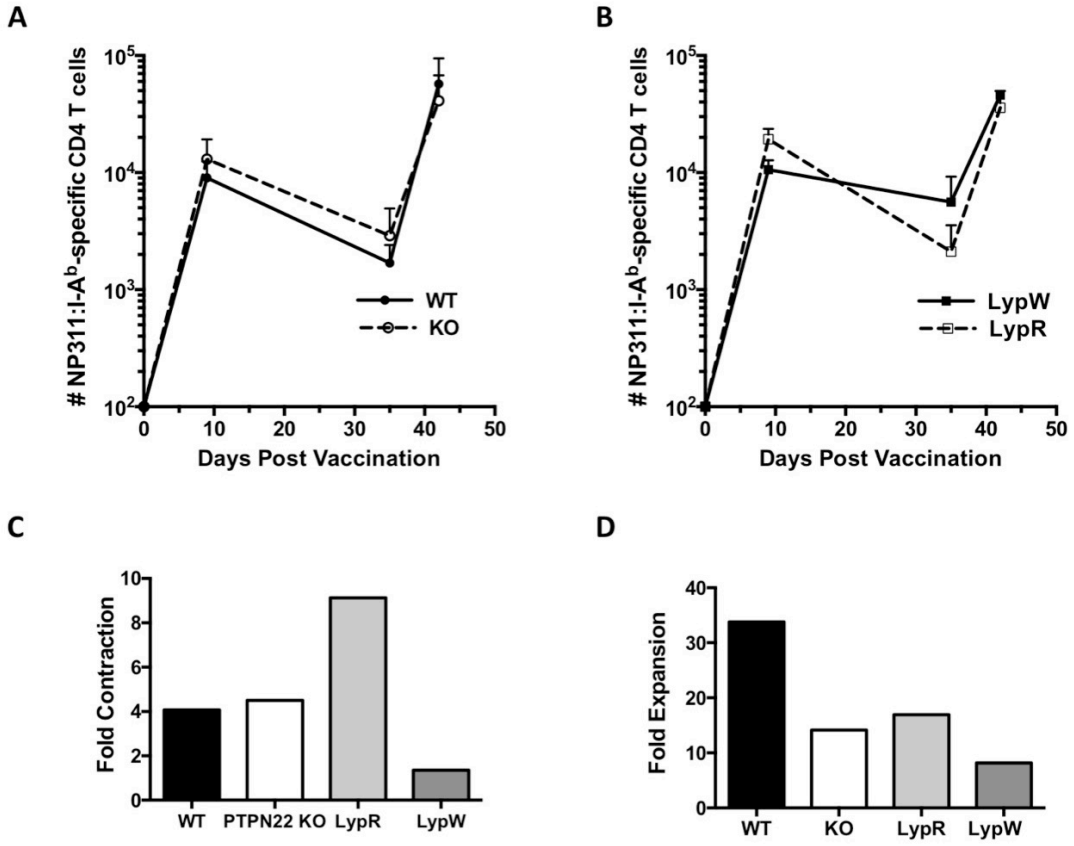

Figure 3-4: Antigen-experienced PTPN22 KO and LypW CD4 T cells display decreased expansion

(A) WT and PTPN22 KO or (B) LypR and LypW transgenic mice were immunized with whole-inactivated influenza vaccine, and NP311:I-A^b-specific CD4 T cells were monitored at primary (Day 9) and memory (Day 35) phases before re-challenge with Lm-NP311 at Day 35. Secondary expansion of NP311:I-A^b-specific CD4 T cells was measured at Day 7 post re-challenge. (C) Fold contraction (Day 10/Day 35) and (D) fold expansion (Day 42/Day35) of NP311:I-A^b-specific CD4 T cells in WT, PTPN22 KO, LypR and LypW mice.

specific CD4 T cells (Figure 3-4 A). Upon secondary challenge with Lm-NP311 there was no significant difference in absolute number of NP311:I-A^b-specific CD4 T cells in WT and *Ptpn22* KO mice (Figure 3-4 A). The mean NP311:I-A^b-specific CD4 T cell number at day 35 and day 9 post immunization and day 7 post Lm-NP311 challenge was used to calculated the fold-contraction and fold-expansion of antigen-specific T cells in WT and *Ptpn22* KO mice. While *Ptpn22* KO and WT mice had similar rates of contraction (Figure 3-4 C), the fold expansion of *Ptpn22* KO CD4 T cells was half that of the WT (Figure 3-4 D). This suggests that *Ptpn22* is important for efficient T cell expansion in antigen-experienced T cells.

We assessed the CD4 T cell polarization profiles of the WT and *Ptpn22* KO mice at each stage of the experiment, but found that there were no statistically significant differences in the frequency or absolute number of T_{reg}, T_{H1}, T_{FH} or T_{H17} cells at primary (Figure 3-5 A) memory (Figure 3-5 C) or secondary challenge to antigen (Figure 3-5 E). This corroborates our earlier findings that CD4 T cell polarization is unaffected in intact *Ptpn22* KO mice.

In order to understand whether the human *PTPN22* variant LypW has a similar effect on antigen-experienced T cell expansion, we performed the same primary and secondary challenge on transgenic mice expressing either the human LypW gene variant, or the LypR major allele as a control. Similar to the *Ptpn22* KO

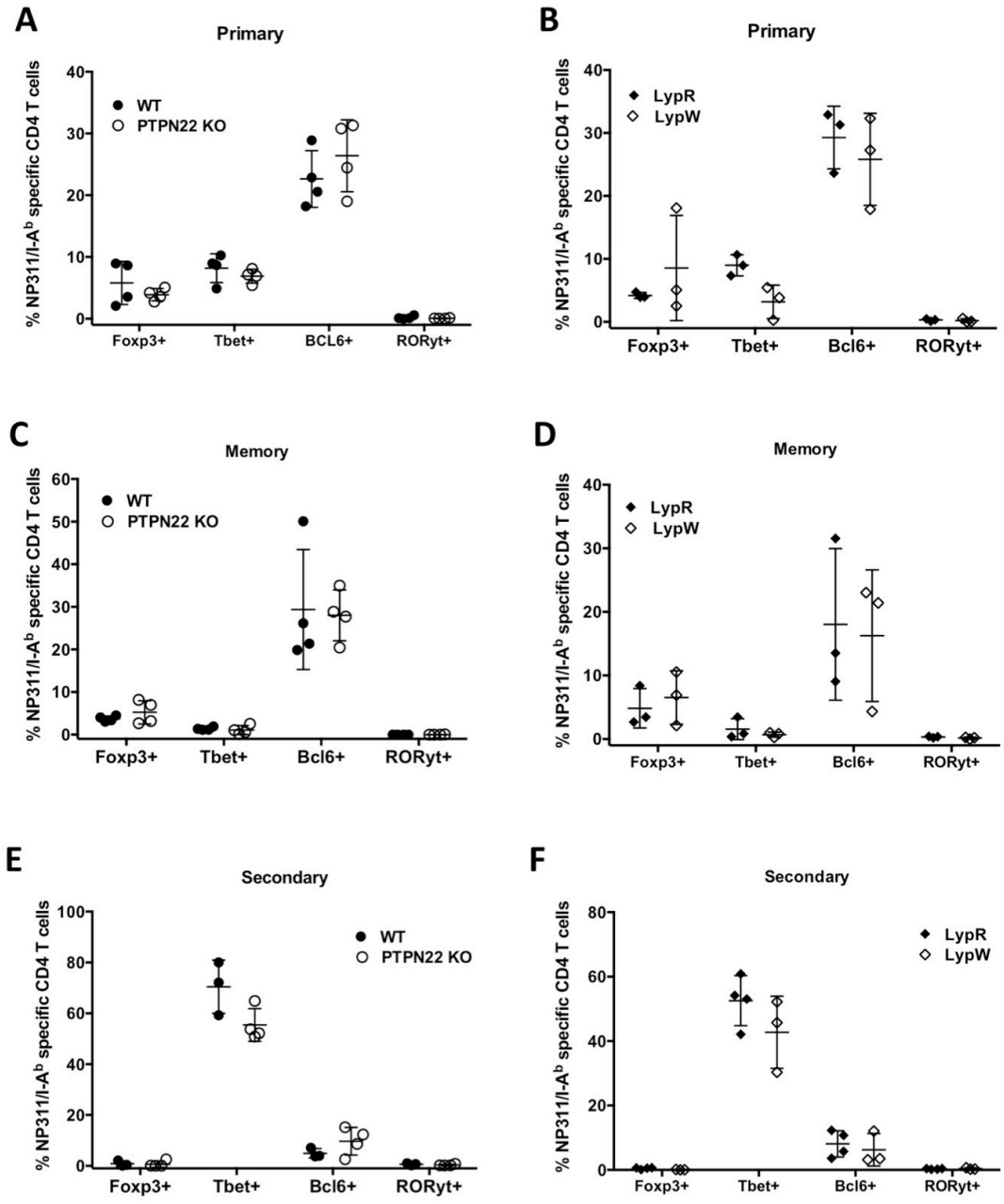

Figure 3-5: PTPN22 KO and LypW transgenic mice do not have altered T_{Helper} polarization.

Frequency of NP311:I-A^b-specific CD4 T cells in WT and PTPN22 KO (A, C, E) or LypR and LypW transgenic (B, D, F) mice immunized with whole-inactivated influenza vaccine and challenged with Lm-NP311 at Day 35 post immunization. (A-B) Day 9 post immunization. (C-D) Day 35 post immunization. (E-F) Day 7 post Lm-NP311 challenge. Mann-Whitney, Mean ± S.D.

mice, LypW mice had no significant difference in absolute numbers of NP311:I-A^b-specific CD4 T cells at day 9 or day 35-post primary immunization (Figure 3-4 B), nor at day 7-post secondary challenge with Lm-Np311 (Figure 3-4 B). However, the LypW variant mice exhibited a very low rate of contraction (1.4 fold) compared to that of the LypR mice (9.1-fold) (Figure 3-4 C). This suggests that LypW may function to promote survival during the normal contraction phase of effector CD4 T cells after immunization. Similar to the *Ptpn22* KO, upon secondary challenge LypW mice exhibited a decreased expansion rate (8.2 fold) that was about half the expansion rate of the LypR control mice (16.9-fold) (Figure 3-4 D). This suggests that LypR normally functions to promote T cell expansion in antigen-experienced cells, but the LypW variant loses that function.

We assessed the CD4 T cell polarization profiles of the LypR and LypW transgenic mice at each stage of the experiment, and found the same results as the WT and *Ptpn22* KO mice. No significant differences in T_{reg}, T_{H1}, T_{FH} or T_{H17} cells were found at primary (Figure 3-5 B), memory (Figure 3-5 D) or secondary challenge to NP311 antigen (Figure 3-5 F). Thus like *Ptpn22* KO, intact LypW transgenic mice display no defect or difference in CD4 T cell polarization during response to antigen stimulation

Antigen-experienced *Ptpn22* KO CD4 T cells may have T cell-intrinsic defect in expansion

One caveat for interpreting the T cell proliferation capacity in intact *Ptpn22* KO

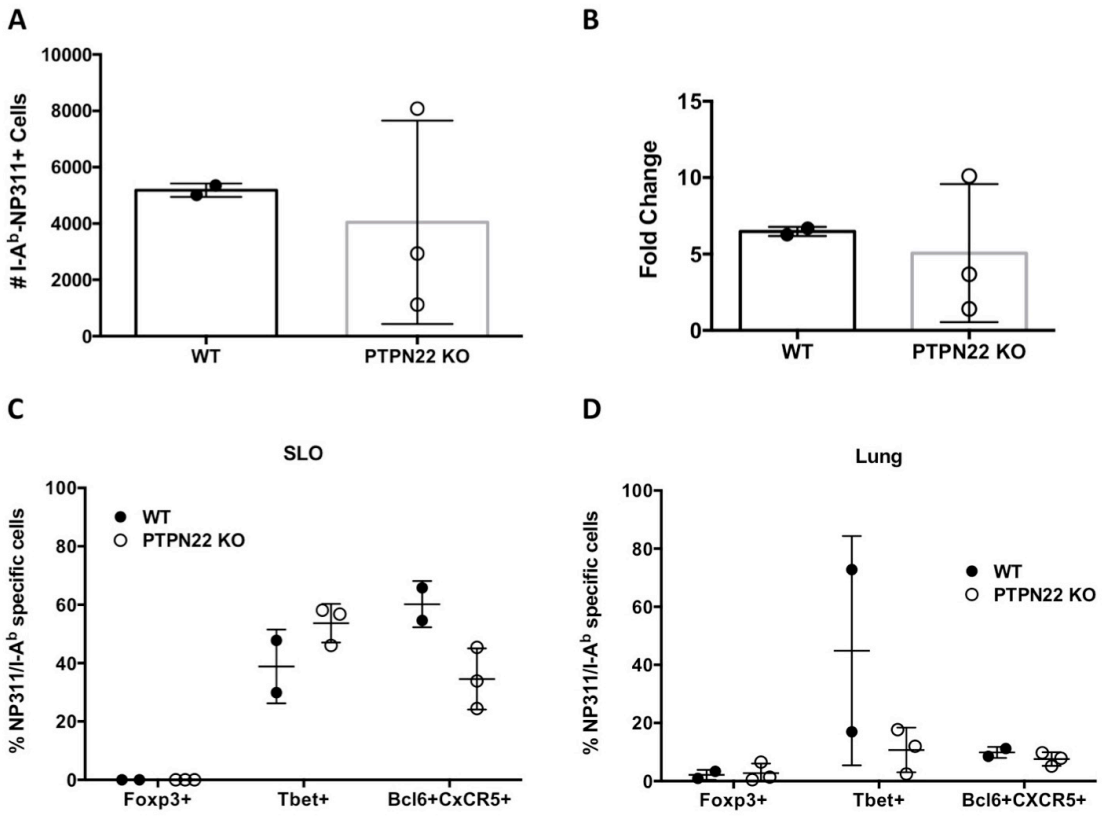

Figure 3-6: Antigen-experience PTPN22 KO CD4 T cells may have altered proliferation during secondary challenge to antigen.
WT and PTPN22 KO mice were infected i.v. with Lm-NP311 and memory NP311:I-A^b-specific CD4 T cells were isolated at Day 35. Purified NP311:I-A^b-specific T cells were adoptively transferred into WT CD45.1 recipients, which were then infected with influenza virus PR8. (A) Number and (B) fold expansion of NP311:I-A^b-specific CD4 T cells Day 7 after PR8 infection. Frequency of NP311:I-A^b-specific CD4 T cells expressing Foxp3, Tbet, or Bcl6 and CXCR5 in the (C) secondary lymphoid organs (SLO) and (D) lungs of infected mice. Mean ± S.D.

and LypW mice is whether the loss of *Ptpn22* function in other cells contributes to the T cell expansion phenotype observed in antigen-experienced T cells. In order to assess the T cell-intrinsic role of *Ptpn22* deletion we performed adoptive transfers of memory antigen-specific CD4 T cells into WT recipients. WT or *Ptpn22* KO mice were infected with *Listeria monocytogenes* expressing the NP311 epitope of influenza virus (Lm-NP311) and at day 35 post-infection memory CD4 T cells were isolated and an equal number of NP311:I-A^b-specific CD4 T cells were transferred into CD45.1 recipient mice. Following adoptive transfer, recipients were infected with influenza virus via intranasal inoculation. A park rate of 1% for transferred cells was assumed, and NP311:I-A^b-specific CD4 T cell expansion of transferred cells was enumerated at day 7 post-infection. Unfortunately, three of the recipient mice succumbed to viral infection on day 6, two of which received WT CD4 T cells and one of which received *Ptpn22* KO CD4 T cells. This resulted in insufficient *N* to perform statistical analysis of T cell expansion, however some interesting results were observed that do inform future studies of the role of *Ptpn22* in T cell expansion. Both recipients that received WT memory CD4 T cells reached similar numbers of NP311:I-A^b-specific CD4 T cells ($5.0x10^3$ and $5.3x10^3$) at day 7 post-infection (Figure 3-6 A). The three recipients that received *Ptpn22* KO memory CD4 T cells exhibited a bimodal distribution of T cell expansion, with two animals having lower absolute numbers of NP311:I-A^b-specific CD4 T cells ($1.1x10^3$ and $2.9x10^3$), and one animal having a higher absolute number of NP311:I-A^b-specific CD4 T cells ($8.0x10^3$)

(Figure 3-6 A). A similar trend was found for fold-expansion, with WT antigen-specific memory CD4 T cells exhibiting an average fold change of 6.48±0.3 (Figure 3-6 B), while two of the *Ptpn22* KO recipients had lower fold-expansion of memory CD4 T cells (1.4 and 3.7-fold) and one exhibited a higher expansion rate (10.1-fold) (Figure 3-6 B). The low *N* of the experiment, and the bimodal distribution of the *Ptpn22* KO results preclude a definitive conclusion, however they suggest that *Ptpn22* likely plays a T cell-intrinsic role in modulating the expansion of memory T cells upon encounter with antigen.

While unable to calculate statistics due to the low experimental *N*, there are nevertheless notable trends in the T_{Helper} polarization profiles in WT and *Ptpn22* KO memory CD4 T cells. In the secondary lymphoid organs (SLO) there was a much greater frequency of T_{H1} and T_{FH} cells in both groups than was present in the lung (Figure 3-6 C-D). The *Ptpn22* KO NP311:I-A^b-specific CD4 T cells displayed a trend of lower T_{FH} frequencies and higher T_{H1} frequencies after PR8 infection when compared to WT (Figure 3-6 C-D). However, the polarization of NP311:I-A^b-specific CD4 T cells after primary infection with Lm-NP311 was not assessed before adoptive transfer, which complicates conclusions about the role of *Ptpn22* in altering T_{Helper} polarization in memory CD4 T cells upon encounter with antigen.

Discussion

We show here the first demonstration of the effect of *Ptpn22* on polyclonal, antigen-specific CD4 T cell responses to TCR stimulation *in vivo*. These studies reveal that loss of *Ptpn22* has no effect on the proliferation of naïve antigen-specific CD4 T cells responding to immunization. Alternatively, loss of *Ptpn22* does affect the proliferation response of memory CD4 T cells when re-stimulated with cognate antigen. However whether the effect is a gain of proliferative function or a loss of proliferative function remains to be conclusively shown. Additionally, evidence from mixed bone marrow chimeras indicates that T cell-intrinsic function of *Ptpn22* may serve to regulate the differentiation of CD4 T cells into different T_{Helper} subsets.

The adoptive transfer experimental results bring out a conundrum with regard to *Ptpn22* function in T cell responses. The bimodal results in *Ptpn22* KO T cell fold expansion suggest two possibilities, that absence of *Ptpn22* in memory cells results in more rapid proliferation to antigen stimulation, or the lack of *Ptpn22* in memory T cells serves to repress proliferative responses. The former possibility seems more likely in the context of the scientific literature pointing to *Ptpn22* as a repressor of TCR signaling. However two out of the three replicates actually displayed decreased proliferative responses to antigen-stimulation, suggesting that the latter explanation is a possibility. One important caveat to the interpretation of these results is the assumption of an equal park rate amongst

adoptively transferred cells. If *Ptpn22* deficiency has an effect on the ability of memory CD4 T cells to establish a niche within a fully immunocompetent mouse, that could dramatically alter the calculations of fold expansion. Thus replication of these adoptive transfer experiments, and analysis of the effect, if any, of *Ptpn22* deficiency on park rate of the adoptively transferred cells will be necessary to gain clarity.

Studies of antigen re-stimulation in mice expressing the human *Ptpn22* major LypR variant and autoimmune-associate LypW variant reveal that LypW seems to exert a gain of function as a TCR signaling repressor. These results are consistent with studies of human antigen-specific CD4 T cell responses to influenza immunization in human LypW carriers, which displayed defects in CD4 T cell expansion (80). An important future direction is to investigate the T cell-intrinsic role of LypW on antigen-specific CD4 T cell proliferation responses. Using adoptive transfer of memory antigen-specific CD4 T cells into WT recipients, similar to what has been done with *Ptpn22* KO T cells, we can determine whether LypW alterations of TCR signaling result in decreased proliferation.

One surprising observation was the effect of LypW on CD4 T cell contraction after T cell priming. The decreased rate of contraction in LypW CD4 T cells has not been described before, however it is known that both animal

knockins of the R619W orthologue and human LypW carriers exhibit increased accumulation of effector/memory phenotype cells over time (22,24). One favored hypothesis for this accumulation was that it reflected an increased rate of priming, due to more sensitive TCR signaling. However our observation of normal priming of naïve CD4 T cells and decreased contraction after antigen encounter would suggest that LypW is exerting increased survival signals to the cell. A similar mechanism has been shown in human B cells from LypW carriers, where stimulation of the B cell receptor resulted in higher levels of Bcl2 expression, and resistance to apoptosis (33). Future work into possible mechanisms by which LypW exerts a reduced rate of contraction on CD4 T cells should investigate whether T cells express higher levels of the pro-survival factor Bcl2 and whether that results in increased resistance to apoptosis.

Chapter 4: Discussion

PTPN22 variant R620W in human vaccine immunity

This work has characterized the first effort to measure the effect that the PTPN22 autoimmune-associated gene variant LypW exerts on *in vivo* response to immunization in humans. Carriers of the LypW variant display decreased antigen-specific CD4 T cell expansion and defective antibody affinity maturation in response to the trivalent influenza vaccine (TIV). These data support the conclusion that in regard to lymphocyte antigen receptor signaling, the LypW variant acts as a gain of function as a signal repressor. High levels of influenza-specific CD4 T cells and high affinity antibodies to the influenza HA protein correlate with increased protection to influenza infection (46,56), thus carriers of LypW are hypothesized to have an increased risk of influenza infection after immunization with TIV. Future studies of human LypW carriers should seek to determine if LypW confers increased risk for infection with influenza virus in immunized individuals, as well as whether LypW carriage confers increased risk to infection with influenza in general. Because the influenza vaccine does not induce a measurable response in CD8 T cells, it remains to be shown whether the LypW variant exerts similar defects in CD8 T cell proliferation and effector function responses to immunization. To understand the role of LypW in human CD8 T cell responses it will be important to use a vaccine that induces robust CD8 T cell immunity. One candidate is the Yellow Fever vaccine (86). Studying

Yellow Fever vaccination in human LypW carriers will not only allow determination of whether LypW exerts repressive function on CD8 T cell immune responses, but will also provide an opportunity to study the effect of LypW on innate immune responses *in vivo.* Unlike the influenza vaccine, the Yellow Fever vaccine is a live-attenuated virus, which allows for intracellular replication. This in turn provides important ligands for induction of innate TLR and other PRR signaling to induce a strong T1 IFN response. Thus it is likely that human LypW carriers will exhibit defective innate T1 IFN responses and adaptive CD8 T cell responses to Yellow Fever immunization. Studies of the effect of LypW on different vaccine formulations are necessary to establish whether human LypW carriers are at an increased risk of infection due to defective or incomplete response to immunization.

Ptpn22 and the LypW variant in mouse models of immunization

Studies of immunization responses in human LypW carriers provide critical insight into the mechanisms by which LypW alters immune function, however there are several limitations of human research that make it difficult to tease apart the multiple effects of LypW in different immune cell types and how those cell types interact with one another in an immunization response. This is where studying immunization responses in mice offer several benefits. Mouse models avail the use of multiple genetic and cellular manipulations in order to pinpoint the effects that *Ptpn22* exerts on each cell type individually and the ways

in which those individual effects converge on one another in a complex biological system. To that end, the work described here in *Ptpn22* KO mice and LypR and LypW transgenic mice shows that *Ptpn22* deficiency and the LypW variant seem to exert repression of memory T cell proliferation responses to antigen re-stimulation. While this work is still preliminary, it will be important to replicate the adoptive transfer experiments of memory *Ptpn22* KO T cells to understand the effect of *Ptpn22* on memory T cell responses to antigen stimulation. Of even more significance will be examining the role of the LypW variant on memory CD4 T cell responses to antigen encounter, and thus adoptive transfer of memory LypW T cells should be pursued. These studies will provide important mechanistic insight about the function of LypW on memory T cell responses, which will help to clarify whether the CD4 T cell defects observed in human LypW carriers are due to T cell-intrinsic function of LypW.

Generation of new *Ptpn22* genetic models in mice

The *Ptpn22* KO and LypR and LypW transgenic mouse models currently available have been helpful in describing the function of *Ptpn22* in cellular immune responses to immunization and infection. However even these systems have limitations which preclude understanding the complex cellular interactions that *Ptpn22* affects. It is difficult to understand whether a defect in T cell proliferation is a product of the increased TCR signaling repression of a *Ptpn22* genetic variant, or whether it is attributable to a defect in the dendritic cell

presenting antigen and costimulation to the T cell during priming. To better address these issues experimentally, it is necessary to generate mouse models with conditional expression of *Ptpn22* and genetic variants. The first of these would be to generate *Ptpn22* conditional knockout. This is achieved by addition of *loxP* sites around the *Ptpn22* genetic locus, which are recognized and excised by the Cre recombinase. Such a mouse could be crossed with current mouse lines that express Cre recombinase under the control of different cell specific promoters, such as CD4-Cre for the deletion of *Ptpn22* in T cells or CD11c-Cre for the deletion of *Ptpn22* in Dendritic cells. Immunization studies in these conditional knockout mice would allow clearer characterization of the intrinsic effect of *Ptpn22* deficiency on each cell type and how that deficiency contributes to altered immunization responses.

Of even greater interest would be generation of a mouse line that has conditional expression of the LypW variant. This would entail engineering *loxP* sites around the LypR gene locus such that Cre recombinase would excise the LypR gene, and introduction of a *loxP*-Stop-*loxP* cassette into the promoter region of the LypW gene, which would prevent expression of the LypW gene until the *loxP*-Stop-*loxP* cassette is excised by Cre recombinase. These two mouse lines could be crossed, and then combined with different cell-specific Cre-recombinases to generate cell-specific expression of LypR and LypW within the same mouse. For example, the LypR $^{fl/fl}$x LypW-Stop$^{fl/fl}$ mice could be crossed

with CD4-Cre to generate mice that express the LypW variant exclusively in T cells and the LypR major variant in every other cell type of the immune system.

Studies of immunization responses utilizing the conditional expression of *Ptpn22* and human *PTPN22* genetic variant models described above would be useful for characterizing the cell-specific effects of *PTPN22* function. One area of particular interest would be to understand the role of *PTPN22* in modulating germinal center reactions for the production of high affinity antibodies to vaccination. Experiments looking the individual contributions of *Ptpn22* or LypW in B cells, CD4 T cells, and myeloid cells such as follicular dendritic cells would paint a clearer picture of how each of these cells interacts to modulate antibody production. This would be helpful in understanding the decreased antibody affinity maturation observed in human LypW carriers after influenza immunization, as well as the elevated serum IgM levels.

Studies of human immunization responses in LypW carriers, as well as the study of specific immune mechanisms by which LypW alters immunization responses in animal models all aim to characterize the function of LypW in the immune system. A thorough characterization of the effects of LypW on myeloid cells, B cells and T cells and how this alters their functional capacity will lead to better understanding of how the LypW variant predisposes individuals to autoimmune inflammation and the breakdown of tolerance to self.

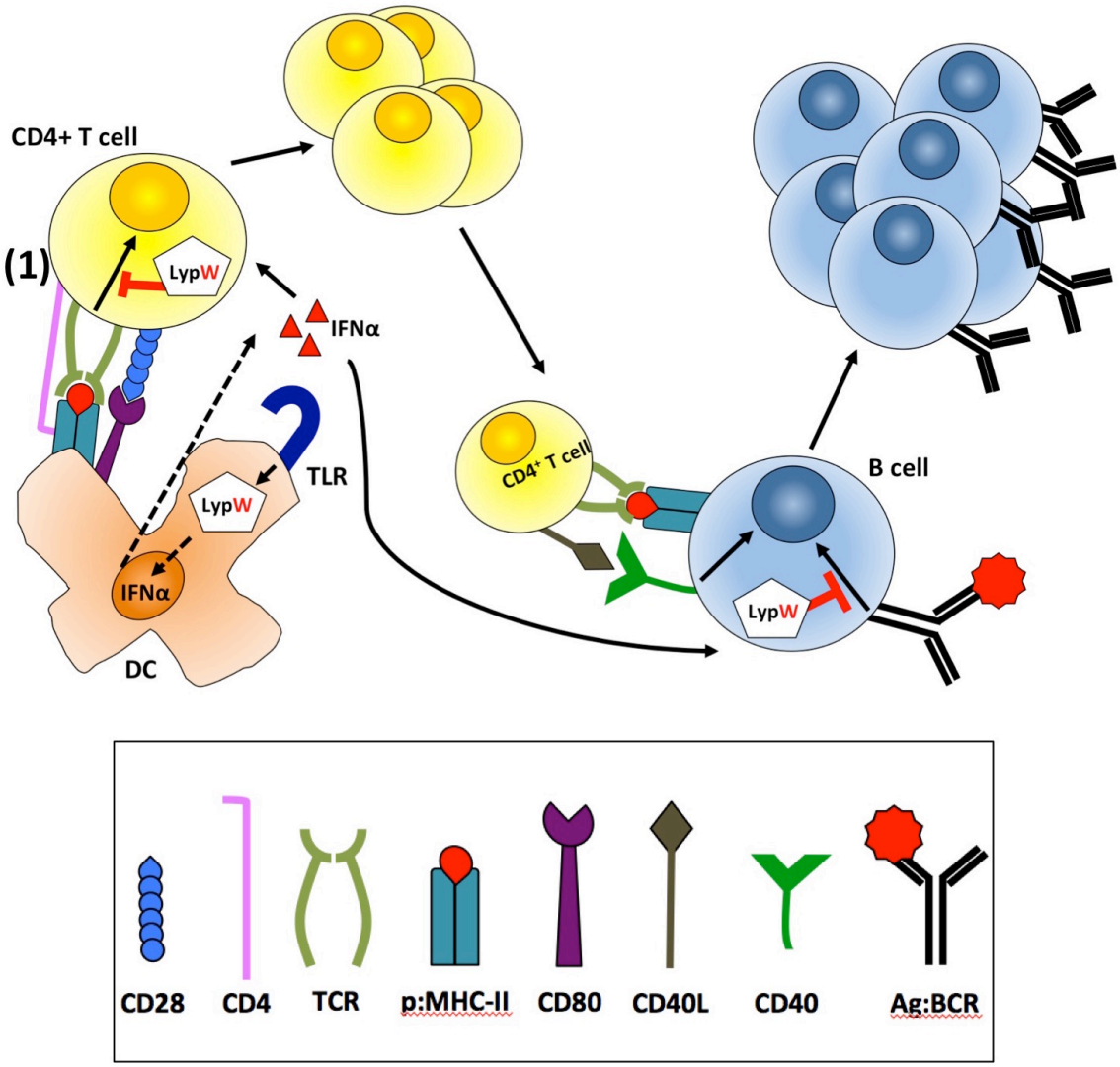

Figure 4-1: The *PTPN22* variant LypW likely acts as a gain-of-function for TCR signal repression
(1) LypW acts as a repressor of TCR signaling in CD4 T cells, resulting in decreased proliferation in response to antigen-stimulation.

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