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Overexpression of the *PTPN22* Autoimmune Risk Variant LYP-620W Fails to Restrain Human CD4+ T Cell Activation

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Abstract

A missense mutation (R620W) of *Protein Tyrosine Phosphatase, non-receptor type 22* (*PTPN22*), which encodes Lymphoid-tyrosine Phosphatase (LYP), confers genetic risk for multiple autoimmune diseases including type 1 diabetes (T1D). LYP has been putatively demonstrated to attenuate proximal T and B cell receptor signaling. However, limited data exist regarding *PTPN22* expression within primary T cell subsets and the impact of the T1D risk-variant on human T cell activity. Herein, we demonstrate endogenous *PTPN22* is differentially expressed and dynamically controlled following activation. From control subjects homozygous for the non-risk allele, we observed 2.1 ($P<0.05$) and 3.6-fold ($P<0.001$) more *PTPN22* transcripts in resting $CD4^+$ memory and regulatory T cells (Tregs), respectively, over naïve $CD4^+$ T cells, with expression peaking 24 hours post-activation. When LYP was overexpressed in conventional $CD4^+$ T cells (Tconv), T cell receptor (TCR) signaling and activation were blunted by LYP-620R ($P<0.001$) but only modestly affected by the LYP-620W risk-variant versus mock-transfected control, with similar results observed in Tregs. LYP overexpression only impacted proliferation following activation by antigen presenting cells, but not anti-CD3 and anti-CD28 coated microbeads, suggesting LYP modulation of pathways other than TCR. Notably, proliferation was signifacanlty lower with LYP-620R than with LYP-620W overexpression in Tconv, but as similar in Treg. These data indicate that the LYP-620W variant is hypomorphic in the context of human $CD4^+$ T cell activation and may have important implications for therapies seeking to restore immunological tolerance in autoimmune disorders.

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Author Contributions

DJP researched/analyzed the data and wrote the manuscript, PSL, LDP, LZ, and ZH researched the data and reviewed/edited the manuscript, CEM, CHW, and MAA contributed to discussion and reviewed/edited the manuscript, TMB conceived of the study and reviewed/edited the manuscript.

INTRODUCTION

Genome Wide Association Studies (GWAS) have identified over 60 genetic variants that are thought to confer varying degrees of susceptibility for the T cell-mediated autoimmune disease, Type 1 Diabetes (T1D) (1, 2). *Protein tyrosine phosphatase, non-receptor type 22* (*PTPN22*), which encodes Lymphoid-tyrosine Phosphatase (LYP), is one such candidate gene bearing the strongest T1D risk association after the HLA class II and INS-IGF2 loci (3, 4). A missense C1858T single nucleotide polymorphism (SNP) in the *PTPN22* coding sequence results in an LYP variant with an Arginine to Tryptophan substitution at position 620 (R620W) that is associated with increased risk for a number of autoimmune conditions including T1D, rheumatoid arthritis, systemic lupus erythematosus, Graves' Disease, vitiligo, and myasthenia gravis (3, 5–10). Most early studies of *PTPN22*-associated risk centered around lymphocytes, given their central role in autoimmune pathogenesis. However to date, no consensus has been reached regarding whether LYP R620W constitutes a gain- or loss-of-function for phosphatase activity (11, 12). Moreover, it remains poorly characterized how the risk variant influences human regulatory T cell (Treg) versus conventional T cell (Tconv) function in a manner that facilitates autoimmune disease development.

Ptpn22 was originally identified in the mouse (13) and later in humans (14) as a cytoplasmic phosphatase predominantly expressed in lymphoid cells. It is composed of a catalytic domain responsible for its phosphatase activity as well as a series of proline rich domains that regulate activity and protein interactions, most notably, with C-terminal Src kinase (CSK) (15). In T cells, LYP dephosphorylates several proximal T cell receptor (TCR) signaling molecules including LCK, CD3ε, CD3ζ, zeta-chain associated protein kinase 70 (ZAP-70), and VAV to down-modulate TCR signaling and is therefore, predicted to have a central role in T cell selection, activation, and differentiation (16). It is known that LYP also functions in B cells, as well as myeloid cells (17–21). As such, LYP has been shown to modulate B cell receptor signaling (22), Toll-like receptor (TLR) signaling (23–25), NLRP3 signaling (19), and outside-in integrin signaling (26, 27). Hence, LYP serves many functions across the cellular immune landscape, and it is possible that modulation of LYP expression levels may play an important role during immune cell development and activation.

In murine models, *Ptpn22* deficiency has been associated with increased Treg numbers in both the thymus and the periphery as well as with protection against the induction of experimental autoimmune encephalomyelitis (EAE) (28, 29). Similarly, *Ptpn22* deficiency resulted in reduced disease severity and incidence in the SKG mouse model of rheumatoid arthritis as well as enhanced tolerance of allogeneic islet transplants (30, 31). Conversely, while *Ptpn22* deficiency does not confer overt autoimmune disease, it has been shown to facilitate autoimmunity when combined with other genetic risk factors, such as hyperactive CD45 E613R mutation, BXSB background, and the KBxN arthritis model (32–34). Similar to its known association with human T1D susceptibility, *Ptpn22* has been identified as the candidate gene for the *Insulin-dependent diabetes 18.2* (*Idd18.2*) diabetes susceptibility locus in the non-obese diabetic (NOD) mouse model (35). Interestingly, while silencing *Ptpn22* expression in NOD mice resulted in reduced incidence of diabetes (36), expression of the orthologous human risk variant in the endogenous *Ptpn22* locus promoted autoimmunity in the NOD strain (37, 38). Altogether, these observations support the notion

that LYP likely influences T cell development and selection in the thymus as well as activation in the periphery, suggesting that the autoimmune-associated variant may confer multifaceted effects on host immunity.

T cells isolated from the peripheral blood of human T1D patients carrying the LYP-620W variant exhibited reduced proliferation and calcium flux in response to polyclonal stimulation (39), and heterozygosity for LYP-620W was associated with reduced IL-10 production after stimulation of memory $CD4^+$ T cells (17). There is conflicting evidence as to whether the LYP-620W variant impairs interactions of LYP with CSK, which are important for regulating TCR signal strength (32, 40, 41). Further, it is unclear whether the LYP/CSK interaction strengthens or weakens inactivation of the Src family kinase LCK (40, 41). Thus, there is a need to investigate the complex effects of the LYP-620W variant. In this study, we used a lentiviral gene delivery system to stably overexpress the LYP-620R non-risk and LYP-620W risk variants in human Tconv and Treg. Our objective was to assess the cell-intrinsic effect conferred by LYP-620W in the $CD4^+$ T cell compartment while mitigating epistatic biological variability inherent in studies of genotype-selected donors. The methods herein, may also be applied to other cell types, risk variants, or combinations thereof, to facilitate pathway-directed therapies for autoimmune disorders.

MATERIALS AND METHODS

Genotyping

PTPN22 genotype was determined using a Taqman Genotype Assay (ThermoFisher) for rs2476601 per manufacturer instructions. In this report, we refer to the reverse strand genotype (rs2476601 C>T) to match the *PTPN22* gene orientation.

Donors

Healthy control donors (median age 30.9, range 23.6–38.0 y; N=8) that were homozygous for the non-risk (C/C) *PTPN22* variant at rs2476601 were selected from the UFDI Biobank. Fresh venous blood was collected in sodium-heparinized Vacutainer tubes (BD Biosciences) after informed consent in accordance with the University of Florida Institutional Review Board approved protocol (IRB201400703). Leukopack blood donations were also obtained from rs2476601 C/C homozygous individuals under IRB-exempt protocols from Life South Blood Centers (N=9) in sodium citrate. All studies involving human samples were conducted in accordance wth the Declaration of Helsinki.

Cell Enrichment and Sorting

For fluorescence-activated cell sorting (FACS) experiments, whole blood or leukopaks were pre-enriched by $CD4^+$ T cell negative selection with RosetteSep (Stemcell Technologies) prior to density gradient centrifugation. $CD4^+$ T cells were labeled with fluorescent antibodies against CD4 (RPA-T4), CD45RA (HI100), CD197 (G043H7), CD127 (A019D5) and CD25 (BC96) as previously described (42). Treg were FACS-purified as $CD4^+CD25^+CD127^{lo/-}$, while naïve Tconv were FACS-purified as non-Treg $CD4^+CD127^+CD45RA^+CD197^+$, and memory Tconv were FACS-purified as non-Treg $CD4^+CD127^+CD45RA^-$ on a FACS Aria III (BD Biosciences) as previously described (43).

PTPN22 Transcript Quantification

Leukopak enriched $CD4^+$ T cell subsets were used for endogenous *PTPN22* expression kinetic studies. RNA was prepared from 10^6 naïve Tconv, memory T cells, and Treg either immediately after sorting, or at various time points following activation. Tconv were activated with a 1:1 bead to cell ratio of Dynabeads Human T-Activator CD3/CD28 (ThermoFisher), and Treg were activated with 4:1 ExpAct beads (Miltenyi). Cells were supplemented with growth factor cytokines every 2–3 days: 300U/mL IL-2 for Tregs, 20U/mL IL-2 for Tconv, and after 7 days, 5 ng/μL IL-7 was also added for Tconv. RNA was Qiashredded and extracted using RNeasy Plus Kit (Qiagen) and reverse-transcribed into cDNA using Protoscript First Strand cDNA Synthesis kit (New England Biolabs). The expression level of *PTPN22* was measured using an absolute quantification real-time PCR (RT-qPCR) approach on a Lightcycler 480 platform (Roche Diagnostics). Each reaction well contained 1μL *PTPN22* TaqMan® Gene Expression Assay solution (ThermoFisher), 2μL LightCycler480 Probe Master Mix (Roche), and 3μL cDNA in a 20μL reaction. To create the DNA standards for the RT-qPCR assay, the pUC57.LYP620R.fuT2A.eGFP (see Lentiviral Vector Design and Construction) plasmid containing *PTPN22* coding sequence (CDS) was serially diluted 10 fold. Lambda DNA was used to bring all standards to equivalent concentrations of total DNA (1.2ng/μL). Six concentrations were prepared ranging from 1.2fg/μL to 12ng/μL of plasmid, in addition to an NTC consistiong of 1.2ng/μL Lambda DNA only. 3μL of standard DNA was used in reactions as above. Standards were converted to transcript copy number by dividing the mass of plasmid per reaction in grams by the plasmid formula weight, where 650g/mol was used as the average nucleotide pair formula weight times the plasmid length. Copy number of each standard per well was log-transformed and plotted against the corresponding Ct value to form a standard curve. The standard curve $R^2>0.998$ for each batch, and all samples were within range of the standard curves. *PTPN22* expression level of each sample was extrapolated from the standard curve as previously done (44).

Lentiviral Vector Design and Construction

Design of the LYP620R.fuT2A.eGFP construct began with the human *PTPN22* full length isoform coding DNA sequence (CDS) containing the non-risk variant (C at position 1858 of the CDS). Two endogenous EcoRI restriction enzyme sites (GAATTC) within the CDS were synonymously mutated, and the stop codon was removed (Supplemental Figure 1A–B). This was followed by a furin cleavage site, a T2A self-cleaving peptide sequence (45), and an enhanced green fluorescent protein (eGFP) coding sequence (stop codon included). The LYP620R.fuT2A.eGFP was synthesized in a pUC57 cloning vector by GenScript (Piscataway, NJ). Site-directed mutagenesis by PCR with pfu DNA polymerase followed by DpnI digest was performed to generate the LYP620W.fuT2A.eGFP construct, which contains the *PTPN22* C1858T variant that encodes the LYP R620W amino acid change. These constructs were then subcloned into the pFUGW 3rd generation lentiviral vector (46). pFUGW expressing only eGFP served as the control vector. Lentiviral preparations were conducted as previously described (47). All constructs were validated by restriction enzyme digest and sequence analysis.

Cell Transduction

FACS-isolated naïve Tconv were activated with a 1:1 bead to cell ratio of Dynabeads Human T-Activator CD3/CD28 (ThermoFisher), and Treg were activated with 4:1 ExpAct beads (Miltenyi). On day 2, the cultures were infected with 0.5 titered units of lentivirus per cell in the presence of 8μg/mL protamine sulfate (Sigma), and spinnoculated as previously described (47). The cultures were then allowed to expand and rest for at least 21 days after activation, during which they were supplemented with growth factor cytokines every 2–3 days at dosages of 300U/mL IL-2 for Tregs and 20U/mL IL-2 and for Tconv. After 7 days, 5ng/μL IL-7 was also supplemented every 2–3 days with IL-2 for Tconv. Portions of the cultures were FACS-isolated into stably transduced ($eGFP^+$) and mock-transduced ($eGFP^-$) for cytokine and suppression assays. All other assays used the unsorted fractions of the cultures so that the results from stably transduced cells could be normalized to mock-transduced cells.

Western Blotting

FACS-isolated transduced ($eGFP^+$) and mock-transduced ($eGFP^-$) fractions were lysed with cold lysis buffer (Cell Signaling Technologies) containing protease inhibitors (Sigma). Protein was quantified via BCA assay (Thermo Scientific). 5μg of protein was denatured with Laemmli sample buffer at 95°C for 5 minutes and run on mini protean TGX gels (BioRad). Transfers were performed using the Transblot Turbo Transfer System (BioRad) and Transblot Turbo Transfer Packs (BioRad). Membranes were washed with 1X TBST, blocked with 5% milk in TBST for 1 hour, and incubated with primary antibody (rabbit anti-human LYP (clone D6D1H), β-actin (polyclonal) or GFP (clone D5.1), Cell Signaling Technologies) overnight at 4°C. Membranes were then washed with TBS-T, and incubated with anti-rabbit IgG-HRP secondary antibody (1:1000, Cell Signaling Technologies) for 1 hour at room temperature. Membranes were subsequently washed, developed with Western DuraSignal Substrate (Thermo Scientific), and imaged on the GeneGnome XRQ (Syngene). Western blot band quantification was performed in ImageJ (48). Serial blots were stripped with Restore Western Blot Stripping Buffer (ThermoFisher Scientific) between probes for 15 minutes at room temperature per manufacturer protocol.

Calcium Flux

Stably transduced Tconv or Treg, and their mock-transduced internal control cells, were labeled with 2.5μg/mL FuraRed calcium responsive dye (ThermoFisher) in 1% BSA/HBSS at 37°C for 10 minutes, followed by labeling with Live/Dead Near IR viability dye (ThermoFisher). Cells were then resuspended in HBSS (with Ca^{2+}) containing 10μg/mL goat anti-mouse IgG $F(ab')_2$ crosslinking antibody (Jackson ImmunoResearch). Next, cells were warmed to 37°C, and fluorescence at 605 nm by a 405 nm laser and at 660 nm by a 532 nm laser was collected on a LSRFortessa (BD Biosciences) to indicate high and low calcium, respectively (Supplemental Figure 3A–C). After recording 30 seconds of basal fluorescence, anti-human CD3 (OKT3) was added at 1μg/mL, and fluorescence was acquired for two minutes. FlowJo software (FlowJo LLC) was used to analyze the mean fluorescence intensity (MFI) kinetics of FuraRed for the high and low $[Ca^{2+}]$ channels after first gating on live cells by forward and side scatter morphology and viability dye exclusion.

The percent change from mock in fluxed calcium was calculated as the percent difference between curves of the mock and transduced cells (Supplemental Figure 3D–G).

pERK Signaling

Lentiviral-transduced Tconv or Treg and their mock-transduced internal control cells were labeled with viability dye, then washed into assay buffer (RPMI base medium) and plated at 10^6 cells per well in 96-well V-bottom plates in the presence of 10μg of anti-mouse IgG crosslinking antibody (Jackson ImmunoResearch). Anti-CD3 (2μg of OKT3) was added at times −10, −5, and −2 minutes. At time 0, wells were fixed with 4% formalin for 10 minutes. Cells were then washed, permeabilized with methanol, and stained with PE-conjugated anti-phospho-ERK (anti-pERK; clone 197G2, Cell Signaling Technology) for 60 minutes. Data was acquired by flow cytometry on an LSRFortessa (BD Biosciences), and pERK MFI of live cells was assessed using FlowJo software (Flowjo LLC).

Proliferation and Suppression Assays

Lentiviral-transduced ($eGFP^+$) and internal control mock-transduced ($eGFP^-$) cells were labeled with Cell Trace Violet (CTV, ThermoFisher) cell tracking dye and activated with either beads, autologous antigen presenting cells (APCs), 10ng/mL phorbol 12-myristate 13-acetate (PMA) plus 1 μM ionomycin, or K562 cell line artificial APCs (aAPCs) which do not express MHC class I or class II molecules but do express murine Fc' receptor (CD64) and human CD86 (49). In brief, beads were used at a 1:1 bead to cell ratio as described in Cell Transduction above. Autologous APCs, generated from PBMCs that were CD3-depleted (RosetteSep) and exposed to 3000 Rad of gamma irradiation, were cultered 1:1 with T cells in the presence of 2μg/mL anti-CD3 and 1μg/mL anti-CD28. To generate aAPCs, K562 cells (human chronic myeloid leukemia (CML) line) expressing murine CD64 and human CD86 were incubated with various ratios of mouse anti-human CD3 (OKT3) and isotype antibody, and then irradiated with 3000 Rad. The ratios of isotype to OKT3 used were 1:0, 9:1, 1:1, and 0:1, such that the resulting aAPCs were loaded with 0, 10, 50, and 100% anti-CD3, respectively. aAPC were similarly labelled to include mouse anti-human CD4 (OKT4) in ratios of 1:0:0, 1:0.5:0.5, and 0:0.5:0.5 (isotype:OKT3:OKT4), such that the resulting aAPCs were loaded with 0, 5, 25, and 50% each activating antibody. These were cultured at a 1:4 aAPC to T cell ratio.

Suppression assays were performed as previously described (50). Briefly, titering proportions of Tregs labelled with Cell Proliferation Dye eFluor670 (CPD670, ThermoFisher) were co-cultured with autologous CTV-labelled responder Tconv (Tresp) and unlabelled APCs ranging from 1:1:1 to 0:1:1 Treg:Tresp:APC ratios. Suppression assay cultures were set up in triplicate in round-bottom 96-well plates and activated with 2μg/mL anti-CD3 and 1μg/mL anti-CD28. Two versions of the suppression assays were performed. The first, which was designed to assess the response of LYP-modulated Tconv to Treg suppression, consisted of a combination of stably-transduced and mock-transduced Tconv (not eGFP-sorted) being suppressed by unmodulated Treg (mock-transduced sorted $eGFP^-$). The second, which was designed to assess suppression by LYP-modulated Treg, consisted of unmodulated Tconv (mock-transduced sorted $eGFP^-$) being suppressed by stably-transduced Treg (sorted GFP^+).

Proliferation and suppression assay data were acquired on an LSRFortessa (BD Biosciences), and analyze using FlowJo software (Flowjo LLC). Live T cells were gated by forward and side scatter morphology, followed by viability dye exclusion. Proliferation of live-gated cell-tracking dye$^+$ cells was assessed by expansion index. Suppression was calculated as the percent decrease in Tresp proliferation when co-cultured with Tregs as compared to when cultured without Tregs.

Surface Marker Activation Kinetics

Stably transduced (eGFP$^+$) and internal control mock-transduced (eGFP$^-$) cells were activated with irradiated autologous APCs at 1:1 in the presence of 2μg/mL anti-CD3 and 1μg/mL anti-CD28. The cultures were harvested at 0h, 4h, 24h, 48h, 72h, and 144h and stained with Live/Dead Near-IR viability dye (ThermoFisher). Next, the cells were washed into 2% FCS in PBS and labelled with CD278 (C398.4A, BioLegend), CD69 (FN50, BioLegend), CD4 (RPA-T4, BioLegend), CD226 (11A8, BioLegend), CD25 (BC96, BioLegend), CD279 (EH12.2H7, eBiosciences), CTLA4 (L3D10, BioLegend). The cells then were fixed in 1% formaldehyde in PBS. Data was acquired on an LSRFortessa (BD Biosciences) and was analyzed using FlowJo software (Flowjo LLC). Live lymphocytes from CD4$^+$ T cell cultures were gated by forward and side scatter morphology, followed by viability dye exclusion before extracting surface marker expression. Fluorescence minus-one (FMO) controls were used to set gates for marker positivity.

Cytokine Detection

Stably transduced (eGFP$^+$) T cells were activated with irradiated autologous APCs at 1:1 in the presence of 2μg/mL anti-CD3 and 1μg/mL anti-CD28. Supernatants were collected at the specified time points, and cytokines were detected via multiplexed bead-based ELISA (42).

Statistics

Data were analyzed by one-way ANOVA with Bonferroni's post-hoc analysis or by two-way ANOVA with Bonferroni's posttest, and graphs were prepared using GraphPad Prism software v6. $P < 0.05$ was considered significant. Subject matching was used to compare donors' cells across experimental conditions (biological replicates).

RESULTS

PTPN22 expression varies by lymphocyte subset and activation state.

We examined endogenous *PTPN22* expression in human naïve, memory, and regulatory CD4$^+$ T cell subsets at rest and following *in vitro* activation. CD4$^+$ T cells were sorted from leukopacks determined to be homozygous for the T1D non-risk allele, which encodes LYP-620R. Since the transcriptional profile of activated cells is dramatically altered from that of the quiescent state, such that the standard array of housekeeping genes is broadly upregulated, we employed absolute quantification to assess *PTPN22* expression. At rest, naïve Tconv expressed significantly less *PTPN22* (387±83 transcripts/ng total RNA) than memory Tconv cells (741±169 transcripts/ng total RNA, $P < 0.05$) and Tregs (1014±120 transcripts/ng total RNA, $P < 0.001$) (Figure 1A). Following polyclonal activation, all three subsets rapidly increased *PTPN22* expression, peaking at 24 hours (Figure 1B). Naïve CD4$^+$

Tconv increased 12.00±5.45 fold from the quiescent state whereas memory Tconv increased 4.01±0.89 fold ($P < 0.001$ vs. naïve) and Treg increased 2.85±1.31 fold ($P < 0.01$ vs. naïve). Following the initial increase, *PTPN22* expression decreased and plateaued around day 7. Taken together, we found *PTPN22* expression to differ across T cell subsets at rest and in response to activation.

Overexpression of *PTPN22* decreases TCR signaling.

Next, we examined whether LYP-620R and −620W may differentially modulate T cell function in Tconv and Treg populations. Naïve $CD4^+$ Tconv and Treg were sorted from PBMCs from normal healthy control donors that were homozygous for the T1D non-risk *PTPN22* allele (C/C at rs2476601 encoding LYP-620R). Cells were then activated and transduced with a lentivirus to express bicistronic constructs of the non-risk LYP-620R or the risk LYP-620W variant with an eGFP reporter following a 2A element, or with eGFP alone for a vector control condition (Supplemental Figure 1A–B). The cultures were expanded, followed by an extended rest period for a total of 21–28 days to allow for reversion of activation programs. We confirmed stable overexpression of *PTPN22* transcripts and LYP protien for each variant (Supplemental Figure 1C–E and Supplemental Figure 2).

The proximal signaling events resulting from TCR ligation lead to assembly and activation of the linker for activation of T cells (LAT) signalosome, followed by diverging downstream signaling pathways. These include calcium signaling and MAPK/ERK pathways (51). We sought to determine the functional impact of the non-risk and risk LYP variants on these downstream signaling pathways in Tconv and Treg. We first assessed Ca^{2+} flux by comparing stably transduced cells to non-transduced ($eGFP^-$) internal culture control cells (Supplemental Figure 3). As expected, LYP-620R expressing Tconv exhibited 25.8±3.7% less Ca^{2+} flux than non-transduced Tconv, whereas transduction with eGFP alone had no effect (Figure 2A). A similar effect was observed for LYP-620R Tregs in which Ca^{2+} flux was diminished by 40.3±13.3% compared to non-transduced Tregs (Figure 2B). Conversely, LYP-620W expression only resulted in 4.9±1.6% and 7.9±2.7% less Ca^{2+} flux in Tconv and Treg, respectively (Figure 2A–B). These differences were not different from T cells transduced with eGFP alone. Thus, the risk LYP-620W variant less effectively downregulated TCR-induced Ca^{2+} flux.

We next assessed the impact of LYP modulation on the MAPK/ERK pathway via pERK signaling following TCR ligation. As with Ca^{2+} signaling, we observed decreased pERK relative to mock transfected cells in both Tconv and Tregs when LYP-620R was overexpressed (Figure 2C–D). Once again, the impact of LYP-620W on TCR signaling was diminished in both subsets when compared to LYP-620R. Taken together, these results indicate that the LYP risk variant has a diminished capacity to regulate TCR signaling activity.

The risk variant of *PTPN22* permits increased T cell activation.

In response to Ca^{2+} flux and pERK signaling, T cells express receptors on their surface to direct activation. We further explored the impact of LYP variants by examining the experssion of a set of these activation markers. Stably transduced T cells were activated with

autologous APCs, and surface expression of activation markers was assessed at 0, 4, 24, 48, 72, and 144 hours by flow cytometry. As expected, CD69, ICOS, CD25, CD226, and PD-1 were observed to be dynamically expressed following Tconv activation (Supplemental Figure 4A–D) (52–56). Likewise, Tregs also exhibited activation-induced expression kinetics for CD69, ICOS, CD226, CTLA-4 (Supplemental Figure 4E–F) (57). Interestingly, while most activation marker kinetics involved an intensification followed by a reduction of surface expression, the kinetics of CD226 on Tregs waned before increasing to a level higher than baseline (Supplemental Figure 4H). When normalized to internal mock-transfected controls, the activation marker kinetics on both Tconv (Figure 3A–E) and Tregs (Figure 3F–J) were blunted by overexpression of LYP-620R, with the exception of CD226 on Tconv (Figure 3D). Similar to what was observed with Ca^{2+} and pERK signaling, cells expressing the LYP-620W variant were less impacted, supporting the notion that the R620W variant confers a loss-of-function for LYP in terms of modulating of T cell activation.

The *PTPN22* risk variant permits proliferation in Tconv, but not in Treg.

We demonstrated that the two LYP variants differentially modulate TCR signaling and expression of T cell activation markers. We next asked whether overexpression of the LYP variants would differentially modulate T cell proliferation. Activation-induced proliferation of Tconv and Treg cells was assessed by dilution of cell tracking dye following *in vitro* activation. Transduced cells ($eGFP^+$) were cultured with mock-transduced ($eGFP^-$) internal culture control cells. The cultures were activated with either anti-CD3 and anti-CD28 with irradiated APCs, anti-CD3 and anti-CD28 bound to microbeads, or with PMA and ionomycin. While expression of the eGFP reporter did not affect proliferation, overexpression of LYP-620R reduced APC-mediated proliferation of Tconv and Treg by 35.32±15.30% and 19.80±17.26%, respectively (Figure 4A–B). In Tconv, the LYP-620W variant was once again deficient in its ability to downregulate activation, as it did not impede proliferation to the same degree that was observed for the LYP-620R variant (Figure 4A). However, the LYP-620W variant was capable of blocking Treg proliferation to a similar degree as the LYP-620R variant (Figure 4B), indicating a differential impact of *PTPN22* on Tconv and Tregs.

As expected, mitogenic activation by PMA and ionomycin was not altered by LYP overexpression, as all transduced Tconv and Treg proliferated to a comparable degree as their internal mock control cells (Figure 4A–B). Unexpectedly however, proliferation was also unaltered in transduced cells that were activated by microbeads (Figure 4A–B). To assess whether this was due to TCR signal strength, we repeated the experiment with K562 aAPCs loaded with titrated ratios of activating and isotype antibodies. The aAPCs did not induce proliferation when loaded with 0% anti-CD3 (100% isotype), whereas 10% and 50% anti-CD3 induced incrementally more proliferation, with a plateau from 50% to 100% anti-CD3 (Supplemental Figure 5A). In all cases, the proliferation of transduced cells was similar to their internal controls (Supplemental Figure 5B). Similar results were found when CD4 was included in the activation signal to allow for LYP-LCK interactions (Supplemental Figure 5C–D) (30, 58). This suggests that optimized signal strength, as well as fluid membrane interface are not sufficient to recapitulate the differential inhibition of proliferation by the two LYP variants, and that other factors supplied by natural APCs (e.g.,

adhesion molecules, soluble factors, and/or co-stimulatory and co-inhibitory ligand and downstream signaling interactions) may be required (30, 58).

Overexpression of *PTPN22* influences T cell cytokine production.

$CD4^+$ effector T cells modulate host immune responses after encountering cognate antigens, in part, through the production of an array of cytokines. Thus, we sought to assess the impact of LYP variant expression on the secretion of cytokines important in driving Th1 and Th2-associated immunity. Sorted transduced Tconv cells were activated with APCs, anti-CD3, and anti-CD28, and culture supernatants were assayed for cytokines over a 72-hour time period. The net accumulation of IL-2 in this system is a function of activation-induced secretion and of consumption via the IL-2 receptor (IL-2R) because the high-affinity component, CD25, is dramatically upregulated in this time period (Supplemental Figure 4) (59). We found that the net accumulation of IL-2 was not altered by overexpression of the LYP-620R variant, but was increased at the 24-hour time point by the LYP-620W variant (Figure 5A). The secretion of IL-4, IL-5, IL-9, IL-10, and IL-13 was robustly inhibited for the first 48 hours by expression of either LYP variant. At the72-hour time point, inhibition occurred to a somewhat lesser degree by LYP-620W, thought this difference was not significant between variants (Figure 5B–F). Finally, and somewhat surprisingly given the documented Th1 signature implicated in several of the autoimmune disorders that are associated with the rs2476601 risk variant (60, 61), IFNγ secretion was not altered by overexpression of either variant of LYP (Figure 5G). This result is in agreement with studies of *Ptpn22* knockout mice (28). Taken together, these data demonstrate that both LYP variants result in similar levels of effector cytokine secretion.

Treg-mediated suppression is altered by overexpressed *PTPN22.*

Our data demonstrated that *PTPN22* is more highly expressed in Treg as compared to Tconv (Figure 1A). We therefore hypothesized that the *PTPN22* risk variant may influence disease risk by altering Treg-mediated suppression. Hence, we assessed whether transduced Treg were deficient in their suppressive capacity and whether transduced Tconv were refractory to Treg suppression. In order to determine the effect of overexpressed LYP variants on Tresp, irradiated autologous APCs and titered amounts of Treg were co-cultured with transduced Tconv ($eGFP^+$) and mock-transduced ($eGFP^-$) internal control Tconv. We found that, similar to our previous results (Figure 4A), overexpressed LYP-620R significantly repressed Tresp proliferation while repression by LYP-620W was dramatically reduced across the Treg:Tresp range (Figure 6A). However, the relative suppression of transduced Tresp did not differ significantly from their internal mock controls (Figure 6B). This suggests that the Tconv-intrinsic susceptibility conferred by LYP-620W is due to reduced control over activation, proliferation, and effector mechanisms, rather than a defect in the ability of effector cells to be suppressed by Treg.

Finally, we assessed the effect of the LYP variants on Treg suppressive capacity by co-culturing titered amounts of sorted transduced ($eGFP^+$) Treg with irradiated autologous APCs and untransduced Tresp. We found suppression by Tregs overexpressing LYP-620R to be similar to eGFP reporter Tregs, while suppression by Tregs expressing the risk LYP-620W variant was slightly increased (Figure 6C). Consistent with our previous result

(Figure 4B), Treg proliferation in the suppression assay co-culture was similar for LYP-620R and LYP-620W (Figure 6D). This indicates that the difference in suppressive capacity was not due to differences in Treg proliferation, and suggests a Treg intrinsic impact of LYP-620W to enhance suppressive function.

DISCUSSION

To date, much of the literature has attempted to categorize rs2476601, the *PTPN22* C1858T autoimmune-associated missense mutation, as a loss- or gain-of-function variant (11, 12). Because the LYP-620W variant confers risk for T cell-mediated autoimmune diseases (3, 5–8), we were interested in its allotypic effects on TCR signaling and function in the Tconv and Treg subsets. We first evaluated endogenous *PTPN22* expression in primary human T cells homozygous for the T1D non-risk allele encoding LYP-620R. We then employed a lentiviral gene delivery system to induce constitutive overexpression of the T1D non-risk variant, LYP-620R, or T1D risk variant, LYP-620W, in primary human $CD4^+$ Tconv and Tregs. There are a few advantages to this approach. First, it enables expansion of primary cell material that stably expresses either the non-risk or risk variant for long-term assays. Second, by isolating LYP variant overexpression to $CD4^+$ T cells, the complicating effects of the LYP variant from other immune subsets are removed so that $CD4^+$ T cell-intrisic effects can be studied. Finally, it controls for epistasis and the associated biologic variability by assessing the functional differences of the LYP variants within the same subjects.

Our observation that endogenous expression of *PTPN22* varies by T cell subset and is dynamic throughout activation suggests that SNP-related functional effects are multifaceted and may differ across cell types in a manner that is subject to temporal control and activation state in the periphery. Importantly, our assay measured both splice variants which encode the 85 kD and 105 kD LYP isoforms that are differentially expressed in T cells at rest and after activation, respectively (14), thereby measuring total endogenous *PTPN22* expression. Due to the MAF of rs2476601, we were unable to assess *PTPN22* expression kinetics of the 620W variant using the methods herein. We will persue this question using scRNA-Seq technology, which requires many fewer cells, thus enabling time course examination of genotype-selected, cryopreserved PBMCs. We next demonstrated that, as expected, overexpression of LYP-620R decreases T cell Ca^{2+} flux, pERK signaling, surface expression of activation markers, and proliferation in both Tconv and Tregs. However, with the exception of Treg proliferation, overexpression of LYP-620W had little effect on TCR activation-induced responses. We therefore conclude that LYP-620W is hypomorphic in terms of $CD4^+$ T cell responses to TCR activation.

Interestingly, proliferation was only affected by *PTPN22* modulation in the context of APC activation, indicating a requirement for a natural immune synapse or specific costimulatory signaling. Burn et al. showed that primary human T cells with the endogenous LYP-620W variant had increased pERK1/2 induced by LFA-1/ICAM-1 outside-in signaling as compared to T cells expressing the LYP-620R variant (27). This resulted in stronger integrin-mediated adhesion that was resistant to shear forces, which may also apply to cell-cell interactions. Thus, whereas interactions between autoreactive T cells and APCs may normally be transient (62), expression of the LYP-620W variant may stabilize the immune

synapse allowing for more efficient activation and initiation of autoreactivity. Similar experiments comparing LYP variants in the APC are necessitated to determine if the reported effect on synapse stability occurs bidirectionally.

Treg proliferation was blunted by LYP overexpression, but contrary to Tconv, Treg proliferation was not differentially impacted by the two LYP variants. This was the case when cultured alone with APCs as well as with APCs and Tconv in the context of suppression assays. Overall suppressive capacity was not affected for LYP-620R Tregs and was enhanced for LYP-620W Tregs. The data presented here further indicate that certain mechanisms of suppression, perhaps IL-2 consumption or regulatory cytokine production, may not be affected by LYP expression, while other mechanisms, such as contact-dependent co-inhibitory receptor interactions, may only be affected by the LYP-620W variant. It was previously shown that Tregs from *Ptpn22* deficient mice had improved suppressive function due to increased and prolonged LFA-1 interactions between Tresp and Tregs (26). This is in line with the finding that LYP-620W is a loss-of-function variant with respect to outside-in integrin signaling, which results in sustained LFA-1 interactions (27). We previously showed that Tregs expressing a higher affinity TCR are more potent suppresors (63). Sustained Treg interactions conferred by LYP-620W may be functionally similar to higher affinity TCR engagement, resulting in enhanced suppressive capacity per cell (Figure 6C). Nevertheless, enhanced Treg suppressive capacity does not support a mechanism for autoimmune pathogenesis. In fact, suppression assays from rs2476601 T/T individuals were recently shown to exhibit lower suppression than those from C/C subjects (64). Thus, we posit that progression to autoimmunity may occur when the LYP-620W deficit in restraint of Tconv proliferation overcomes the LYP-620W enhancement of Treg suppression.

In terms of cytokine production, IL-2 accumulation was not altered in T cells overexpressing LYP-620R, but was increased by the LYP-620W variant. This finding is consistent with observations of the murine Pep-R619W ortholog knock-in (37). While further studies are required to determine if this is attributable to increased production or reduced consumption, this result may corroborate the observed differential effects of LYP variants on Tconv proliferation. The secretion of effector cytokines (IL-4, IL-5, IL-9, IL-10, and IL-13) was inhibited by overexpression of either variant, but this effect appeared to wane in LYP-620W Tconv by the 72-hour time point. IFNγ production by Tconv was not altered by overexperession of either LYP variant. This is in contrast to observations in a murine model of *Ptpn22* overexpression, which exhibited decreased IFNγ production in effector T cells (65). We also did not observe increased IFNγ production in in LYP-620W expressing cells relative to LYP-620R expressing cells. Conversely, Anderson et al. recently reported increased IFNγ production and activation marker expression in *PTPTN22*-deficient human $CD4^+$ T cells after 48 hours of reactivation (66). Those results suggests that a loss-of-function variant may also result in enhanced IFNγ secretion. While, the reason for these observed differences between experimental model systems is not clear at this point, we posit that constitutive overexpression and CRISPR knock-out systems impact LYP stoichiometry with TCR signaling molecules in an opposing manner. As such, cytokine regulation may not be affected in the same manner as activation and proliferation.

Faced with inconsistent reports in the current literature (12, 17, 39), our data nonetheless support the notion that the risk LYP-620W variant is generally a loss-of-function variant in Tconv, as TCR signaling, activation, and proliferation are less affected by its overexpression compared to LYP-620R. Still, the fundamental question of whether the LYP-620W functional decrease results from abrogated phosphatase activity or altered localization remains outstanding. While altered localization may also expose a distinct set of targets to dephosphorylation, it likely also disengages LYP-620W from the TCR signalosome, where its modulatory capacity is specifically tied to weaker TCR signaling (67, 68). In this regard, differences in activation threshold, signal strength, and costimulation between Tconv and Treg may make them differentially amenable to LYP modulation (69, 70). In the context of recurrent autoreactive T cell responses, which tend confer weaker low-affinity TCR signaling in memory T cells (62, 71), the net effect may be the outgrowth of autoreactive Tconv relative to Treg. Future studies will examine the nature of our observed loss of function.

Despite the advantages of the lentiviral gene delivery methods utilized herein, there are caveats to consider when interpretating our results. First, while constitutive overexpression induced by our lentiviral constructs is useful for unmasking differences between variants, it does not provide equivalent kinetics to those observed for endogenous *PTPN22* expression. Hence, more careful control of expression may be required for mechanistic studies of temporal LYP activity. Second, in this experimental system we are examining the the effect of LYP variant expression in the context of endogenous LYP-620R. In that regard, these data are analogous to comparing homozygous non-risk to heterozygous risk LYP variants, with the caveat of continual and mild overexpression (3–6 fold higher LYP protein expression, Supplemental Figure 1E) from the LV construct. However, a qPCR assay could have been designed for a more thorough analysis of exogenous vs endogenous expression. This is an important consideration because amount of LYP relative to its binding parters, most notably CSK, influences the degree of dephosphorylation of TCR signaling (32, 72). Further studies are required to determine how this modest LYP overexpression relative to CSK impacts the biological outcome. Third, for efficient integration to occur, we transduce actively dividing cells. We are therefore unable to examine LYP functional aspects during primary activation, for example to study naïve vs memory or to determine the impact on polarization. Finally, we have only examined the 620W effect in the full length isoform. Several isoforms of LYP are expressed in human T cells, and variation in phosphatase activity, cellular localization, and association with autoimmune disease have been reported (73, 74).

Considering the various roles for LYP in modulating immune responsiveness of T cells, B cells, APCs, neutrophils and more (11, 17, 23, 24, 29, 75), it is not surprising that the effects of the LYP-R620W are complex, even within the $CD4^+$ T cell compartment. Further, there is a need for understanding the role of LYP in B and T cell central tolerance. Indeed, it is likely that the phenotypes observed here from T cell LYP variant expression would have additional implications *in vivo* and that cell-specific downstream effects of the *PTPN22* SNP (rs2476601) may work in concert for altered effector function. Hence, there is a need moving forward for additional investigation of SNP-mediated *in vitro* versus *in vivo* effects. We plan to address these questions through ongoing efforts to build isogenic cellular systems to model multiple cell-cell interactions utilizing gene editing technologies (76). It is clear

that rs2476601 contributes susceptibility for a number of autoimmune diseases including T1D (3, 4), likely related to functional consequences within the T cell compartment. We demonstrated, herein, that the risk variant imparts a hypomorphic phenotype marked by impaired ability to down-regulate T cell activation and effector function, supporting the notion that *PTPN22* represents an important target for therapies aimed at preventing or reversing autoimmune disorders, including T1D.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Guarantor Statement

Todd M. Brusko is the guarantor of this work and as such, assumes full responsibility for the ethical acquisition and reporting of data.

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KEY POINTS

- The *PTPN22* autoimmune risk allele confers loss-of-function in human T cells
- Natural APC activation is required for PTPN22 to inhibit $CD4^+$ T cell proliferation
- Inhibition of proliferation by the PTPN22 risk variant is not defective in Treg

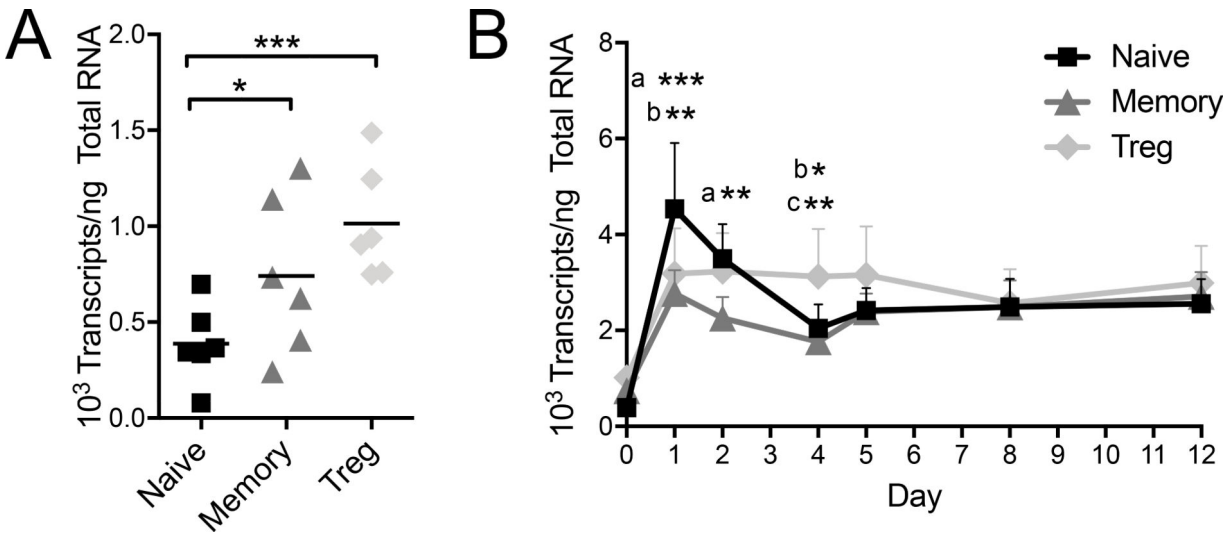

Figure 1. Endogenous *PTPN22* is differentially expressed in CD4 T cell subsets.

Primary naïve Tconv, memory Tconv and Treg subsets were separated by FACS from leukopack donations and *PTPN22* transcripts were quantified by absolute real time PCR. (*A*) In the resting state (freshly isolated on day 0), naïve Tconv have lower *PTPN22* gene expression compared to memory Tconv or Tregs (One-way ANOVA using subject matching with Bonferroni's posttest). (*B*) Following *in vitro* activation with surface-fixed anti-CD3/CD28 beads, naïve Tconv, memory Tconv, and Treg *PTPN22* expression was quantified at d1, d2, d4, d5, d8, and d12. (n=6, Two-way ANOVA using subject and timepoint matching with Bonferroni's posttest, *p<0.05, **p<0.01, ***p<0.001, a: Naïve vs Memory, b: Naïve vs Treg, c: Memory vs Treg)

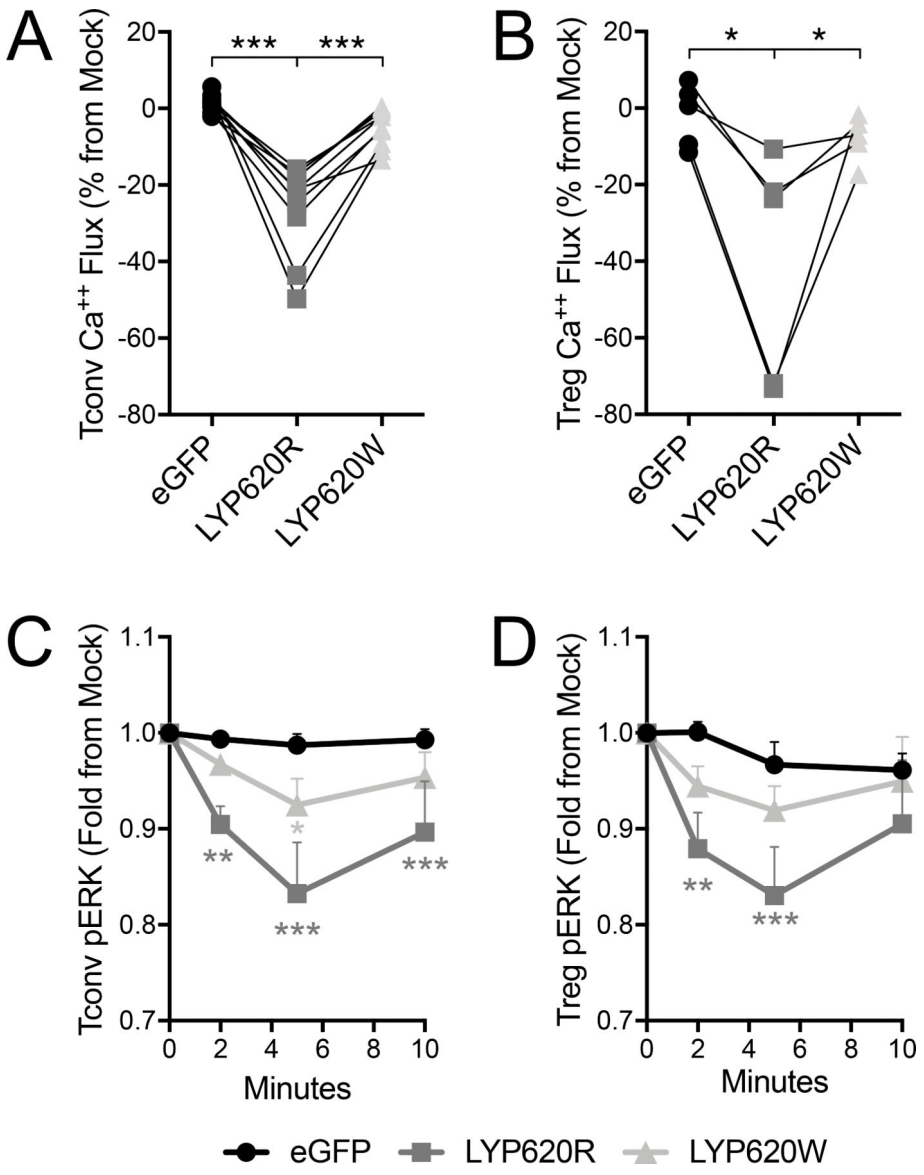

Figure 2. The risk variant of *PTPN22* has a weaker impact on distal TCR signaling than the non-risk variant.

Primary human CD4 Tconv and Treg were transfected to express LYP-620R, LYP-620W risk variant, or eGFP as a control. Calcium flux by (*A*) Tconv and (*B*) Treg in response to soluble anti-CD3 and cross-linker was assessed by ratiometric fluorescent dye. The area under the curve during TCR activation was quantified (see Supplemental Figure 3) and then normalized to internal mock control cells ($eGFP^-$). Individual donors are shown with lines connecting their matched T cell transductants (Tconv donors n=10, Treg donors n=5, One-way ANOVA using subject matching with Bonferroni's posttest). Phospho-ERK (pERK) signaling by Tconv (*C*) and Treg (*D*) in response to soluble anti-CD3 and cross-linker was assessed by intracellular phospho-ERK staining. The mean fluorescence intensity (MFI) for each time point was quantified and then normalized to internal mock control cells ($eGFP^-$). Normalized pERK means±SEM are shown with lines connecting the T cell transductant group over the timecourse (Tconv donors n=8, Treg donors n=5, Two-way ANOVA using

subject and timepoint matching with Bonferroni's posttest, *p<0.05, **p<0.01, ***p<0.001, shaded to indicate significance for that group versus eGFP).

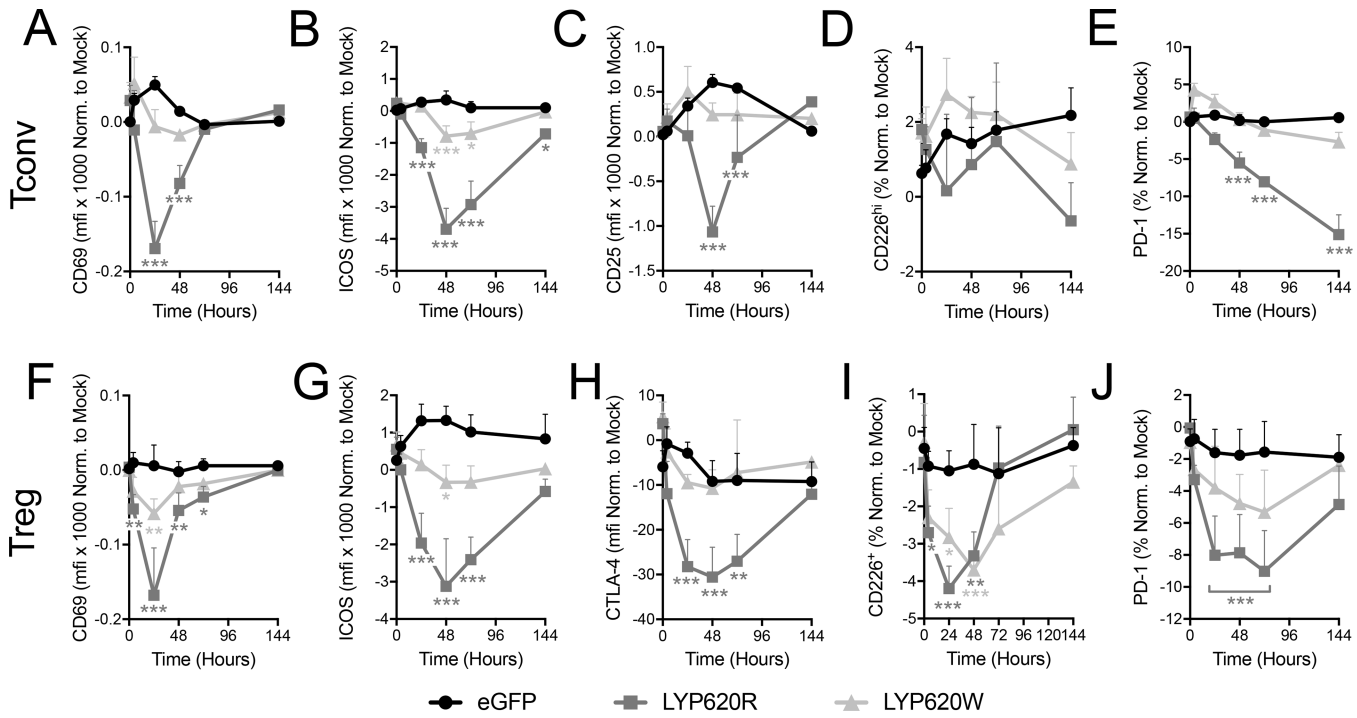

Figure 3. The risk variant of *PTPN22* less effectively inhibits activation.

Activation marker expression on Tconv (*A*-*E*) and Tregs (*F*-*G*) in response to co-culture with antigen presenting cells (APC) and soluble anti-CD3 and anti-CD28 was assessed by surface staining and flow cytometry at 0, 4, 24, 48, 72, and 144 hours post activation. Tconv overexpressing LYP-620R, LYP-620W, or eGFP were assessed for CD69 (*A*), ICOS (*B*), CD25 (*C*), CD226 (*D*), and PD-1 (*E*). Treg overexpressing LYP-620R, LYP-620W, or eGFP were assessed for CD69 (*F*), ICOS (*G*), CTLA-4 (*H*), CD226 (*I*), and PD-1 (*J*). The mean fluorescent intensity (MFI) or percent positive was quantified and then normalized to internal mock control cells ($eGFP^-$). (Tconv donors n=5, Treg donors n=5, Two-way ANOVA using subject and timepoint matching with Bonferroni posttest, *p<0.05, **p<0.01, ***p<0.001, shaded to indicate significance for that group versus eGFP at that time point).

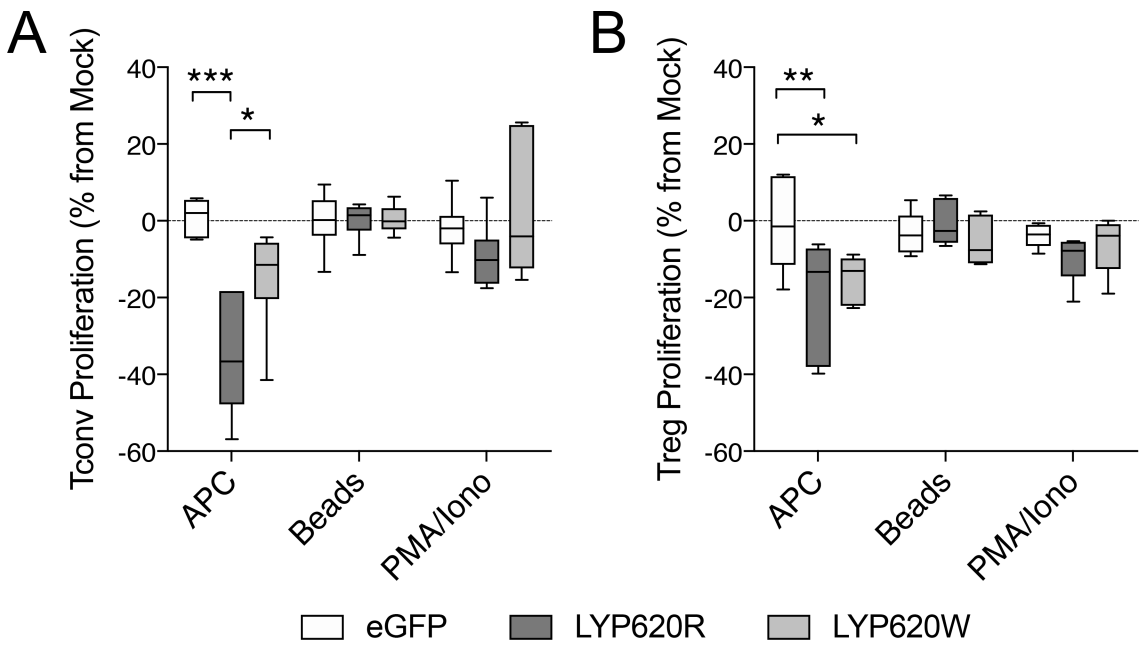

Figure 4. PTPN22–mediated abrogation of proliferation requires APC activation.

Proliferation was assessed for Tconv (*A*) and Tregs (*B*) overexpressing the LYP-620R variant, the LYP-620W risk variant, or eGFP transfected controls. Proliferation assessed by cell tracking dye dilution and was compared to internal mock control cells (GFP^-). Three activation stimuli were compared: autologous antigen presenting cells (APC) along with soluble anti-CD3 and anti-CD28, surface-fixed anti-CD3/CD28 beads, and mitogenic compounds that bypass TCR signaling (PMA/Ionomycin). (Tconv donors n=6, Treg donors n=5, Two-way ANOVA using subject and treatment matching with Bonferroni posttest, *p<0.05, **p<0.01, ***p<0.001).

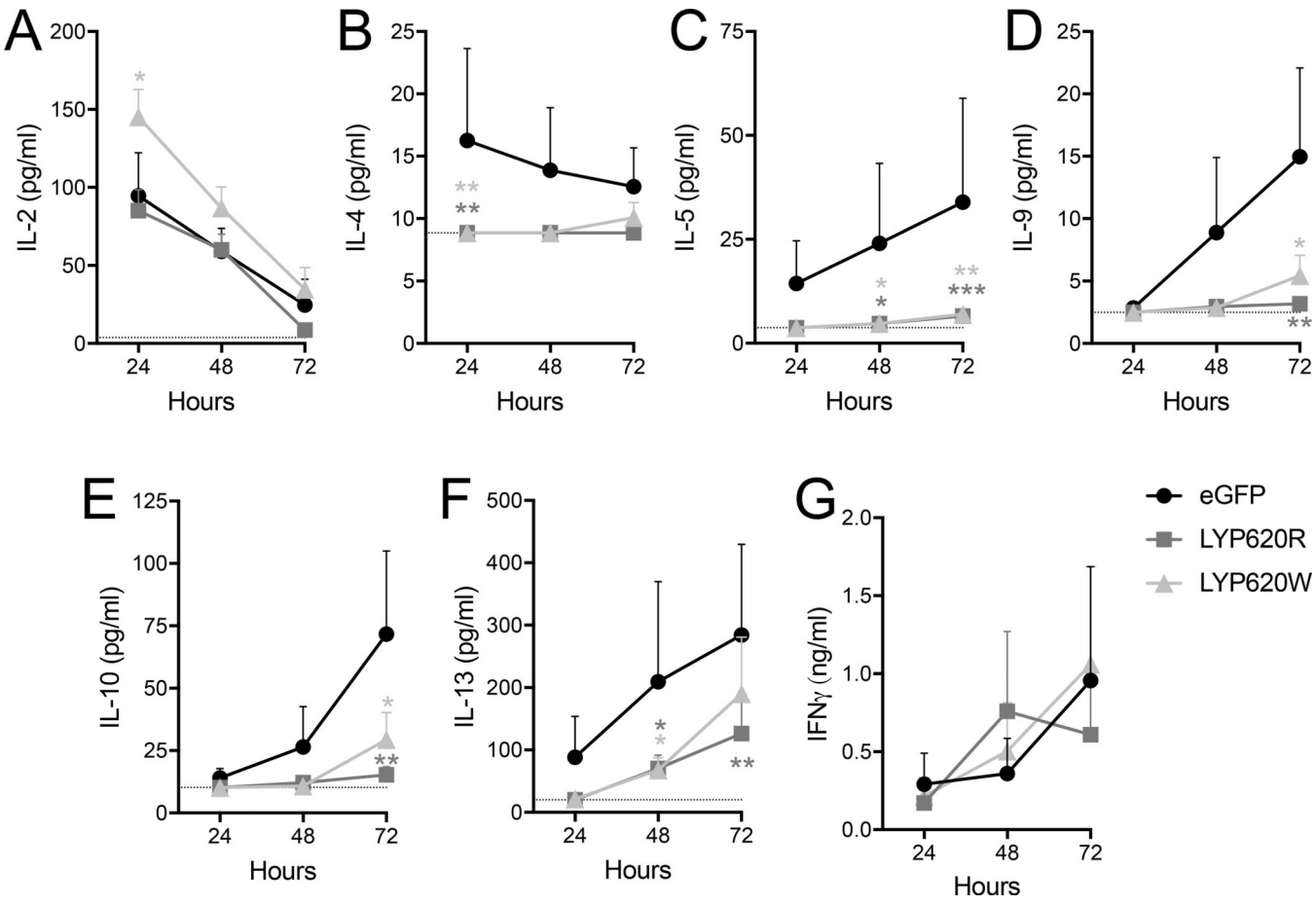

Figure 5. Cytokine production by PTPN22 modulated CD4 T cells.

Tconv were sorted for stable transductants (eGFP^{+}) and activated by antigen presenting cells (APCs) with soluble anti-CD3 and anti-CD28. (*A*) IL-2, (*B*) IL-4, (*C*) IL-5, (*D*) IL-9, (*E*) IL-10, (*F*) IL-13, and (*G*) IFNγ production were assessed by multiplexed immunoassay of culture supernatants at 24, 48, and 72 hours. The limit of detection for each assay is indicated as a dotted line. (Donor n=5, Two-way ANOVA using subject and timepoint matching with Bonferroni's posttest, *p<0.05, **p<0.01, ***p<0.001, shaded to indicate significance for that group versus eGFP at that time point).

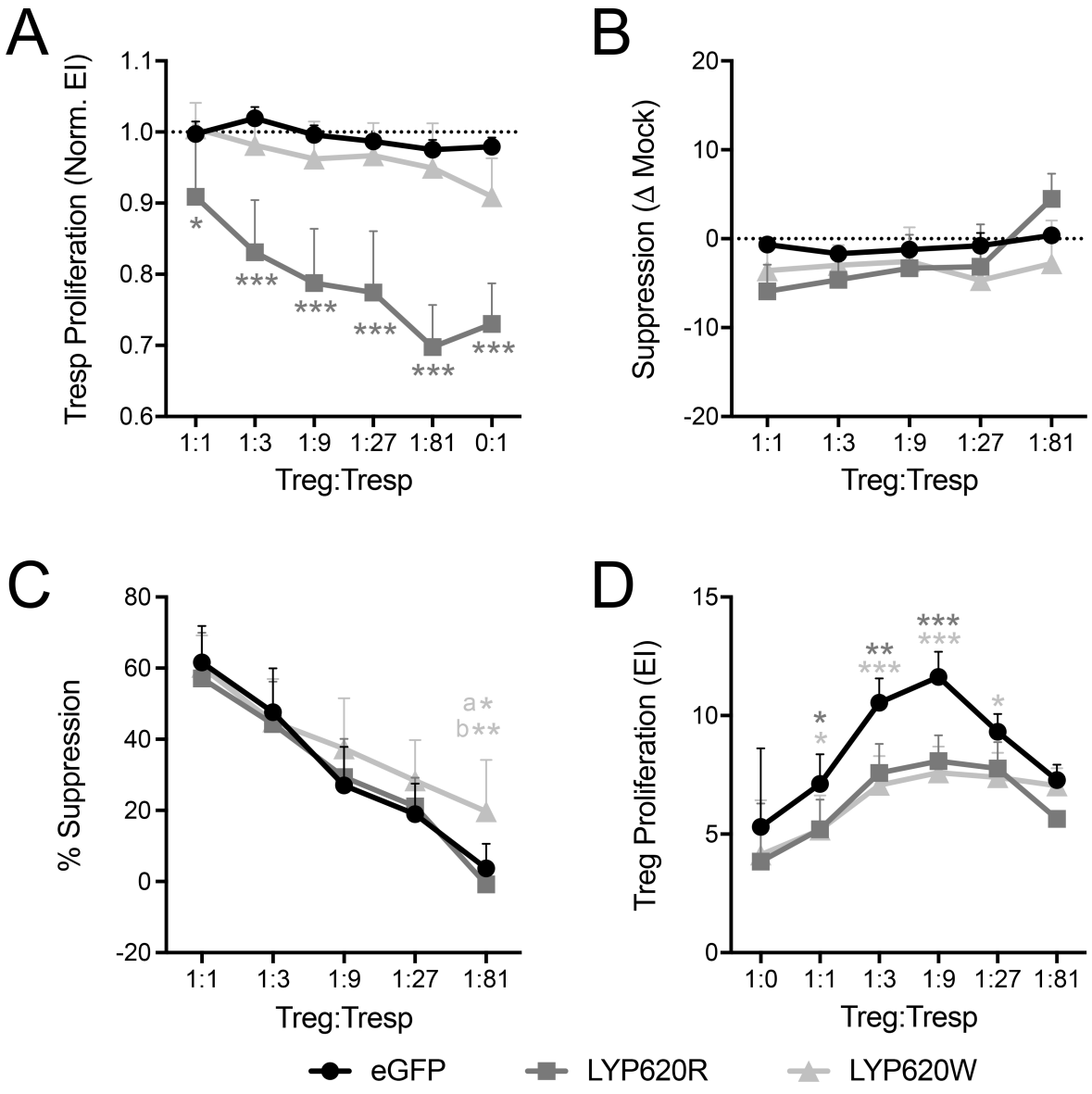

Figure 6. Suppression of modulated CD4 T cells and suppression by modulated Tregs is not affected by *PTPN22* modulation.

(*A*, *B*) Un-modulated regulatory T cells (Tregs, sorted eGFP$^-$) were titrated and co-cultured with Tconv overexpressing LYP-620R, LYP-620W, or eGFP and their respective mock-transduced (eGFP$^-$) internal control Tconv. APCs, anti-CD3, and anti-CD28 were used for activation for 4 days (Donor n=5). (*A*) Proliferation was assessed as Expansion Index (EI) by cell tracking dye dilution for transduced responder T cells (Tresp) cells and then normalized to the proliferation of internal control mock cells. (*B*) Percent suppression was calculated for the transduced cells and then normalized to the percent suppression of the mock-transduced cells. (*C*, *D*) Tregs overexpressing LYP-620R, LYP-620W, or eGFP (sorted eGFP$^+$) were titrated and co-cultured with un-modulated Tconv (sorted eGFP$^-$), followed by activation with APCs, anti-CD3, and anti-CD28 for 4 days (Donor n=4). (*C*) Tresp proliferation was assessed and used to calculate percent suppression. (*D*) Treg proliferation

was assessed by cell tracking dye dilution. (Two-way ANOVA using subject and Treg:Tresp matching with Bonferroni posttest *p<0.05, **p<0.01, ***p<0.001, shaded to indicate significance for that group versus eGFP at that time point. In (A) we limited the statistical annotation to the comparisons to eGFP. The following were also significant for LYP-620R vs LYP-620W: 1:1 p<0.05, 1:3 p<0.001, 1:9 p<0.001, 1:27 p<0.001, 1:81 p<0.001, 0:1 p<0.001. In (*C*) a: LYP-620W vs eGFP, b: LYP-620W vs LYP-620R).