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CYCLE XIX

“ROLE OF PTPN22 IN TYPE 1 DIABETES”

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A. SOMMARIO

Il polimorfismo missense C1858T del gene *PTPN22* e' associato con diverse malattie autoimmuni umane, incluso il diabete autoimmune tipo 1, l'artrite reumatoide, il lupus eritematoso sistemico, la malattia di Graves, l'artrite giovanile idiopatica, e la vitiligine generalizzata. Diversi studi hanno mostrato che la associazione del polimorfismo *PTPN22* C1858T con l'autoimmunita' e' primaria e presente in diverse popolazioni. Il gene *PTPN22* codifica per la tirosin fosfatasi linfoide LYP, che e' espressa solo in cellule emopoietiche, ed e' un importante regolatore negativo della attivazione dei linfociti T. Il meccanismo molecolare utilizzato da LYP per ridurre la segnazione attraverso il recettore delle cellule T (TCR) include la formazione di un complesso fra LYP e la chinasi Csk, anche essa un inibitore della attivazione del TCR. L'allele 1858T di *PTPN22* codifica per la variante LYP-W620 della fosfatasi, che non puo' legare Csk. Questo studio e' stato condotto per elucidare il meccanismo di azione della variante LYP-W620 nella trasduzione del segnale del TCR. Abbiamo trovato che cellule T di soggetti portatori della variante LYP-W620 producono meno IL-2 dopo stimolazione del TCR, e che la fosfatasi –W620 ha una incrementata attivita' enzimatica. I nostri dati suggeriscono che la variante di LYP associata alla autoimmunita' umana possiede incrementata funzione ed e' un piu' potente inibitore della attivazione dei linfociti T.

Parole chiave

PTPN22, Diabete autoimmune, Genetica, Polimorfismo, Immunologia, Trasduzione del segnale

A. SUMMARY

A missense single-nucleotide polymorphism, C1858T in the *PTPN22* gene, is associated with several human autoimmune diseases, including type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, Graves' disease, juvenile idiopathic arthritis, generalized vitiligo, and others. Genetic studies have shown that the *PTPN22* C1858T polymorphism is primarily associated with autoimmunity in different populations. The *PTPN22* gene encodes the lymphoid tyrosine phosphatase LYP, which is expressed only in white blood cells and acts as a gatekeeper for T lymphocyte activation. The molecular mechanism by which LYP tempers T lymphocyte activation through the T cell receptor (TCR) involves the formation of a complex between LYP and the negative regulatory kinase Csk. The autoimmune-predisposing *PTPN22* T1858 allele encodes the phosphatase variant LYP-W620, which cannot bind Csk. This study was undertaken in order to elucidate the mechanism of action of LYP-W620 in TCR signaling. We found that T cells from carriers of the predisposing allele produce less interleukin-2 upon TCR stimulation, and the encoded phosphatase has higher catalytic activity and is a more potent negative regulator of T lymphocyte activation. We conclude that the autoimmune-predisposing allele is a gain-of-function mutant.

Keywords

PTPN22, Type 1 diabetes, Genetics, Polymorphism, Immunology, Signal transduction

B. INTRODUCTION

B.1. TCR signaling and autoimmunity

Autoimmune diseases are common diseases of complex etiology, in which a combination of genetic and environmental factors cause a loss of central and/or peripheral immune tolerance, leading to an attack against self-antigens [1]. Twin-concordance and family-based studies have demonstrated a strong genetic component in many autoimmune diseases, and numerous candidate loci have been found during the last decades both in human and murine models of type 1 diabetes (T1D), systemic lupus erythematosus (SLE), and others [2-5].

Although the pathological and clinical picture tends to be different and only partially overlapping between different autoimmune diseases, it is now generally accepted that some of the pathogenic mechanisms leading to the loss of immune tolerance are shared by different autoimmune diseases [6]. In other words, there are common general autoimmunity pathways, while additional disease-specific pathogenic pathways lead to the specific pathology of each autoimmune disease. Genes involved in common autoimmunity pathways tend to be associated with different autoimmune diseases, and might be responsible for the co-occurrence of different autoimmune diseases in families [6]. Anomalies in development and/or differentiation of T cells are important candidates as general autoimmunity mechanisms. T cells have been reported to play a variable pathogenic role in different autoimmune diseases, but they are almost always involved in autoimmunity [7]. In some autoimmune diseases, like T1D, T cells of both

$CD4^+$ and $CD8^+$ subpopulations play a key role in the autoimmune destruction of target tissues [8].

The development and differentiation of T cells, as well as their effector functions, are finely controlled by the activation of several intracellular signaling pathways in response to triggering of the T cell receptor (TCR) and receptors for co-stimulatory molecules [9,10]. Anomalies in TCR signaling pathways can result in autoimmunity, through effects on T cell proliferation, apoptosis, cytoskeletal changes, cytokine production, differentiation, or anergy, to mention only few of the processes regulated by the TCR in thymic and peripheral T cells [11]. TCR signaling is therefore the object of much investigation in several autoimmune diseases. In the NOD mouse model of T1D, peripheral T cells are hyporesponsive to TCR engagement [12]. This phenotype appears early in the course of the disease, and appears to have pathogenic relevance. TCR hyporesponsiveness of thymocytes due to a mutation in ZAP-70 (one of the LYP substrates) causes rheumatoid arthritis (RA) in mice [13]. Similar studies of peripheral T cell signaling in humans are complicated by the different genetic backgrounds of different individuals, which tend to increase the noise-to-signal ratio. Nevertheless a hyporesponsiveness of peripheral T cells to engagement of the TCR has been reported in human T1D and RA [14,15]. Genetic studies on molecules involved in T cell signaling pathways also point to an important role of T cell signaling in the etio-pathogenesis of different autoimmune diseases. For example, the gene encoding for the T cell co-stimulatory molecule CTLA-4 has been isolated as a strong candidate for T1D and other organ-specific autoimmunity [16].

B.2. Protein tyrosine phosphatases in TCR signaling

Tyrosine phosphorylation of cellular proteins is a key mechanism for signal transduction and is a result of the balanced action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) [17]. Engagement of the TCR leads to a spatially and temporally regulated chain of PTK/PTP activation/deactivation events, which in turn result in tyrosine phosphorylation and dephosphorylation of numerous cytosolic and transmembrane proteins [18]. PTPs are very active mediators of TCR signaling; they can be finely regulated in their activity and subcellular location, and they play both positive and negative roles in various signaling pathways activated by engagement of TCR and/or co-stimulatory receptors [18]. Due to their important roles in regulation of TCR signaling, several PTPs are currently the objects of investigation as candidate genes in autoimmunity [19]. Genetic manipulations of PTPs like CD45 and SHP1 in mouse models demonstrate the importance of PTPs in many key aspects of the immune system, including central and peripheral tolerance [20,21]. Genetic association data already suggest that functional polymorphisms in PTPs are associated with human organ-specific or systemic autoimmunity [22,23].

B.3. The tyrosine phosphatase LYP/PTPN22 – an important negative regulator of TCR signaling

This study is focused on a PTP with a critical negative regulatory role on TCR signaling, the lymphoid phosphatase LYP [24], which is encoded by the *PTPN22* gene on chromosome 1p13.3-13.1. The mouse ortholog was isolated in 1992 under the name PEST-enriched phosphatase (PEP) [25].

The 105kDa LYP/PEP is a Class I PTP [26], which contains an N-terminus catalytic PTP domain and a long C-terminus domain with four proline-rich motifs, termed P1 - P4. A shorter splice form of LYP also exists, called LYP2 [24 and see Section C.3]. Through the P1 motif, LYP and PEP bind to the Src homology 3 (SH3) domain of the Csk PTK, an important negative regulator of TCR signaling [24,27]. In mouse and human T cells, approximately 20% of Csk in T lymphocytes is in complex with ~50% of PEP/LYP [28]. The complex between PEP and Csk exerts a synergistic inhibition on TCR signaling by acting on the PTKs (Lck, Fyn, and ZAP-70) that initiate TCR-triggered T cell activation [28,29]. A model has been forwarded in which both enzymes in the PEP/Csk complex act on Lck and Fyn by targeting different tyrosines: while Csk phosphorylates the negative regulatory tyrosine in the C-terminus of Lck and Fyn, PEP dephosphorylates the positive regulatory site in the 'activation loop' of Lck and Fyn [28,29]. The structural basis for the interaction between Csk and PEP was clarified by co-crystallization of the SH3 domain of Csk with a PEP-derived P1 peptide [30]. Human LYP has also been found to be a powerful inhibitor of TCR signaling in several studies [31,32].

Although it is clear that LYP and PEP are potent negative regulators of early TCR signaling events, there are many open questions that need to be addressed. For example, it seems that PEP is not in lipid rafts at any time during TCR signaling [33], while only a very small proportion of LYP is recruited to lipid rafts in human T cells (T. Mustelin, personal communication). This surprising finding suggests that the majority of LYP may not associate with the pool of Csk that is recruited to lipid rafts. Thus,

the mechanism of recruitment and action of LYP may be more complex than expected.

Because of its role in regulating critical early steps of TCR signaling, and because of its restricted leukocyte expression, LYP is an ideal candidate for involvement in autoimmunity, especially in diseases in which T cells play an important pathogenic role. A role for LYP in T cell-mediated autoimmunity is also suggested by the phenotype of the recently reported [34] PEP$^{-/-}$ mouse. Deletion of the phosphatase in this mouse led to no effects on B cell receptor signaling, but affected thymic selection, and led to increased differentiation and responsiveness of T cell effector/memory cells [34].

B.4. Genetic polymorphisms in *PTPN22* associate with human autoimmunity

We recently found a single-nucleotide polymorphism (SNP) in the *PTPN22* gene that encodes for LYP, in which a C to T substitution at position 1858 in the coding region of LYP changes arginine 620 (R620) to a tryptophan (W620) within the P1 motif [35]. We demonstrated that while the more common LYP-R620 binds Csk (as discussed above), the LYP-W620 variant was almost unable to bind. Finally, we found that LYP-W620 is associated with T1D both in the North-American and Sardinian populations [35]. Our findings in T1D were subsequently confirmed by us [36] and by several other groups in different populations [37,38, reviewed in ref. 39]. The same polymorphism was subsequently found to also be associated with RA [31,40-42], SLE [43], Graves' disease [44,45], autoimmune Addison's disease [44], and other autoimmune diseases [46,47], suggesting that

PTPN22 is a general autoimmunity gene in humans [48]. Recent further genetic studies have shown that the *PTPN22* C1858T SNP is primarily associated with human disease in different populations, and thus plays a true causal role in the development of autoimmunity [49,50]. Considering the known role of LYP in TCR signaling, it is reasonable to speculate that the association between *PTPN22* and human autoimmunity is mediated by the effects of the phosphatase on TCR signaling, which affects T cell development, differentiation and activation and, in turn, both central and peripheral tolerance.

B.5. Advantages of genetic studies in the Sardinian population

The discovery of variants involved in complex autoimmune traits such as T1D is complicated by many factors. These are essentially represented by the small sizes of individual genetic effects compounded by low penetrance of most of the disease variants. Further complications might be created by interlocus and allelic heterogeneity, interactions between different disease loci, and interactions between disease loci and environmental factors. Some of the aforementioned problems might be alleviated in special populations such as that from the Mediterranean island of Sardinia. With a disease incidence of 36.8 cases/100,000 population/year in children 0-14 years of age, Sardinia has together with Finland, the highest incidence of T1D in the world [51]. Importantly, children with Sardinian parents, who live on the Italian mainland, where T1D is considerably less frequent, were found to have about the same incidence of T1D as the Sardinian children living on the island [52]. These data suggest that powerful founder T1D susceptibility alleles are prevalent in the Sardinian population. The present time

population, while still in the European range of genetic variation, is the result of several founder effects with a fixation of alleles and haplotypes, which are rare or absent elsewhere [53]. This genetic differentiation is extremely useful for pinpointing the etiologic polymorphisms. For instance, the population of Sardinia has already helped to provide key support in the identification of the *HLA-DRB1* locus as a primary etiologic determinant of T1D by virtue of the presence in this population of distinct HLA-DR4 haplotypes (DR4 is one of the two main predisposing haplogroups) with several different allelic combinations at the *DQB1* and *DRB1* loci [54].

The uniqueness of the Sardinian population is further supported by the distinctive distribution of variants for most of the genetic systems so far studied; it is highly possible that these founder effects will also be detected for SNPs that predispose to T1D. In other words, the particular genetic make-up of this population can influence the disease gene architecture (both in terms of allelic frequencies and effect sizes of the predisposing variants) and improve the power of a study to detect disease associations. An additional, relevant feature of the Sardinian population is its remarkable lack of population sub-structure. This reduces the potential confounding effects of locus and allelic heterogeneity. Finally, the lack of large-scale heterogeneity in Sardinia is also useful because it reduces the risk of artifacts due to population admixture, which can seriously complicate case-control association studies from mixed populations [53].

C. METHODS AND RESULTS

C.1. Rationale for the project

We were the first to report that a missense single-nucleotide polymorphism, C1858T in the *PTPN22* gene is associated with a human autoimmune disease, type 1 diabetes (T1D) [35]. Our observation has now been confirmed by several other laboratories [36-39] and expanded to rheumatoid arthritis (RA) [31,40-42], systemic lupus erythematosus (SLE) [43], Graves' disease [44,45], and other autoimmune diseases [46-49]. Further genetic studies have shown that the *PTPN22* C1858T polymorphism is indeed primarily associated with autoimmunity in different populations [49,50, and see Section C.2]. The *PTPN22* gene encodes the lymphoid tyrosine phosphatase LYP, which is expressed only in white blood cells and acts as a gatekeeper of T lymphocyte activation. The molecular mechanism by which LYP tempers T lymphocyte activation involves the formation of a complex between LYP and the negative regulatory kinase Csk. We have reported that the autoimmune-predisposing LYP-W620 variant cannot bind Csk [35]. Our current working hypothesis is that the autoimmune-predisposing *PTPN22* C1858T polymorphism leads to anomalies in LYP activity by affecting the interaction between the phosphatase and Csk. A thorough analysis of the functional effects of the LYP R620W polymorphism is a first necessary step for clarifying *PTPN22* mechanism of action in human autoimmunity.

The aim of this study is to characterize the effects of the autoimmune-associated *PTPN22* C1858T polymorphism on TCR signaling and on LYP activity in T cells.

C.2. Genetic sample sets and confirmation of association by TDT

For our genetic analyses, we chose the Sardinian population, which is a founder population showing one of the highest incidence of autoimmunity in the world. We have available large sample sets sufficient to assure statistical power to the genetic analyses we carried out. We have assembled, and have DNA from 759 T1D families. These include 625 simplex families (1,196 parents, 625 affected siblings, 597 unaffected siblings), 22 multi-generational extended families, 16 of which are complete (60 affected and 93 healthy individuals), and 112 ASP families (194 parents, 241 affected siblings, 126 unaffected siblings). Particular attention has been paid to the ethnic origin of these individuals in order to ensure a Sardinian origin in all parental lines in at least the last four generations. Using the TDT [55], on our sample set of 648 T1D Sardinian families, we could confirm that the LYP R620W polymorphism is significantly associated with T1D (transmission of the *W620 allele from heterozygous parents to affected children was 68.6%, $p<0.001$). In the same Sardinian families, four additional *PTPN22* polymorphisms (a SNP [rs1235005] and a [gaat] repeat located 5' to the R620W SNP, and another SNP [rs2476599] and a [ca] repeat located 3' to the R620W SNP) in a 75 Kbp range around the R620W SNP, were also genotyped, but did not show any association with the disease. These results are in line with the data of Begovich *et al.* [49] and suggest that the association between the LYP-R620W and T1D is not due

to linkage disequilibrium and the polymorphism has a direct causal role in the disease.

C.3. Plasmids, antibodies, and other reagents

We have cloned LYP-R620 and LYP-W620 cDNAs into the pEF-HA and pcDNA3.1 eukaryotic expression vectors. We have also cloned in the pEF-HA vector the corresponding variants of mouse PEP (PEP-R619 and PEP-W619). So far, the human and mouse genes appear to be indistinguishable and mouse PEP-R619, but not PEP-W619, binds to human Csk when expressed in human T leukemia cells (data not shown). We also made shorter constructs containing the P1 motif (residues 603-710) with either R620 or W620 and with an N-terminal S-tag in the pET30c prokaryotic expression vector [35].

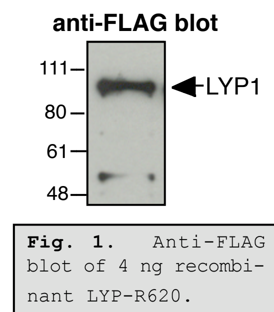

Fig. 1. Anti-FLAG blot of 4 ng recombinant LYP-R620.

Flag-tagged LYP constructs were also made in the baculovirus expression vector pFastBAC-HTa vector (Invitrogen, Carlsbad, CA), including the R620 and W620, as well as the catalytically inactive C227S mutants. We have now optimized the expression and recovery of these proteins from *Sf*9 insect cells. As an example, **Fig. 1** shows LYP-R620 isolated using Flag-antibody-conjugated beads and eluted using 0.2 mg/ml multi-Flag peptide. By silver staining, the purity of the protein was over 85% and it was catalytically active against *p*-nitrophenyl phosphate (*p*NPP) and the phospho-peptide ARLIEDNEpYTAAREG (derived from Lck auto-phosphorylation site) *in vitro* (data not shown).

We have a polyclonal antibody against mouse PEP [29], which works well in immunoprecipitation (IP), western blotting, and immunofluorescence staining, and we recently generated a monoclonal antibody, termed A2H3, against human LYP. The A2H3 mAb reacts with the peptide QQRETKEVDSKENFC corresponding to residues 457-470 of human LYP and immunoprecipitates LYP from lysates of Jurkat cells (**Fig. 2**). It also works in immunoblots. We recently conjugated this antibody with FITC and are presently testing it for use in immunofluorescence and flow cytometry.

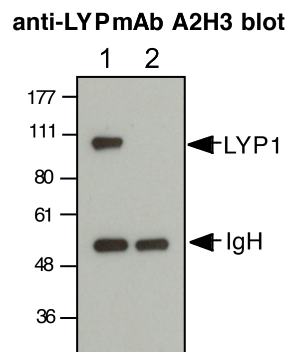

Fig. 2. Anti-LYP IPs from lysates of Jurkat cells. Lane 1: IP using the anti-Lyp mAb A2H3. Lane 2:IP using anti-LYP mAb C4G10 (clone negative at ELISA screening).

C.4. Autoimmune-associated LYP-W620 encodes for a gain-of function phosphatase

We carried a series of experiments to see if the disease-predisposing variant of LYP, LYP-W620, impacts T cell behavior in a way that can be measured by functional or biochemical assays.

First, we analyzed the activation of primary T lymphocytes from genotyped T1D patients. T cells isolated from 5 unrelated patients carrying the autoimmunity-predisposing

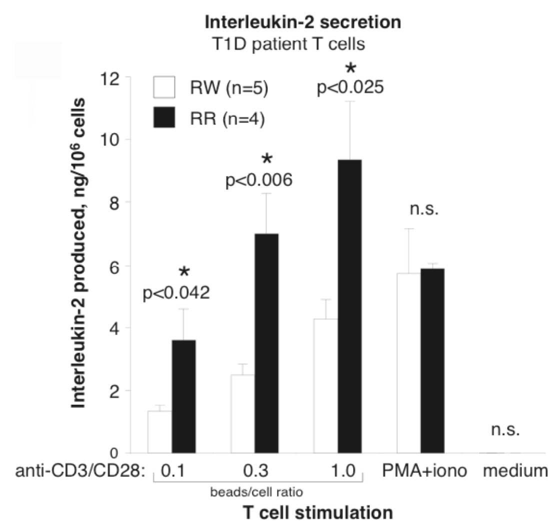

Fig. 3. Reduced interleukin-2 production of primary T lymphocytes from T1D patients of the RW genotype compared to patients of RR genotype. T lymphocytes were isolated from venous blood and stimulated with anti-CD3epsilon and anti-CD28 mAb-coated beads at the indicated bead/cell ratio, or with 40 nM phorbol 12-myristate,13-acetate plus 10 µM ionomycin, or medium alone. Interleukin-2 in the culture supernatant was measured in triplicate by ELISA. Graph represents mean ± SEM of data from 4 RR patients (black bars) and 5 RW patients (white bars). The statistical significance of the differences between RW and RR was calculated by Student's t-test. n.s., not significant.

LYP*W620 allele (RW genotype) secreted significantly less interleukin-2 (IL-2) than T cells from 4 unrelated patients of RR genotype (**Fig. 3**). This was seen at three different ratios of anti-CD3/CD28 antibody-coated beads to cells in the T cell activation assays. As an important control, the response to phorbol ester plus ionomycin was similar between the two groups. Thus, there was a clear difference in the response to TCR stimulation between the carriers of LYP*W620 and non-carriers, but not in response to pharmacological activation that bypasses early TCR signaling events.

Analysis of different T cell subpopulations revealed that patients of the two genotypes had very similar numbers of naïve versus memory T cells, $CD4^+$ versus $CD8^+$ T cells, as well as $CD4^+CD25^+$ regulatory T cells (data not shown), excluding the possibility that the differences in IL-2 production reflected any skewing in T cell lineages.

Next, we analyzed the activation of primary T lymphocytes transfected with physiological amounts of LYP-W620 or LYP-R620. Transfection efficiency using the nucleofection technique (Amaxa Inc., Germany) was consistently 60 – 80% of cells, allowing us to analyze the cells directly without sorting. Stimulation of these cells with anti-CD3epsilon plus anti-CD28 mAbs led to production of IL-2, which was reduced by LYP (**Fig. 4**). At similar amounts of expressed protein, LYP-W620 consistently inhibited the IL-2 response to a higher extent. Thus, the disease-predisposing LYP-W620 was a more efficient inhibitor of T cell activation.

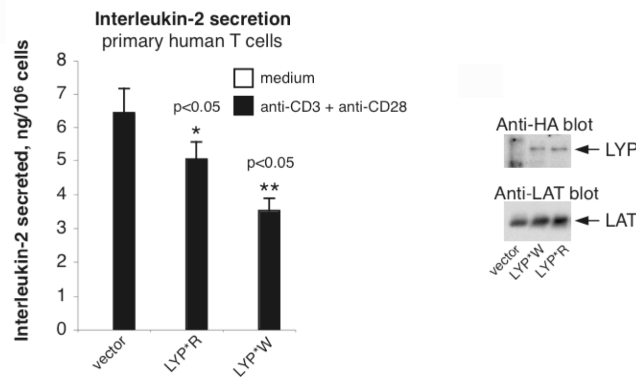

Fig. 4. Interleukin-2 secretion by primary human T lymphocytes transfected with empty vector or HA-tagged LYP*R620 or LYP*W620 and then stimulated with anti-CD3epsilon and anti-CD28 mAbs coated beads at bead to cell ratio 1:1. Data represent mean ± SD from triplicate cell cultures in one of three independent experiments with similar results.

Similar results were obtained with expression of the disease-predisposing LYP-W620 or the normal LYP-R620 in primary T cells (**Fig. 5**) or Jurkat T cells (data not shown) together with a luciferase reporter gene driven by the nuclear factor of activated T cells (NFAT)/activator protein-1 (AP-1) transcription factor complex. Stimulation of these cells through the TCR revealed that both LYP variants inhibited the response in a dose-dependent manner. However, when normalized for expression levels, the dose-response for LYP-W620 was clearly shifted to the left compared to that for LYP-R620, suggesting again that LYP-W620 was a more efficient negative regulator of T cell signaling than LYP-R620.

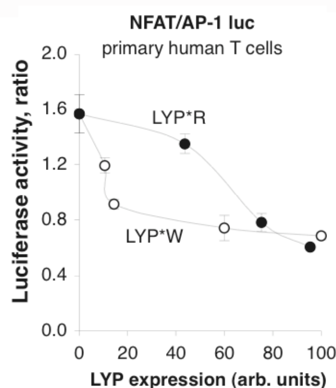

Fig. 5. Activation of the NFAT/AP-1-luciferase reporter in primary human T lymphocytes transfected with a range of doses of LYP*R620 (filled circles) or LYP*W620 (open circles) expression plasmids and then stimulated with anti-CD3epsilon and anti-CD28 mAbs coated beads at bead to cell ratio 1:1. The data points represent ratio between firefly and *renilla* luciferase (mean ± SD, n=3) and were normalized for LYP expression (arbitrary units). Data are representative of 3 independent experiments with similar results. When error bars are not seen, they are within the resolution of the points; lines are nonlinear fits of experimental data.

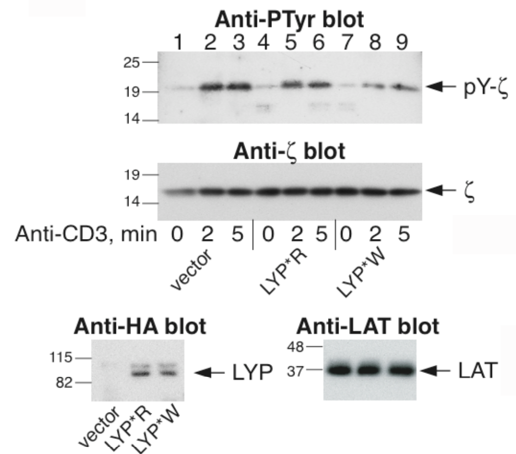

Fig. 6. Tyrosine phosphorylation of TCRzeta (upper panel) in primary T lymphocytes nucleofected with LYP*W620 or LYP*R620 and activated with anti-CD3epsilon mAb and $F(ab)_2$ fragments of anti-mouse Ig for 0, 2, or 5 min. Control blot for total TCRzeta (middle panel), anti-HA blot for LYP expression (left bottom panel), and anti-LAT blot (right bottom panel) as loading control for the LYP blot. Data shown are representative of four independent experiments.

To address this more directly, we studied the tyrosine phosphorylation of proteins involved in the earliest events of TCR signaling.

In cells expressing LYP-W620, the Lck-mediated phosphorylation of the TCRzeta chain was clearly reduced, compared to cells expressing LYP-R620 (**Fig. 6**). The activation of the Erk2 kinase was also lower in cells with LYP-W620 than in cells with LYP-R620 (data not shown), despite equal levels of expression. Similarly, LYP-W620 reduced the tyrosine phosphorylation of LAT and the phosphorylation of Lck at Y394

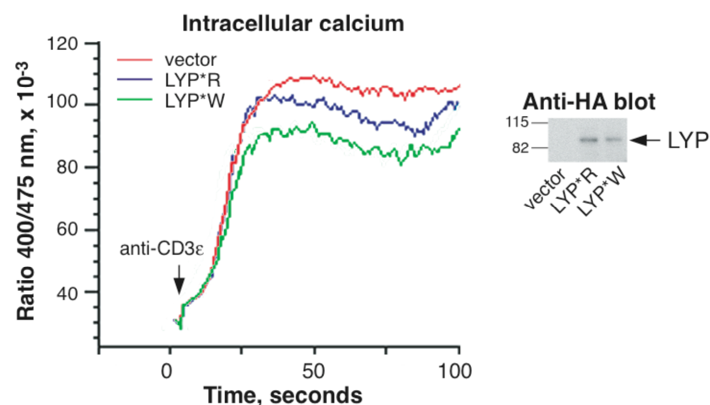

Fig. 7. Calcium mobilization in response to anti-CD3epsilon mAb in Jurkat T cells co-transfected with LYP and GFP. Cells were loaded with indo-1, and indo-1 fluorescence followed in several thousand GFP+ cells. Graph shows data from vector control cells (red), and cells expressing LYP*R620 (blue), or LYP*W620 (green). A control anti-HA blot of the same cells shows equal LYP expression.

more efficiently than LYP-R620 (data not shown).

TCR-induced calcium mobilization was also inhibited more by LYP-W620 than by LYP-R620 (**Fig. 7**). Thus, LYP-W620 again was more efficient than LYP-R620 when compared at equal levels of expression.

Finally, we measured the catalytic activities of LYP-W620 and LYP-R620 immunoprecipitated from cells. As substrate, we used the ARLIEDNEpYTAAREG phosphopeptide, modeled after the autophosphorylation site of Lck, a physiological substrate [29]. Immunoprecipitated LYP-R620 dephosphorylated 6.312 ± 1.596 pmoles of substrate/min/10^6 transfected cells (n=5), while LYP-W620 displayed a 57.2% (± 9.8%, n=5) higher specific activity when corrected for amount of protein (**Fig. 8**), despite not binding Csk (data not shown). These direct measurements provided the final proof that the *W620 allele encodes a more active phosphatase.

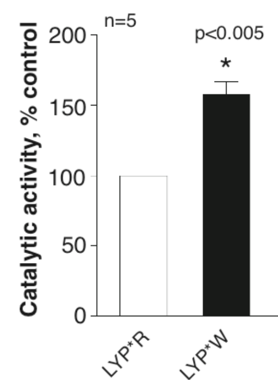

Fig. 8. Dephosphorylation of an Lck phosphopeptide by immunoprecipitated HA-tagged LYP*W620 and LYP*R620 in 100 mM Bis/Tris, pH 6.0, 150 mM NaCl, 1 mM dithiotreitol. Nonenzymatic hydrolysis of the peptide was corrected by measuring the control with addition of heat-inactivated (100°C, 10 min) immunoprecipitates. Another set of controls was obtained by adding 5 mM sodium orthovanadate to the assays, and gave comparable results. The graph represents LYP*W620 activity relative to the activity of LYP*R620 and shows the mean ± SEM from five independent experiments. Statistical analysis on the absorbance data normalized for LYP expression (arbitrary units) showed that differences in the activity between LYP*W620 and LYP*R620 were significant in each one of the five experiments (paired t-test, df=2-4, two-tailed p values were between 0.000025 and 0.019).

Staining of transfected Jurkat T cells with an Alexa-fluor conjugated anti-HA antibody did not show any differences in subcellular localization between LYP-R620 and LYP-W620. The two HA-tagged LYP variants showed also similar pattern of fractionation into detergent-insoluble

fraction in Jurkat cell fractionation experiments carried as described in ref. 56 (data not shown).

C.5. Preliminary studies on the mechanism of LYP-W620 gain-of-function

To test if the lack of binding to Csk might affect the binding of LYP to other proteins, which mediates the increase in phosphatase activity, we performed pull-down experiments in Jurkat T cell lysates using equal amounts of the S-fusion-proteins encompassing residues 603-720 of LYP with either R620 or W620. Staining of the gel with Sypro Ruby revealed that both fusion proteins bound a few common bands, but that the R620-containing peptide bound a prominent 50 kDa band, which was much weaker in the W620-cintaining peptide lane (**Fig. 9**). The molecular weight and the preferential binding to LYP-R620 suggest that this protein is Csk. Instead, the W620 peptide bound a protein of ~ 55 kDa, which was undetectable in the R620 peptide lane.

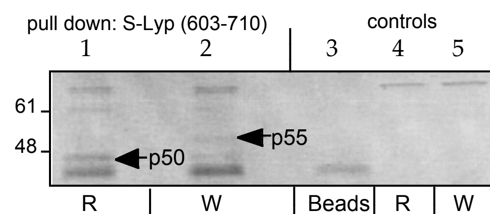

Fig. 9. "Differential" pull-down using R620 and W620 proteins. Pull-down assays were performed on lysates of Jurkat T cells using S-LYP (603-710)-R620 (lane 1) or S-LYP (603-710)-W620 (lane 2). Lane 3 shows a pull-down performed with S-agarose alone. Lane 4 and 5 are the S-proteins alone. The figure shows a polyacrylamide gel stained with SYPRO-RUBY.

D. DISCUSSION

Together, all these results suggest that LYP-W620 is a gain-of-function variant of the phosphatase. LYP-R620 and W620 mRNAs have also been recently shown to be expressed more or less at the same level in T cells from genotyped individuals [57], thus excluding effects of the polymorphism on transcription or mRNA stability. The gain-of-function phenotype of LYP-W620 was an unexpected finding in view of the previous published data about PEP regulation in T cells. Nevertheless, our observation makes sense in the light of the current reviewed data about TCR signaling in the pathogenesis of autoimmunity. Our data are also compatible with the above mentioned observations that TCR signaling is reduced in T cells in both murine and human T1D and rheumatoid arthritis [12-15]. Increased LYP activity could lead to a reduction in TCR signaling during negative selection in the thymus and might therefore lead to increased survival of autoreactive T cells, which would have been deleted in individuals with LYP-R620. It is also possible that the R620W polymorphism affects development or function of regulatory T cells (Tregs), thus impairing peripheral tolerance. Recently *PTPN22* was found to be a major target of the FoxP3 transcription factor in $CD4^{+}CD25^{+}$ “natural” Tregs [53]. Interestingly FoxP3 reduces expression of LYP in Tregs, by interfering with NFAT-mediated induction of the gene. It seems that the expression of *PTPN22* is physiologically low in resting and stimulated Tregs. A gain-of-function of LYP could lead to anomalously high levels of activity of this phosphatase in Tregs, thus interfering with development/function of this important T cell subpopulation [58].

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ELSEVIER

Association of the Single Nucleotide Polymorphism C1858T of the *PTPN22* Gene With Type 1 Diabetes

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ABSTRACT: The *PTPN22* (protein tyrosine phosphatase N22) gene encodes the protein tyrosine phosphatase Lyp. One function of Lyp is downregulation of T-cell signaling through its interaction with the negative regulatory kinase C-terminal Src tyrosine kinase (Csk). A single nucleotide polymorphism in the *PTPN22* gene, C1858T, encodes products with different Csk binding affinities. Disease association of the PTPN22 1858T allele has been reported in case-control studies of three different autoimmune disorders: type 1 diabetes (T1D), rheumatoid arthritis, and systemic lupus erythematosus. In this study, a set of 341 white, multiplex T1D families were genotyped for the C1858T single nucleotide polymorphism of PTPN22, and transmission disequilibrium test analysis revealed significant association ($p = 0.005$) of the T allele with T1D. No effects of parent of origin, sex of patient, or human leukocyte antigen genotype (high-risk human leukocyte antigen DR3/DR4 vs non-DR3/DR4) were observed. However, transmission of the T allele was significantly increased in the subset of patients who also carried at least one copy of the TCF7 883A allele, another allele that is important in regulating T-cell responses and that is associated with T1D. These results are consistent with the hypothesis that individuals lacking the C allele of PTPN22 may have reduced capacity to downregulate T-cell responses and may therefore be more susceptible to autoimmunity. *Human Immunology 66, 60-64 (2005).*
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KEYWORDS: PTPN22; type 1 diabetes; polymorphism; genetic susceptibility; transmission disequilibrium test

ABBREVIATIONS

Csk	C-terminal Src tyrosine kinase
Grb2	growth factor receptor-bound protein 2
HLA	human leukocyte antigen
PTPN22	protein tyrosine phosphatase N22
SNP	single nucleotide polymorphism
T1D	type 1 diabetes

INTRODUCTION

Insulin-dependent type 1 diabetes mellitus (T1D), an autoimmune disease, is characterized by the destruction of insulin-producing pancreatic β-cells by cytotoxic T cells. Although both genetic and environmental factors appear to be associated with the development of the disease, a clear genetic susceptibility to T1D has been established in the human leukocyte antigen (HLA) region of chromosome 6 (see, *e.g.,* [1]). In addition to the HLA region, other genetic susceptibility regions have been identified by whole-genome screens on large numbers of affected sib pairs [2, 3]. Recently, single nucleotide polymorphisms (SNPs) in several genes have manifested an association with T1D, thus expanding the repertoire of genetic factors that influence its development. Reinforcing the fact that T1D is an autoimmune disease, SNPs in genes involved in the T-cell–mediated immune response have been demonstrated to be associated with its development (*e.g.,*TCF7, CTLA4, IL6) [4–6].

The *PTPN22* (protein tyrosine phosphatase N22) gene maps to chromosome 1p13.3–p13.1 and encodes a lymphoid-specific phosphatase known as Lyp. Lyp dephosphorylates the kinases Lck, Fyn, and ZAP-70, all known to be important in T-cell signaling. An addi-

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tional function of Lyp is to downregulate activation of T cells by binding to C-terminal Src tyrosine kinase (Csk). Csk is an important suppressor of kinases that mediate T-cell activation [7]. In addition, Lyp has been demonstrated to bind to the adaptor molecule Grb2 (growth factor receptor-bound protein 2), and this interaction is thought to play a negative regulatory role in T-cell signaling [8]. Recently, an allelic variation of the *PTPN22* gene, C1858T, was revealed to be associated with T1D in a non-Hispanic, white population from North America and an Italian population [9]. The infrequent 1858T allele changes the amino acid at position 620 from an arginine (R) to a tryptophan (W), disrupting the proline-rich binding motif PxxPxR that is important for Lyp binding to both Csk and Grb2 [8–10]. The 1858T allele has also been reported to be associated with both rheumatoid arthritis and systemic lupus erythematosis [10, 11]. In the present study, we genotyped the PTPN22 C1858T SNP in 341 families with at least two siblings affected with T1D. This set includes 282 families previously genotyped for all HLA loci.

SUBJECTS AND METHODS

Subjects

Data were collected from genomic DNA from 1711 individuals from 341 families in the Human Biological Data Interchange (HBDI) repository (Philadelphia, PA). The HBDI was established, in part, for the discovery of T1D susceptibility loci. Samples from the HBDI repository are commercially available to approved researchers. All families had at least two children with T1D. Twenty-two families had one additional affected child, one family had two additional affected children, and one family had three additional affected children. Sixteen families had a parent affected with either T1D or type 2 diabetes. The average age at onset in the affected children was 11.20 (SD 7.6), with a range of 10 months to 36 years.

PTPN22 Genotyping

The PTPN22 C1858T SNP was analyzed by chip-based matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry of primer extension products [12]. Two hundred base pairs of genomic DNA surrounding the PTPN22 C1858T SNP (GenBank accession number rs2476601) were entered into the Sequenom (San Diego, CA) SpectroDesigner software for design of polymerase chain reaction and extension primers. DNA amplifications and MassExtend reactions were performed according to established Sequenom protocols. The final reactions were loaded onto a 384-well SpectroCHIP and analyzed. After analysis of all family members, 37 samples were determined to have unreadable genotypes. For these few samples, 20 ng of DNA was amplified with the same polymerase chain reaction primers that were used for the MALDI-TOF genotyping, and the amplified DNA was digested with *Xcm*I. The 100-bp amplified product containing the C allele is not digested with *Xcm*I, whereas the product containing the T allele is digested with the enzyme, resulting in a 64-bp and a 36-bp fragment. Concordance of the two methods was established by testing 22 samples of known genotype with the restriction digestion method. The tested samples included representatives of all three possible genotypes, and the concordance of genotype calls between the two methods was 100%.

Statistical Methods

The transmission disequilibrium test was used [13] to assess whether one PTPN22 allele was transmitted more frequently than the other from heterozygous parents. An analysis of variance was carried out (S-Plus 6.1; Insightful Corp, Seattle, WA) on the PTPN22 genotype with the following two classifications: the number of T alleles carried (0, 1, or 2), and TT homozygous versus non-TT homozygous patients.

RESULTS

The allele frequencies for the C1858T SNP in the parents were 0.873 for the C allele and 0.127 for the T allele. Although the parents do not represent an unbiased control group, these observed frequencies agree closely with those observed by Bottini *et al.* [9] for 395 North American white healthy controls [f(C) = 0.884, f(T) = 0.116]. The two alleles were in Hardy-Weinberg's equilibrium in the parents. Allele frequency for the T allele was slightly higher in the affected sibs [f(T) = 0.142]. The distribution of the parental genotypes in the 341 families was as follows: 200 CC/CC, 112 TC/CC, 17 TC/TC, nine CC/TT, and three TC/TT.

Transmission of PTPN22 alleles from heterozygous parents to affected children was analyzed for deviation from the expected value of 50%. Of the 682 parents in the sample set, 149 were heterozygous for the PTPN22 C1858T, and transmission of the PTPN22 allele could be assessed for 303 children of these heterozygous parents. Observed transmission of the T allele differed significantly from expected (58%; $p = 0.005$) (Table 1). In addition, the PTPN22 genotype distribution of affected children with two heterozygous parents ($n = 34$) was skewed toward the T allele, with three CC, 19 TC, and 12 TT genotypes. Deviation of this distribution from expected values under the null hypothesis (no effect of PTPN22 C1858T genotype on T1D) did not reach statistical significance ($p = 0.07$) but is suggestive that a significant effect might be seen in a larger study.

TABLE 1 Transmission disequilibrium test (TDT) on PTPN22 alleles

Allele	Transmitted	TDT	p Value	Trans % of T
C	127	—	—	—
T	176	7.92	0.005	58%
Total	303	—	—	—

Previous results from analysis of the C883A SNP in TCF7, a gene encoding a transcription factor that is hypothesized to drive T-cell responses toward a Th1 phenotype, had a significant bias toward paternal transmission of the risk allele [4]. We observed no parent of origin effect on transmission of the PTPN22 1858T allele (Table 2). Because both PTPN22 and TCF7 are important regulators of T-cell activation, PTPN22 C1858T and TCF7 C883A genotypes were analyzed for evidence of epistatic interaction. The genotype distributions of the polymorphic alleles of the two loci appear to be independent; however, the transmission proportion of the PTPN22 1858T allele is higher among individuals carrying at least one copy of the TCF7 883A allele (65.6% [95% CI 54–77]) than among those carrying the TCF7 883CC genotype (53.6% [95% CI 46–61] (data not shown). This predisposing effect of the PTPN22 SNP is only significant in patients who carry at least one copy of the TCF7 A allele, *i.e.,* have an AA or AC genotype ($p = 0.015$ vs $p = 0.34$ for patients with the CC genotype at TCF7 883). This result is consistent with the idea that individuals who have not only decreased ability to downregulate T-cell responses (*i.e.,* carry two copies of PTPN22 1858T) but also increased likelihood of mounting a Th1, rather than Th2, response (*i.e.,* carry at least one copy of TCF7 883A) are at high risk for developing T1D. Clearly, larger studies are needed to address this question.

Loci in the HLA region of chromosome 6, particularly those encoding the DR and DQ proteins, are well established to be major contributors to T1D susceptibility. Genotyping data for the DRB1 and DQB1 loci were available for 282 of the 341 families in this sample set. For the samples with HLA data available, affected children with the highest-risk HLA genotype (DR3/DR4-DQB1*0302) were analyzed separately from those with other genotypes. Data for other loci suggest that the susceptibility effects for loci other than those encoding DR and DQ, *e.g.,* TCF7, DPB1, and IL-4R, are more pronounced in patients who do not carry the high-risk HLA genotype [4, 14, 15]. Data for PTPN22 C1858T have no difference in transmission frequency between HLA high-risk patients and those without the high-risk HLA genotype (Table 2). In addition, transmission frequencies did not differ between affected boys and girls (Table 2).

Genetic susceptibility factors are known to affect age at T1D onset. The high-risk DR3/DR4 genotype, for example, is much more prevalent in those individuals with young age at onset [16]. In addition, the HLA class I allele A*2402 is associated with early age at onset [17, 18]. In this study, the average ages of T1D onset were age 11.37, 11.07. and 8.93 years for patients with the PTPN22 genotypes CC, TC, and TT, respectively. The age at onset is lower for the patients with the TT genotype, although the result is not significant, perhaps because of the small sample size.

DISCUSSION

The *PTPN22* gene product Lyp is an important downregulator of T-cell activation, in part through its physical interaction with Csk and probably through its interaction with the adaptor molecule Grb2 [8, 19]. Csk, when bound to Lyp, suppresses the kinases Lck and Fyn, which mediate T-cell signaling. The binding of Lyp to Csk is dramatically reduced when the Lyp protein has Trp at amino acid residue 620 (allele 1858T) rather than Arg (allele 1858C), as demonstrated by coimmunoprecipitation experiments [9, 10]. The reduced binding capacity of W620 Lyp (from allele 1858T) for Csk is likely to disrupt the function of Csk as a downregulator of T-cell activation. T cells lacking a Lyp-Csk complex are likely to be hyperreactive and more likely to mount an autoimmune response. In support of this hypothesis, RNAi experiments revealed decreased Lyp expression in Jur-

TABLE 2 Transmission disequilibrium test (TDT) on PTPN22 alleles depending on parental origin of the allele, patient HLA genotype, and patient sex

Characteristic	Allele	Transmitted	TDT	p Value	Trans % of T
Parental origin					
Mothers	C	49			
Mothers	T	72	4.37	0.037	59.5%
Fathers	C	59			
Fathers	T	85	4.69	0.030	59.0%
DR3/4					
No	C	69			
No	T	88	2.30	0.129	56.1%
Yes	C	38			
Yes	T	50	1.64	0.201	56.8%
Sex					
F	C	69			
F	T	96	4.42	0.036	58.2%
M	C	58			
M	T	80	3.51	0.061	58.0%

kat's cells and increased T-cell receptor–dependent activation [10]. The results of the present study suggest the hypothesis that individuals having no copies of the C allele may be less able to maintain a state of immune tolerance to self antigens. Consistent with this hypothesis, data in this study suggest that the age at onset for children carrying two T alleles is lower than that for children who are heterozygous CT or homozygous CC. The observation that TT homozygous patients have younger age at onset relative to CT heterozygotes or CC homozygotes suggests that a single copy of the C allele may produce a sufficient quantity of Lyp to effectively bind to Csk and/or Grb and successfully downregulate T-cell responses. Biologic data will be required to test this hypothesis.

Our results confirm recently published data indicating an association of the 1858T allele with T1D [9]. The reported association of the PTPN22 1858T allele with two other autoimmune disorders, rheumatoid arthritis and systemic lupus erythematosus, underscores the importance of the product of the *PTPN22* gene in immune regulation. In addition, the PTPN22 T allele demonstrates significant overtransmission only in patients who carry at least one copy of the TCF7 883A allele, an allele whose product may shift the balance of the T-cell response toward a Th1, rather than Th2, phenotype. Taken together, these results suggest that T-cell hyperactivity, combined with a predisposition to mount T-cell responses of the Th1 type, may lead to an earlier onset of pancreatic β-cell destruction and the presentation of T1D at a young age. Other T-cell factors are likely to influence the onset of the autoimmune state, pancreatic β-cell destruction, and the age at which diabetes first appears. Our preliminary result, revealing a significantly increased transmission proportion of the PTPN22 1858T allele in patients carrying at least one copy of the TCF7 883A allele, emphasizes both the complexity of the autoimmune response and the need for large studies or meta-analyses of data to begin to understand the complex molecular interactions that lead to T1D. Identification and genotyping of other candidate SNPs, and analysis of the combined data, could lead to greater positive predictive value not only for susceptibility to autoimmune diseases but also for risk of early onset of autoimmune disorders.

Acknowledgments
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Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant

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A SNP in the gene *PTPN22* is associated with type 1 diabetes, rheumatoid arthritis, lupus, Graves thyroiditis, Addison disease and other autoimmune disorders. T cells from carriers of the predisposing allele produce less interleukin-2 upon TCR stimulation, and the encoded phosphatase has higher catalytic activity and is a more potent negative regulator of T lymphocyte activation. We conclude that the autoimmune-predisposing allele is a gain-of-function mutant.

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Protein tyrosine phosphatases (PTPs) are important regulators of the immune response[1] and are involved in maintaining the resting phenotype of lymphocytes as well as in controlling signaling from antigen receptors, costimulatory receptors and cytokine receptors. The ability of PTP abnormalities to cause autoimmune disease was first illustrated by the motheaten mouse, in which loss of the SH2-containing PTP-1 (SHP1) causes a severe hyperinflammatory disease and hyper-responsive T and B lymphocytes[2]. Autoimmune disease also develops in transgenic mice with a gain-of-function mutation of the transmembrane phosphatase CD45 (ref. 3), and mice lacking the PEST-enriched phosphatase (PEP) have exaggerated and prolonged effector T cell expansion[4].

We recently found that the SNP 1858C→T in *PTPN22*, resulting in the amino acid substitution R620W in the encoded lymphoid tyrosine phosphatase (LYP; the human ortholog of PEP), was associated with autoimmune type 1 diabetes (T1D) in two different populations[5]. This observation has now been confirmed by many laboratories[6,7] in Northern European populations and has also been expanded to include rheumatoid arthritis[8], systemic lupus erythematosus, Graves disease and other autoimmune diseases[9]. The positive association of 1858C→T with T1D and other autoimmune disorders in different populations, in which the patterns of linkage disequilibrium around this variant are expected to be different, suggests that the 1858C→T polymorphism is primarily associated with T1D and other autoimmune disorders. But proof of disease causality of any variant with primary disease association has to be corroborated by additional biochemical, structural and functional evidence.

Because LYP is expressed in lymphocytes and T1D is caused by β-cell destruction by cytotoxic T lymphocytes with help from $CD4^+$ T cells[10], disease predisposition could be caused by altered T lymphocyte function. T cells also have a central role in the initiation of rheumatoid arthritis, lupus and Graves disease. Therefore, we asked whether the putative disease-predisposing variant of LYP, LYP-Trp620, affects T cell behavior in a way that can be measured by functional or biochemical assays. First, we analyzed the activation of primary T lymphocytes from genotyped individuals with T1D. For this purpose, we genotyped 631 Sardinian families with T1D. In this sample (which was independent of that previously reported[5]), the association of 1858C→T with T1D, evaluated by the transmission disequilibrium test, was significant: transmission of the mutated allele 1858T (encoding LYP-Trp620) was 69.4% (degrees of freedom (d.f.) = 2; one-tailed P value = 0.0033). T cells isolated from five unrelated individuals with T1D carrying the autoimmunity-predisposing allele 1858T (genotype $PTPN22^{1858C/1858T}$) secreted substantially less interleukin-2 than did T cells from four unrelated individuals with T1D of genotype $PTPN22^{1858C/1858C}$ (**Fig. 1a**). We observed this discrepancy in interleukin-2 levels at three different ratios of beads coated with antibody to CD3/CD28 to cells in T cell activation assays. As a control, we measured the response to phorbol ester plus ionomycin and found that it was similar between the two groups. Therefore, we observed a difference in response to TCR stimulation, but no difference in response to pharmacological activation that bypasses early TCR signaling events, in carriers of LYP-Trp620 versus noncarriers. Analysis of different T cell subpopulations showed that individuals with T1D of the two genotypes had very similar numbers of naive versus memory T cells, $CD4^+$ versus $CD8^+$ T cells, and $CD4^+CD25^+$ regulatory T cells (**Supplementary Fig. 1** online), excluding the possibility that the differences in interleukin-2 production reflected a skewing in T cell lineages.

Next, we analyzed the activation of primary T lymphocytes transfected with physiological amounts of LYP-Trp620 or LYP-Arg620. Transfection efficiency using the nucleofection technique was consistently 60–80% of cells, allowing us to analyze the cells directly without sorting. Stimulation of these cells with monoclonal antibodies to CD3ε and to CD28 led to production of interleukin-2, which was reduced by LYP (**Fig. 1b**,**c**). At similar amounts of expressed protein, LYP-Trp620 consistently inhibited the interleukin-2 response to a higher extent. Therefore, the disease-predisposing variant LYP-Trp620 was a more efficient inhibitor of T cell activation.

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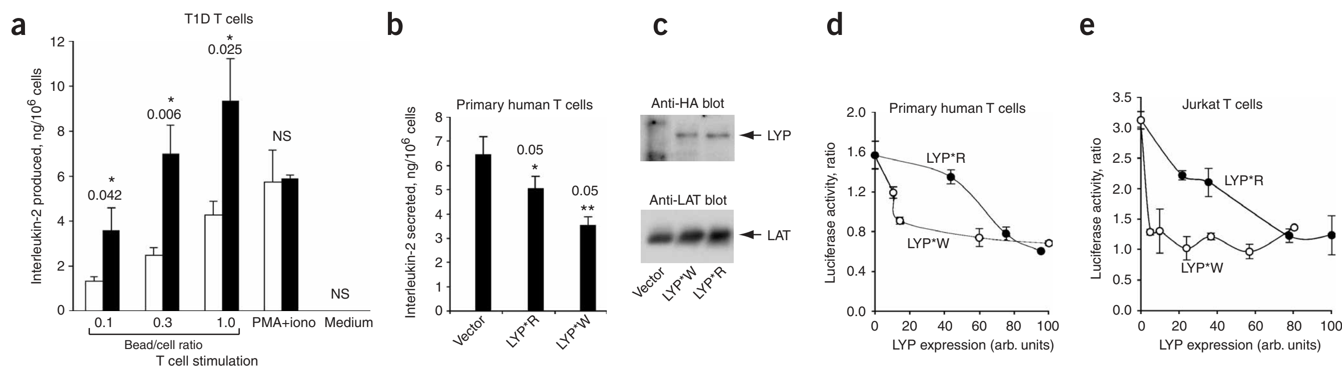

Figure 1 The disease-associated variant LYP-Trp620 inhibits T cell activation more potently than LYP-Arg620. (**a**) Interleukin-2 production was reduced in primary T lymphocytes from individuals with T1D of genotype *PTPN22*$^{1858C/1858T}$ compared with those of individuals with T1D of genotype *PTPN22*$^{1858C/1858C}$. This study was approved by the Institutional Review Board at the University of Cagliari, Italy, and informed consent forms were signed by each affected individual or a parent. T lymphocytes were isolated from venous blood from individuals with T1D by Ficoll (Biochrom AG) gradient centrifugation and further purified by immunodepletion of non-T cells using Pan T cell isolation Kit II human (Miltenyi Biotec Macs). Cells were stimulated with beads coated with monoclonal antibodies to CD3ε and CD28 (Dynal Inc.) for 20 h at the indicated bead/cell ratios; with 40 nM phorbol 12-myristate,13-acetate plus 10 μM ionomycin (PMA+iono); or with medium alone[15]. Interleukin-2 in the culture supernatant was measured in triplicate by ELISA (Quantikine, R & D Systems Inc.). Values represent mean ± s.e.m. of data from four *PTPN22*$^{1858C/1858C}$ individuals (black bars) and five *PTPN22*$^{1858C/1858T}$ individuals (white bars). The statistical significance of the differences between *PTPN22*$^{1858C/1858T}$ and *PTPN22*$^{1858C/1858C}$ individuals was calculated by Student's *t*-test (*P* values shown above bars). NS, not significant. (**b**) Interleukin-2 secretion by primary human T lymphocytes transfected by nucleofection (Amaxa Inc.) with empty vector or hemagglutinin (HA)-tagged LYP-Arg620 (LYP*R) or LYP-Trp620 (LYP*W) and then stimulated for 20 h with beads coated with monoclonal antibodies to CD3ε and CD28 at bead/cell ratio of 1:1 (black bars) or with medium alone (white bars). Values represent mean ± s.d. from triplicate cell cultures in one of three independent experiments with similar results. *P* values are shown above bars. (**c**) Expression of LYP proteins in the same transfectants as in **a** (upper panel) and blotted against LAT as loading control (lower panel). (**d**) Activation of the NFAT/AP-1-luciferase reporter in primary human T lymphocytes transfected by nucleofection with various doses of plasmids expressing LYP-Arg620 (filled circles) or LYP-Trp620 (open circles) and then stimulated as described for **a** for 6 h (ref. 15). Data represent the ratios between firefly and *Renilla* luciferase (mean ± s.d., $n = 3$) and were normalized for LYP expression (arbitrary units). Data are representative of three independent experiments with similar results. Some error bars are within the resolution of the points and are not visible. Lines are nonlinear fits of experimental data. (**e**) Similar NFAT/AP-1-luciferase assay in Jurkat cells transfected by electroporation with different amounts of LYP expression plasmids, then stimulated with the optimal concentration (150 ng ml^{-1}) of monoclonal antibody to CD3ε for 6 h and otherwise treated as described in **d**. Data are representative of seven independent experiments.

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We obtained similar results with expression of the disease-predisposing variant LYP-Trp620 or normal LYP-Arg620 in primary T cells (**Fig. 1d**) or Jurkat T cells (**Fig. 1e**) together with a luciferase reporter gene driven by the nuclear factor of activated T cells (NFAT)/activator protein-1 (AP-1) transcription factor complex. Stimulation of these cells through the TCR showed that both LYP variants inhibited the response in a dose-dependent manner. When normalized for expression levels, however, the dose response for LYP-Trp620 was shifted to the left relative to that for LYP-Arg620 (**Fig. 1d,e**), suggesting again that LYP-Trp620 was a more efficient negative regulator of T cell signaling than LYP-Arg620.

To address this possibility more directly, we studied the tyrosine phosphorylation of proteins involved in the earliest events of TCR signaling. Lck-mediated phosphorylation of the TCRζ chain was reduced in cells expressing LYP-Trp620 compared with cells expressing LYP-Arg620 (**Fig. 2a**). Activation of the Erk2 kinase was also lower in cells with LYP-Trp620 than in cells with LYP-Arg620 (**Fig. 2b**), despite their equal levels of expression. Similarly, LYP-Trp620 reduced the tyrosine phosphorylation of LAT and the phosphorylation of Lck at Tyr394 more efficiently than did LYP-Arg620 (**Supplementary Fig. 2** online). TCR-induced calcium mobilization was also inhibited more by LYP-Trp620 than by LYP-Arg620 (**Fig. 2c**). Therefore, LYP-Trp620 was more efficient at dephosphorylation than LYP-Arg620, even at equal levels of expression.

Finally, we measured the catalytic activities of LYP-Trp620 and LYP-Arg620 immunoprecipitated from cells. As substrate, we used a phosphopeptide modeled after the autophosphorylation site of Lck, a physiological substrate[11]. Immunoprecipitated LYP-Arg620 dephosphorylated 6.312 ± 1.596 pmol of substrate per min per 10^6 t ransfected cells ($n = 5$). LYP-Trp620 had a 57.2% (± 9.8%, $n = 5$) higher specific activity when corrected for amount of protein (**Fig. 2d**), despite not binding Csk (**Fig. 2e**). These direct measurements provided the final proof that the 1858T allele encodes a more active phosphatase.

We conclude that the LYP variant that causes autoimmune disease is a gain-of-function form of the enzyme. Further studies are needed to establish the role of the increased phosphatase activity of LYP-Trp620 in the pathogenesis of human autoimmunity. Although a simplistic model of autoimmunity would predict that T cells with defects that augment TCR signaling would be likely to cause disease, experiments have shown that peripheral T cells from individuals with T1D are hyporesponsive to *in vitro* stimulation with antibodies to CD3 (ref. 12). Thymocytes from nonobese diabetic mice are also hyporesponsive to TCR-mediated activation and proliferation[13], and indirect evidence suggests that thymocyte hyporesponsiveness due to anomalies in early TCR signaling has a causative role in autoimmune disease[14]. Therefore, the increased efficacy of LYP-Trp620 to inhibit TCR signaling may lead to weaker signaling and a failure to delete autoreactive T cells during thymic selection or insufficient activity of regulatory T cells. This would explain why LYP-Trp620 increases susceptibility to a number of diseases and why even one mutated allele confers predisposition to T1D and other autoimmune disorders.

If the LYP-Trp620 gain-of-function phenotype has a pathogenic role in human autoimmunity, a specific small-molecule inhibitor could be useful to prevent the emergence, or reappearance, of autoreactive T cells. Because LYP-Trp620 has been implicated in so many prevalent

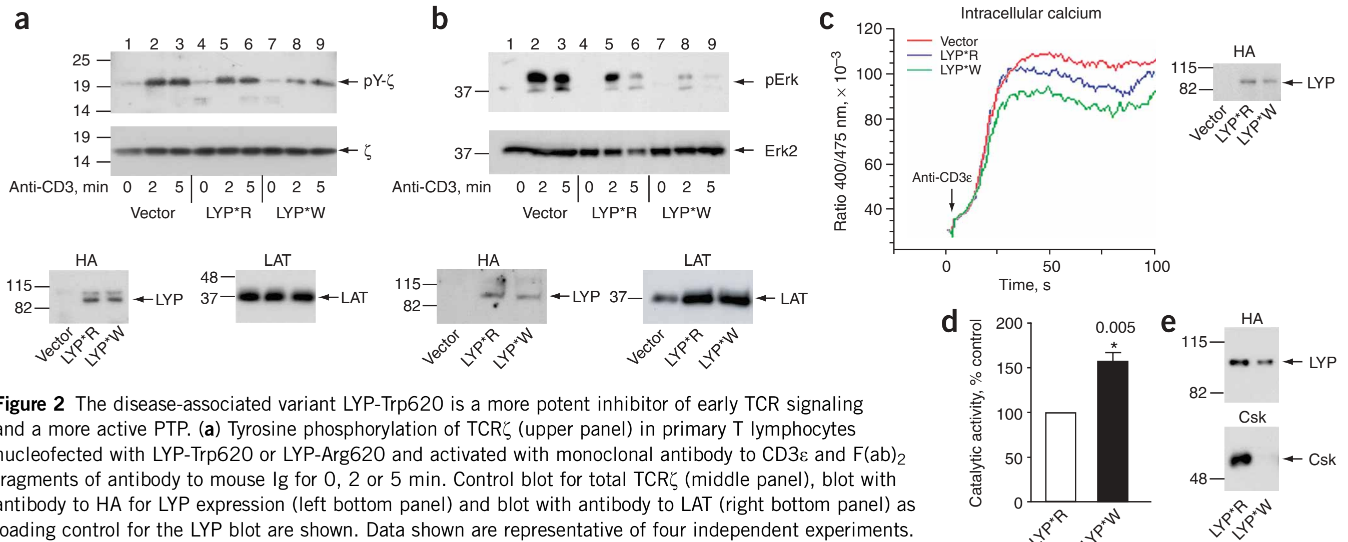

Figure 2 The disease-associated variant LYP-Trp620 is a more potent inhibitor of early TCR signaling and a more active PTP. (**a**) Tyrosine phosphorylation of TCRζ (upper panel) in primary T lymphocytes nucleofected with LYP-Trp620 or LYP-Arg620 and activated with monoclonal antibody to CD3ε and F(ab)$_2$ fragments of antibody to mouse Ig for 0, 2 or 5 min. Control blot for total TCRζ (middle panel), blot with antibody to HA for LYP expression (left bottom panel) and blot with antibody to LAT (right bottom panel) as loading control for the LYP blot are shown. Data shown are representative of four independent experiments. (**b**) Phosphorylation of Erk2 in primary T lymphocytes nucleofected with LYP-Trp620 or LYP-Arg620 and activated as described for **a** (upper panel). Control blot for total Erk2 (middle panel), blot with antibody to HA for LYP expression (left bottom panel) and blot with antibody to LAT (right bottom panel) as loading control for the LYP blot. (**c**) Calcium mobilization in response to monoclonal antibody to CD3ε in Jurkat T cells cotransfected with LYP and GFP. Cells were loaded with indo-1, as described[15], and indo-1 fluorescence was followed in several thousand GFP$^+$ cells. Graph shows data from vector control cells (red) and cells expressing LYP-Arg620 (blue) or LYP-Trp620 (green). A control blot with antibody to HA of the same cells shows equal LYP expression. (**d**) Dephosphorylation of an Lck phosphopeptide (ARLIEDNEpYTAAREG) by immunoprecipitated HA-tagged LYP-Trp620 and LYP-Arg620 in 100 mM Bis/Tris (pH 6.0), 150 mM NaCl and 1 mM dithiotreitol. Nonenzymatic hydrolysis of the peptide was corrected by measuring the control with addition of heat-inactivated (100 °C for 10 min) immunoprecipitates. Another set of controls was obtained by adding 5 mM sodium orthovanadate to the assays; these gave comparable results. The graph represents LYP-Trp620 activity relative to LYP-Arg620 activity and shows the mean ± s.e.m. from five independent experiments. Statistical analysis on the absorbance data normalized for LYP expression (arbitrary units) showed that differences in the activity between LYP-Trp620 and LYP-Arg620 were significant in each of the five experiments (paired *t*-test, d.f. = 2–4, two-tailed *P* values were between 0.000025 and 0.019). (**e**) Anti-HA (upper panel) and anti-Csk (lower panel) blots of a representative anti-HA immunoprecipitate used in **d**.

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autoimmune diseases, such a drug could be of broader value for the treatment of these diseases once the dosage and optimal treatment strategies have been determined.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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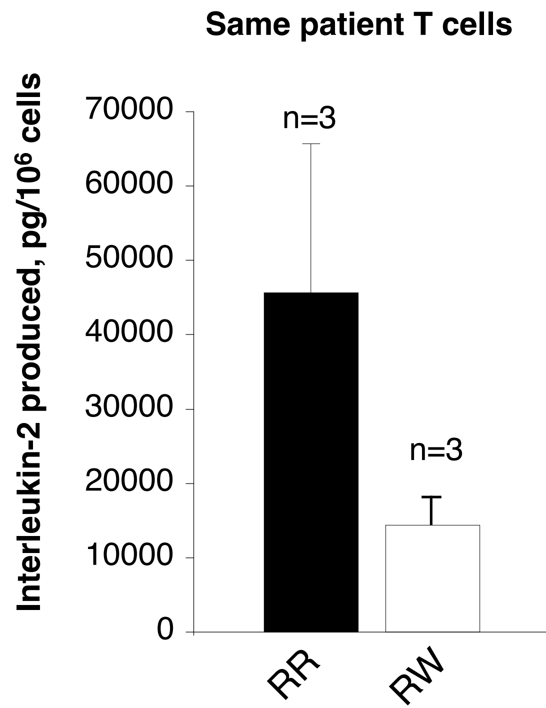
a

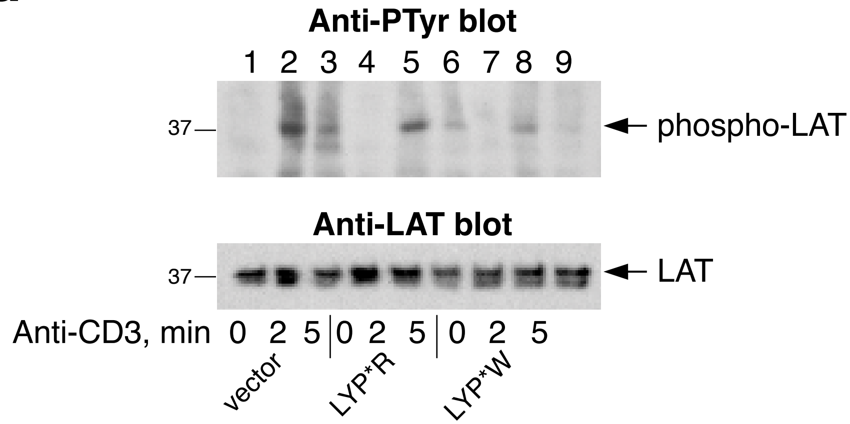
	PATIENTS				
	RW (n=3)		RR (n=3)		*t-test*
	Average	Std. Dev.	Average	Std. Dev.	*P=*
CD3+ (% of CD45+)	69.33	10.41	71.33	6.03	*NS*
CD4+CD3+ (% of CD45+)	41.67	8.08	41.67	5.51	*NS*
CD8+CD3+ (% of CD45+)	22.67	2.08	24	2	*NS*
CD4+CD45RA+ (% of CD3+)	33.04	12.3	28.45	5.12	*NS*
CD4+CD45RO+ (% of CD3+)	24.89	2.28	19.14	2.55	*0.043*
CD45RO+/RA+ (% of CD3+)	0.8	0.2	0.69	0.21	*NS*
CD4+CD44+ (% of CD3+)	57.88	4.13	56.27	0.98	*NS*
CD8+CD44+ (% of CD3+)	31.87	1.79	34.77	1.97	*NS*
CD8+CD44+/ CD4+CD44+ (% of CD3+)	0.55	0.03	0.62	0.04	*NS*
CD4+CD25+ (% of CD3+)	5.81	1.29	5.85	1.42	*NS*

b



Supplementary Figure 1 Analysis of T cell lineages and subpopulations in T1D children of RW or RR genotype. **a)** FACS analysis of peripheral blood T cells from 3 RW and 3 RR patients for the indicated surface antigens. **b)** Reduced interleukin-2 production by primary T lymphocytes from the same 3 patients, which are a subset of the ones shown in **Fig. 1**. IRB approval for the study was obtained at the University of Cagliari, Italy, and informed consent forms were signed by each patient or parent. Statistical analysis was as in **Fig. 1**.

a



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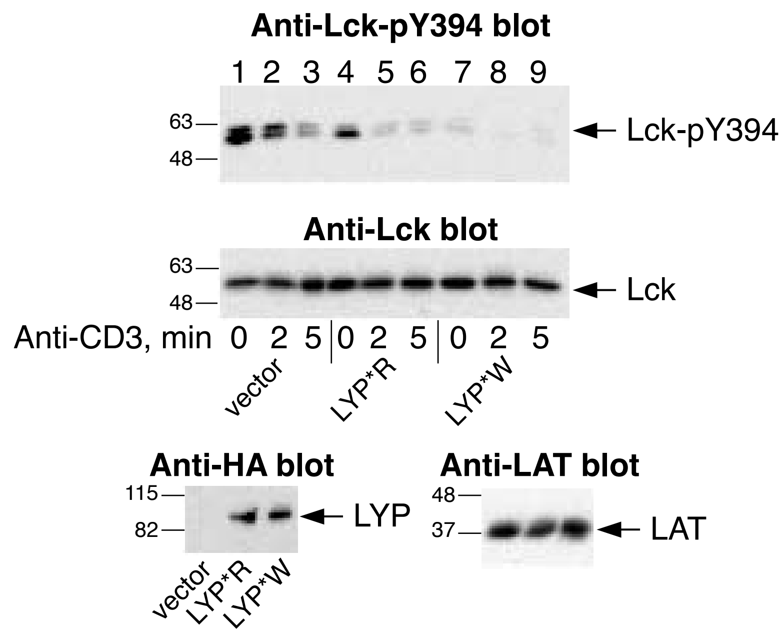

Supplementary Figure 2 The disease-associated LYP*W620 is a more potent inhibitor of early TCR signaling. **a**) Tyrosine phosphorylation of LAT in primary T lymphocytes nucleofected with LYP*W620 or LYP*R620 and activated as in **Fig. 2a** (upper panel). For control blots for total LAT and LYP expression see **Fig. 2b**. Data shown are representative of three independent experiments. **b**) Phosphorylation of Lck at its positive regulatory site, Y394 (upper panel) in primary T lymphocytes nucleofected with LYP*W620 or LYP*R620 and activated with anti-CD3ε mAb and $F(ab)_2$ fragments of anti-mouse Ig for 0, 2, or 5 min. Control blot for total Lck (middle panel) and anti-HA blot for LYP expression (left bottom panel), and anti-LAT blot (right bottom panel) as loading control for the LYP filter. Similar results were obtained in three independent experiments.

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Review

Role of *PTPN22* in type 1 diabetes and other autoimmune diseases

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Abstract

We recently discovered that a single-nucleotide polymorphism (SNP) in the lymphoid tyrosine phosphatase (LYP), encoded by the *PTPN22* gene on chromosome 1p13, correlates strongly with the incidence of type 1 diabetes (T1D) in two independent populations. This findings has now been verified by numerous studies and it has been expanded to rheumatoid arthritis, juvenile rheumatoid arthritis, systemic lupus erythematosus, Graves' disease, generalized vitiligo and other autoimmune disease. Here we review the genetics of the SNP and its association with autoimmunity, discuss the function of the phosphatase in signaling, the biochemistry of the disease-predisposing allele, and the possible mechanisms by which PTPN22 contributes to the development of human disease.
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Keywords: Diabetes; TID; PTPN22; LYP; SNP; Autoimmunity

1. Introduction

Type 1 diabetes (T1D) is a common autoimmune disease of complex etiology, characterized by an autoimmune attack against insulin-producing pancreatic β-cells [1]. The disease is more likely to occur in relatives of an affected person and shows a rapid fall-off in risk with decreased genetic relatedness to affected individuals. Still, the empirical risk for a monozygotic twin of an affected patient is about 50%, thus considerably less than 100%. All together, these data, and the increasing incidence of the disease in many countries over the last 40 years, indicate that disease risk depends on the complex interplay between several co-inherited susceptibility alleles interspersed throughout the genome as well as unknown environmental factors. Indeed, the strong and polygenic component of T1D has been supported by the identification of susceptibility alleles in the HLA class II, insulin (*INS*), *CTLA-4,* and *PTPN22* loci, and many as yet unidentified variants are also required to completely explain disease inheritance [2,3].

T cells of both CD4 and CD8 subpopulations play key roles in the autoimmune destruction of β-islet cells in T1D [4]. The development and differentiation of T cells, as well as their effector functions, are finely controlled by the activation of several intracellular signaling pathways in response to triggering of the T cell receptor (TCR) and receptors for co-stimulatory molecules [5,6]. Anomalies in TCR signaling pathways can result in autoimmunity, trough effects on T cell proliferation, apoptosis, cytoskeletal changes, cytokine production, differentiation, or anergy, to mention only few of the processes regulated by the TCR in thymic and peripheral T cells [7]. TCR signaling is therefore the object of much investigation in T1D and other autoimmune diseases. In the non-obese diabetic (NOD) mouse model of T1D several investigators have reported that peripheral T cells are hyporesponsive to TCR engagement [8–11]. This phenotype appears early in the course of the disease, and appears to have pathogenic relevance. Anomalies in TCR signaling in peripheral T cells from human patients with T1D have been also reported [12,13], although this kind of studies in humans are complicated by the different genetic background of different individuals, which tends to increase the noise-to-signal ratio. Genetic studies of human diabetes also point to an important role of T cell signaling in the etio-pathogenesis of T1D. For example, the gene for the inhibitory T cell molecule CTLA-4 has been isolated as a can-

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didate gene for T1D and other organ-specific autoimmunity [14].

2. The association of *PTPN22* with autoimmune disease

We recently discovered that a single-nucleotide polymorphism (SNP) in the lymphoid tyrosine phosphatase (LYP), encoded by the *PTPN22* gene on chromosome 1p13, correlates strongly with the incidence of type 1 diabetes (T1D) in two independent populations [15]. Within a year of our paper, other studies had confirmed our finding in additional large population samples [16–19]. An association of the same SNP in *PTPN22* was also discovered with rheumatoid arthritis (RA) [20] in the North American Caucasian population. Subsequent studies have replicated the association with RA [21–26] and extended it to juvenile rheumatoid arthritis (JRA) [27], systemic lupus erythematosus (SLE) [28–30], Graves' disease [16,31,32], generalized vitiligo [33], as well as other autoimmune disease. Interestingly, however, there is no significant correlation with inflammatory bowel disease (IBD) [21], celiac disease (CD) [34], and multiple sclerosis (MS) [35].

The numerous genetic studies carried out in different populations showed geographic differences in the T1858 (W620) allele frequencies. In healthy controls the frequency of the W620 allele decreases from Northern to Southern Europe, from around 12.5% in the English and Finnish populations, to around 6% in the Italian and Spanish populations. The T1858 allele is almost absent in African American and Asian populations. Such geographic differences raise interesting questions about the origin of the polymorphism and the selective pressure to which it is subjected in different populations. Studies have now been carried out in enough different populations to reasonably conclude that the association between the C1858T (R620W) polymorphism and autoimmunity is population-independent. Nevertheless future studies should address population-specific patterns of association with single autoimmune diseases (for example with SLE, which seems to be associated with the polymorphism in some populations [29,30]), and the possible presence of population-specific modifier genes or other factors.

The first complete resequencing of the human *PTPN22* gene was completed by the Begovich group in 2005 [36]. The obtained dense map of polymorphisms was tested for RA association and it ruled out that the predisposing effect of the T1858 allele (encoding the W620 variant) was secondary to "hitch-hiking" with a nearby polymorphism. Thus, this study indicated that the association between the T allele and RA is likely to be primary [36]. We also recently completed the resequencing of the untranslated, exonic, and exon–intron boundary regions of the *PTPN22* gene in a large Sardinian sample and after testing a dense map of informative polymorphisms for T1D association and similarly concluded that the T1858 allele is primarily associated with T1D in this population [our unpublished data]. More similar studies in multiple autoimmune diseases are probably forthcoming in the very near future, but the striking functional effect of the SNP and the replication of positive association in various populations that are likely to show different patterns of linkage disequilibrium further point to a true causal role of the C1858T SNP in all the associated autoimmune diseases. Whole gene resequencing efforts are also showing that additional SNPs in *PTPN22* may play a role [36] with patterns of linkage disequilibrium. At least one intronic polymorphism was associated with RA, on a haplotype not carrying the T1858 allele. It will be interesting to see if additional variants play a primary role in T1D and other autoimmune disorders. Functional SNPs in the *PTPN22* gene could have different or multiple effects, and might show association with different and/or overlapping groups of autoimmune diseases. For example a recent study in the German population suggested that while the C1858T polymorphism is not associated with psoriasis, a susceptibility locus for this disease is located somewhere else in the *PTPN22* gene or its vicinity [37]. Thus, the full picture of the role of *PTPN22* in autoimmunity remains to be clarified.

The association of *PTPN22* with multiple human autoimmune diseases places this gene in the small group of shared autoimmunity genes together with *MHC* and *CTLA-4*. However, *PTPN22* and, even more dramatically, *CTLA-4* susceptibility variants, have relatively small genetic effects when compared to susceptibility variants located in the HLA region. For instance, linkage analysis has shown that in T1D the λs of *PTPN22* is 1.05, which assuming a multiplicative model corresponds to a contribution to the familial clustering of disease of ~2%; much lower than HLA (~40%) [38]. Likewise, the population attributable risk related to the presence of the T1858 variant is relatively small, ranging from ~7% in Northern European populations to ~1% in different populations. Still, the disease OR for the T1858 allele in T1D is about 1.5; more than a value of 1.1 detected for the *CTLA-4* predisposing variant in T1D [14].

PTPN22 genotype could perhaps be used as one component of a set of predictor genes, perhaps in combination with additional non-genetic modifier factors for disease prediction. The studies carried out so far did not show any genetic interaction of *PTPN22* with HLA in T1D or RA, but for example a recent study showed a significant interaction of the C1858T polymorphism with the presence of anti-citrullinated peptide antibodies in RA [39]. In this study, the presence of the T1858 allele and of anti-citrullinated peptide antibodies led to a 350 times increased risk of developing RA.

PTPN22 polymorphisms could also be used as prognostic factors, provided they are associated with clinical severity or other disease variables. The association of the C1858T polymorphism with disease variability has been analyzed only in few studies so far. The polymorphism is associated with rheumatoid factor (RF)-positivity in RA [20]. In T1D, one study reported a borderline association between the T1858/T1858 homozygosity and age at onset [17], while no association was found with the development of autoantibodies. More studies are needed in order to clarify if the polymorphism associates with the clinical variability and/or severity of autoimmune diseases, and if determination of the patients' *PTPN22* genotypes could be a useful prognostic factor in the clinics.

3. Structure and function of PTPN22

PTPN22 is located on chromosome 1p13.3–13.1 and encodes a 807-amino acid residue protein referred to as the lymphoid tyrosine phosphatase (LYP) [40]. The mouse ortholog was isolated in 1992 under the name PEST-enriched phosphatase (PEP) [41] and was given the genomic designation *PTPN8*. The designation of the human and mouse orthologs as different genes was based on uncertainty whether they were orthologs or not, because they display a lower degree of identity than what signaling molecules typically show between these species. The two encoded proteins differ particularly in their C-termini to the extent that most antibodies recognize either human LYP or mouse PEP, but not both [our unpublished observation]. However, it is now clear that there is no *PTPN8* in human and no *PTPN22* in the mouse, and that the two genes in fact are orthologs of the same gene, which must have been under differential evolutionary pressures in hominids and rodents. Interestingly, we also find some biochemical differences between human LYP and mouse PEP [unpublished]. It should also be emphasized that much of the published literature concerns mouse PEP and may not be entirely applicable to human LYP.

The predominant splice form of human LYP is a 105-kDa protein with an N-terminal catalytic tyrosine phosphatase domain with a high degree of homology to other strictly tyrosine-specific classical nonreceptor tyrosine phosphatases. The C-terminal 2/3 of the protein is of unknown structure, but has distant similarity with the multidomain presynaptic protein Piccolo and the yeast proteins Flocculin (*Saccharomyces cerevisiae*) and CaO19.4183 (*Candida albicans*), and related lectin-like proteins. There is also an alternatively spliced form of LYP with a somewhat shorter C-terminus [40]. Within the last 200 residues, there are four proline-rich sequence motifs, termed P1–P4, the first of which (PPPLPERTPESFIVV) binds with high affinity to the Src homology 3 (SH3) domain of the Csk tyrosine kinase [42,43], the kinase that suppresses Src family kinases by phosphorylating their C-terminal negative regulatory tyrosine [44,45]. Csk is also an important negative regulator of TCR signaling [46]. Approximately 5% of Csk in mouse T cells is in complex with ∼25–50% of PEP [42], and it was shown that the complex between PEP and Csk exerts a synergistic inhibition on TCR signaling [42,47]. It seems that both enzymes act on the Src family kinases Lck and Fyn, which initiate TCR signaling, by targeting different tyrosines: while Csk phosphorylates the negative regulatory tyrosine in the C-terminus of Lck and Fyn, LYP/PEP dephosphorylates the positive regulatory site in the ‘activation loop’ of Lck [47] and Fyn [48]. While the binding of Csk to the P1 motif was mapped in some detail [43], the structural basis for the interaction was finally clarified by NMR of the SH3 domain of Csk with a LYP-derived P1 peptide [49].

In addition to Csk, LYP has been reported to associate with the adapter protein Grb2 [50] and the c-Cbl proto-oncogene [40]. Although these interactions could steer LYP towards cellular substrates, they are of very low stoichiometry in our hands and therefore of doubtful physiological relevance.

The current picture of LYP/PEP function in T cells is that of a negative regulator of TCR signaling, a task accomplished by direct dephosphorylation of the Src family kinases Lck and Fyn, ITAMs of the TCRζ/CD3 complex, as well as ZAP-70, Vav, valosin-containing protein and other key signaling molecules [47,48,51] (Fig. 1). However, it is already clear that the simplistic model of LYP being targeted to its substrates by Csk and Cbp/PAG is inaccurate: first, mouse PEP is *not* found in complex with those Csk molecules that are bound to Cbp/PAG in lipid rafts [52], as the model would have predicted. A small amount of human LYP can be detected in lipid rafts [our unpublished observation], but its presence appears to be independent of Csk. Second, the mouse knock-out of Cbp/PAG showed no phenotype at all, expect less Csk in lipid rafts [53]. Third, the phenotype of the $PEP^{-/-}$ mouse is less severe than predicted [54]: TCR signaling appears to be completely intact in naïve T cells and Src family kinases are normally phosphorylated at both their negative and positive regulatory tyrosine residues [54]. B cell development and B cell antigen receptor signaling were also unaffected. Only a secondary stimulation of T cells showed some of the expected augmentation and extended duration of tyrosine phosphorylation, as well as hyper-proliferation of T cells [54]. These results indicate that PEP is much less critical for TCR signaling than we anticipated, perhaps due to redundancy with other PTPs (see chapter 6). This is in stark contrast to the ability of a SNP in human *PTPN22* to cause severe and life-long autoimmune diseases.

Clearly, many important questions remain open. If LYP indeed does not accompany Csk to Cbp/PAG in lipid rafts (Fig. 1), how does LYP then target Src family kinases that predominantly reside in this location? How is the small fraction of LYP (but not PEP) in lipid rafts brought there? Where in the cell do LYP-Csk complexes reside and what are they doing? What does nuclear LYP do? Does c-Cbl play a role in LYP function, for example to dock with ZAP-70? LYP contains other Pro-rich motifs than the one binding Csk; what do they bind? Some hints of answers are given by the disease-predisposing W620 allele.

4. Biochemistry of the disease-predisposing LYP*W620

The autoimmunity-predisposing allele of *PTPN22* is a missense C–T mutation at position 1858, which changes amino acid residue 620 from Arg (R) to Trp (W) in the encoded LYP protein. We became interested in this SNP because R620 is a critical residue in the Pro-rich motif in LYP that binds the SH3 domain of Csk [42,43]. Indeed, the LYP*W620 protein fails to bind Csk [15]. Since both LYP and Csk are crucial gatekeepers of T cell antigen receptor (TCR) signaling, we had expect that the threshold for TCR signaling is altered by the SNP. Our biochemical studies on primary human T cells, including T1D patient cells, and Jurkat T leukemia cells showed that the two alleles of LYP indeed behave differently in T cell signaling. Unexpectedly, these experiments unequivocally revealed that the disease-predisposing LYP*W620 is a gain-of-function mutant [55]. It dephosphorylated Lck, TCRζ, and other signaling proteins and reduced calcium mobilization and interleukin-2 gene transactivation considerably more efficiently than LYP*R620 [55]. In response to TCR/CD28 ligation, heterozygous W/R T1D patient T cells produced less interleukin-2

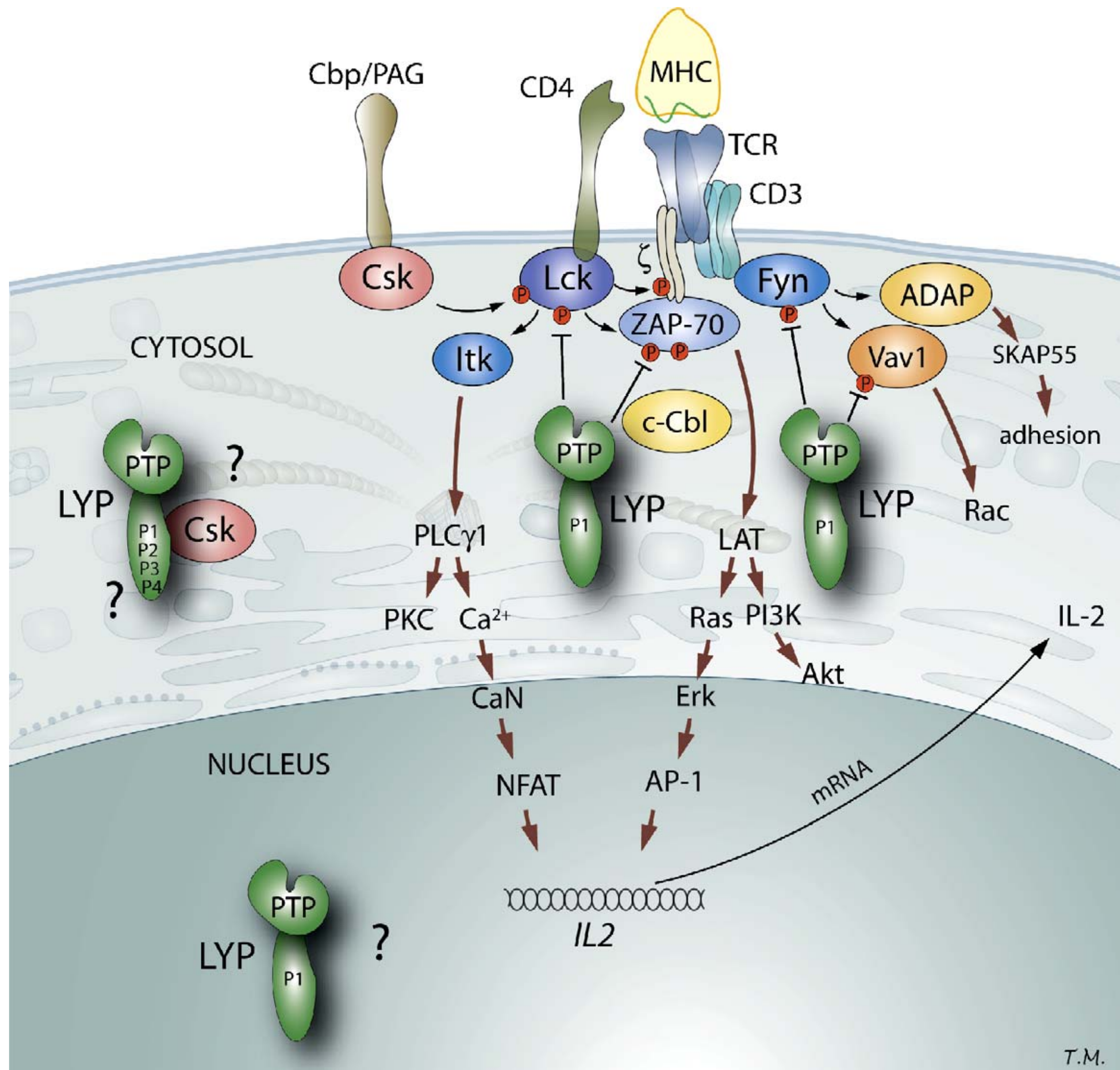

Fig. 1. Schematic representation of the known, presumed, and putative functions of PTPN22 in TCR signaling. Arrows denote positive regulatory events, T-lines denote inhibitory events (e.g. dephosphorylation).

than homozygous R/R patients, while the response to phorbol ester and ionomycin (which bypass early tyrosine phosphorylation events) was equal in the two patient samples. Even recombinant LYP*W620 expressed in Jurkat T cells was nearly twice as active as LYP*R620 against a phosphopeptide modeled after the Lck autophosphorylation site, a physiological substrate [55]. Thus, the disease-predisposing allele encodes for a significantly more active phosphatase, which suppresses TCR signaling better than identical amounts of the 'normal' LYP*R620.

This finding has several interesting implication for the molecular mechanisms by which LYP acts in TCR signaling. First, it demonstrates that association with Csk is not crucial for LYP function, but may, in contrast, restrict the ability of LYP to negatively regulate TCR signaling. Second, it shows that LYP normally exerts only part of its capacity to inhibit TCR signaling, suggesting that this function of LYP could be regulated by posttranslational mechanisms. Third, by immunofluorescence staining and confocal microscopy, the subcellular location of LYP*W620 appears to be identical to that of LYP*R620, mostly perinuclear, largely cytosolic, and some in the nucleus, but very little at the plasma membrane in resting T cells. The possible relevance of nuclear LYP is, at this time, completely unknown. Finally, it is of course fully possible that there are other biochemical consequences than the gain-of-function effect. However, it is important to note that residue 620 is not in the catalytic domain, but resides over 300 residues downstream from the catalytic domain. Thus, the catalytic domain is identical between the two alleles and therefore must have the same intrinsic specificity. However, substrate targeting or other protein–protein interactions could be affected. This hypothesis will need to be tested by specific LYP inhibitors in increasingly complex and physiologically relevant assays. These experiments will reveal whether reducing the activity of LYP*W620 with the inhibitor will eliminate all differences between LYP*W620 and LYP*R620 or not. We are fully open to the latter possibility, which we would view as very interesting and worth pursuing mechanistically. However, there are no indications at present time to suggest that LYP*W620 would have new substrates or new functions and we therefore consider the former possibility much more likely.

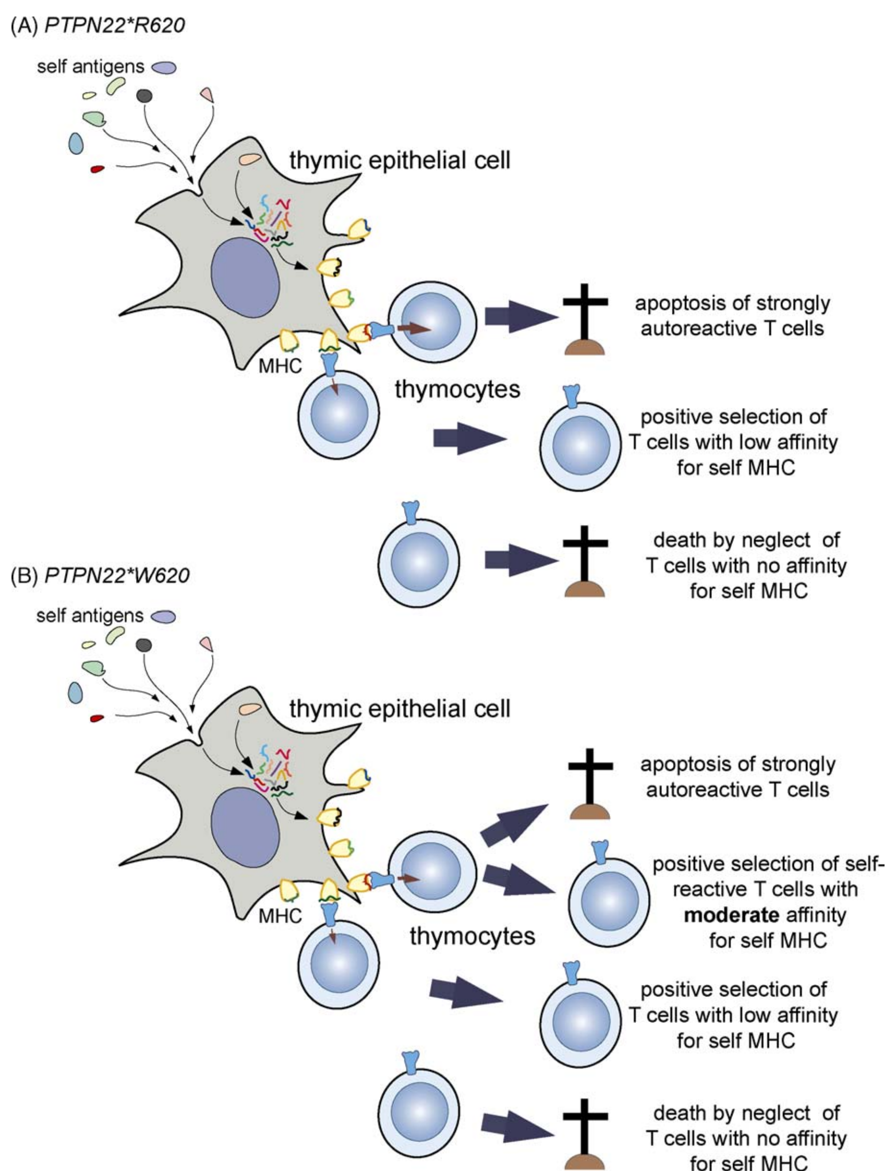

Fig. 2. Model for how the *PTPN22*W620* variant may shift thymic selection to allow for more autoreactive T cells to survive and escape into circulation. The size of the brown arrow inside the thymocytes depict the magnitude of the TCR signal.

5. Possible mechanisms by which LYP*W620 may produce autoimmunity

Based on the observation that LYP*W620 is a gain-of-function variant, our working hypothesis postulates that LYP*W620 predisposes to autoimmune disease because it suppresses TCR signaling more efficiently during thymic development, resulting in the survival of autoreactive T cells that would have been deleted by negative selection in individuals of C1858/C1858 (*R620/*R620) genotype (Fig. 2). This would explain why the susceptibility variant is dominant and not recessive, i.e. heterozygous individuals have an increased T1D incidence. It also explains the discrepancy between the PEP$^{-/-}$ mouse and the human diseases: they are in fact opposite phenomena, loss versus gain of function.

Obviously, the molecular mechanism by which LYP*W620 causes human autoimmune disease may be much more complex. Increased or altered LYP function may affect regulatory T cells to make them less potent in suppressing immune responses against autoantigens. While the phenotype of the PEP$^{-/-}$ mouse was only manifest in the T cell lineage, it is premature to conclude that the SNP in LYP would affect only T cells. LYP is well expressed in B cells, natural killer cells, macrophages, monocytes, and dendritic cells and may well affect the behavior of any or all of these cell types. Indeed, some of the autoimmune disease that associate with *PTPN22* are traditionally not considered T cell-mediated, suggesting that *PTPN22* may well cause disease through alterations in other cell types.

The association of LYP*W620 with multiple autoimmune diseases suggests that the altered function of LYP affects a pathway or mechanism that is shared among these diseases, such as loss of central and/or peripheral tolerance or altered regulatory T cell function. In this respect, the lack of association with MS and IBD is interesting. Presumably, these diseases are less dependent

on the process(es) that involves LYP. It has been suggested that the R620W polymorphism plays a role mainly in diseases characterized by a brisk autoantibody production. In line with this hypothesis, in RA the R620W polymorphism associates with RF-positive RA only, and interacts with the HLA-DRB1 locus in predisposing to the development of specific autoantibodies [56]. However, there is no significant association between the C1858T SNP and the appearance or levels of autoantibodies in T1D. Moreover, a role of LYP in antibody generation could be due either to altered T cell help or to intrinsic B cell effects, or both.

Clearly functional studies at the biochemical and cellular level and in vivo immunology studies in animal models and patient samples will be needed to unravel the mechanism of action of the C1858T SNP and other *PTPN22* polymorphism in autoimmunity. In the absence of a specific animal model, the immune cell types involved in the *PTPN22* pathogenic mechanism(s) remain hypothetical. Based on the papers published so far, we favor the notion that the C1858T polymorphism acts largely in T cell-mediated immunity by affecting TCR signaling in the thymus and/or periphery, but we are open to effects in other leukocyte lineages. This notion is supported by the role that T cells play at least in the initiation phase of the autoimmune disorders that associate with the C1858T polymorphism. In addition, TCR signaling anomalies have been reported in both T1D and RA and in animal models of these diseases, such as the NOD mouse model of T1D, in which peripheral T cells are hyporesponsive to TCR engagement early in the course of the disease [10,11]. TCR hyporesponsiveness of thymocytes due to a mutation in ZAP-70 (which is a substrate for LYP) causes RA in the SKG mouse (see chapter 1). Finally, the other shared autoimmunity genes, *MHC* and *CTLA-4*, are involved in antigen recognition by T cells and the negative regulation of TCR signaling, respectively.

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