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Graduate Course in Immunology and Applied Biothecnologies

XXII CYCLE

Role of the phospatase LYP in T cell fuction. Clinical, molecular and immunological correlates in a cohort of children affected by type 1 diabetes

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Abbreviations

IA-2: Protein tyrosine phosphatase

AP-1: activator protein 1

APC: Professional antigen presenting cells

AT: autoimmune thyroiditis

CD: Celiac Disease

BSA: Bovine serum albumin

CHX: Cycloheximide

DAG: Diacylglycerol

DAISY: Diabetes Autoimmunity Study in the Young

dNTPs: deoxy-nucleotides

GAD: Glutamic acid decarboxylase

Gads: adapter downstream of Shc

GRB2: adapters growth factor receptor-bound protein 2

HLA: Human leucocity antigen

IAA: Anti-insulin antibodies

ITAMs: immunoreceptor tyrosine-based activated motifs

LES: Lupus erythematosus systemicus

LYP: lymphoid protein tyrosine phosphatase

MAPK: The Mitogen-Activated Protein Kinase

MG-132: N-(benzyloxycarbonyl)leucinylleucinylleucina

NFAT: Nuclear factor of activated T-cells

NOD: Non-Obese Diabetic mouse

PBMC: Peripheral Blood Mononuclear Cells

PCR: Polymerase chain reaction

PI3K: phosphoinositide 3-kinase

PKC: Protein kinase C

PLC-γ: phospholipase Cγ1

PMA: Phorbol 12-myristate 13-acetate

PTPN22: Protein tyrosine phosphatase, non-receptor type 22

RA: Rheumatoid Arthritis

SD: Standard Deviation

SFKs: Src family of tyrosine kinases

SI: Stimulation Index

SNP: single nucleotide polymorphism

T1D: Type 1 Diabetes

TCR: T cell receptor

SUMMARY

The *PTPN22* gene encodes the protein tyrosin phosphatase LYP, which is an inhibitor of TCR signal transduction. Recently, a variant of *PTPN22* (1858T) has been associated with the development and progression of Type 1 Diabetes (T1D). This variant is a single nucleotide change at residue 1858 from C to T, which result in a single amminoacid substitution from arginine to tryptophan at position 620 of the LYP protein. Recent studies showed that this LYP variant has an enhanced inhibitory effect on TCR signalling suggesting that the polymorphism confers a gain-of-function form to the protein.

The aim of this study was to further characterize the effect of *PTPN22* C1858T polymorphism on T cell immune function in a cohort of T1D patients from continental Italy.

In the part I of the study we investigated the association between *PTPN22* polymorphism and our cohort of T1D patients. We examined 216 T1D patients and 271 healthy subjects from Rome. We found a higher frequency of *1858T* allele in T1D patients than in healthy controls (11.6% *vs* 5.9%, $p< 0.03$). We found a significantly higher frequency of autoimmune thyroiditis (20% in 1858T carriers vs 12% in non carriers, $p <0.05$). We also observed a significant correlation between the 1858T allele and the DQ2 (A1*0501) HLA genotype.

In the part II of the study we examined the effect of *PTPN22* polymorphism on proliferation and cytokine production of

lymphocytes from healthy controls carrying the C/C polymorphism and from T1D patients carrying the two LYP variants. Monocyte-depleted pheripheral blood mononuclear cells were obtained from 3 C/C healthy children, 16 C/C, 10 C/T and 1 T/T T1D patients and stimulated with or without OKT3/CD28 or PMA/Ionomycin. We observed a defective proliferation of T cells from T1D patients compare to healthy controls irrespective of *PTPN22* polymorphism. We found no differences in T cells proliferation upon TCR activation between T cells from C/C and C/T T1D patients, but a marked defect was observed in T cells from the T1D patient carrying the T/T genotype. Nevertheless, subjects carrying the C/T genotype had significantly lower IL-2 production compared to those with the wild type genotype ($p= 0.002$). Interestingly, in T cells from the T1D patient carrying the T/T genotype the production of IL-2 was totally absent. Furthermore, we observed a marked defect of IL-17 and IFN-γ production by T cells from the T1D patient carrying the T/T genotype.

Since we did not observe the dramatic immunological effect that would have been expected by the gain-of-function of the W620 LYP variant, in the Part III of the study we examined if there are molecular mechanisms that might explain our finding. Interestingly, we found a defect of protein expression in T cells from T1D patients carrying the C/T genotype compared to T1D carrying the C/C genotype. This defect was completely restored after inhibition of the proteasome, suggesting that LYP W620 is more degraded than LYP wild-type. Our hypothesis is that the lack of binding of LYP W620 to Csk, a know LYP-interacting protein, might lead to a greater amount of free protein in the cytosol, could be more susceptible to

degradation. The resulting reduction of the mutated LYP compared to the wild-type LYP, might mask its the gain-of function effect in T cells of heterozygous T1D patients.

SOMMARIO

Il gene *PTPN22* codifica per LYP, una fosfatasi che regola negativamente l'attivazione del recettore delle cellule T (TCR). Recentemente un polimorfismo di *PTPN22* (C1858T) e' stato associato con un maggiore rischio di svluppare il Diabete Tipo 1 (DM1). Questa variante consiste nella sostituzione di una citosina con una timidina nel residuo 1858 del gene, che determina la sostituzione di una arginina con un triptofano in posizione 620 della proteina. Studi recenti hanno mostrato che questa variante di LYP determina una maggiore inibizione sull'attivazione del TCR, suggerendo che il polimorfismo conferisce alla proteina un aumento della sua funzione inibitoria. Lo scopo del nostro studio e' stato quello di caratterizzare l'effetto del polimorfismo C1858T di *PTPN22* sulla funzione delle cellule T in una coorte di pazienti affetti da DM1.

Nella parte I dello studio abbiamo investigato l'associazione tra il polimorfismo di *PTPN22* e il DM1, esaminando 216 e 271 controlli sani. Abbiamo trovato una piu' alta frequenza dell'allele 1858T nei pazienti affetti da DM1 rispetto ai controlli (11.6% *vs* 5.9%, rispettivamente, $p< 0.03$). Abbiamo inoltre evidenziato una prevalenza significativamente maggiore di tiroidite autoimmune nei portatori dell'allele 1858T rispetto ai non portatori (20% *vs* 12%, $p<0.05$). Abbiamo inoltre osservato una correlazione significativa tra la presenza dell'allele 1858T e l'aplotipo DQ2 (A1*0501)dell' HLA.

Nella parte II dello studio abbiamo esaminato l'effetto del

polimorfismo di *PTPN22* sulla capacita' di proliferazione e di produzione di citochine dei linfociti T di soggetti sani con genotipo C/C e di soggetti con DM1 portatori delle due varianti di LYP. Cellule CD14- sono state ottenute da 3 bambini sani con genotipo C/C, 16 bambini affetti da DM1 con genotipo C/C, 10 con genotipo C/T e 1 con genotipo T/T. Le cellule sono state stimolate con o senza OKT3/αCD28 o PMA/Ionomicina.

Abbiamo osservato un difetto di proliferazione delle cellule T di pazienti con DM1 rispetto ai controlli sani indipendente dal polimorfismo di *PTPN22*. Non abbiamo osservato differenze nella capacita' proliferativa delle cellule T dopo attivazione del TCR tra pazienti con genotipo C/C e pazienti con genotipo C/T, ma e' stato evidenziato un marcato difetto di proliferazione nelle cellule T del paziente con genotipo T/T. Inoltre, cellule T di pazienti con genotipo C/T hanno mostrato un significativo difetto di produzione di interleuchina- (IL-2) rispetto alle cellule T di pazienti con genotipo C/C (p= 0.002). In accordo con la ridotta risposta proliferativa, le cellule T del paziente con genotipo T/T erano completamente incapaci di produrre IL-2. Inoltre abbiamo osservato un marcato difetto nella produzione di IL-17 and IFN-γ nelle cellule T del paziente con genotipo T/T.

Non avendo evidenziato un marcato diffetto immunologico, legato all'aumento della attivita' inibitoria della forma mutata di LYP in eterozigosi, ci siamo domandati se questo mancato difetto potesse essere dovuto a meccanismi di compenso messi in atto durante l'attivazione delle cellule T. Per questa ragione, nella parte III dello studio abbiamo ricercato possibili meccanismi molecolari che potessero influenzare la funzione di LYP W620 nelle cellule T.

Abbiamo evidenziato un difetto di espressione dell proteina nelle cellule T dei pazienti con genotipo C/T rispetto a pazienti con genotipo C/C. Questo difetto di espressione e' completamente ricostituito dopo inibizione del proteasoma, suggerendo che la forma mutata di LYP e' piu' soggetta a degradazione rispetto alla forma non mutata. La nostra ipotesi e' che il mancato legame di LYP 620W con Csk, gia' noto in letteratura, comporti un aumento della proteina libera nel citoplasma, rendendola piu' soggetta a degradazione da parte del proteasoma. L'aumento di degradazione della forma mutata di LYP potrebbe cosi' mascherare l'effetto "gain-of-function" nelle cellule T, spiegando in parte la mancata evidenza di un marcato difetto immunologico delle cellule T dei pazienti eterozigoti per il polimorfismo C1858T di *PTPN22*.

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Abstracts

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INTRODUCTION

I TYPE 1 DIABETES

1.1 Pathogenesis

Type 1 diabetes (T1D) is a multifactorial autoimmune disease, characterized by T cell mediated destruction of the insulin secreting β cells of the islets of Langerhans in the pancreas. The destructive process leads to severe insulin depletion and the clinical onset of T1D occurs when more than 80% of the β cells have been destroyed. The rate of β cell destruction is different from patient to patient, but tends to be more rapid in infants and young children (**Couper J et al., 1991**).

The early stages of disease process leading to T1D are characterized by insulitis. Several T1D animal models have shown that the first immune cells to infiltrate the islets are macrophages and dendritic cells (**Yoon JW et al., 2005**). APC first activates the naïve CD4+ T cells via presentation of β cell antigens on their MHC class II molecules, favouring their differentiation in Th1 cells via secretion of IL-12 (**Pearl-Yafe M et al., 2007**). The differentiated Th1 CD4+ T cells, in turns, secrete IL-2 and IFN-γ, which further stimulate APC to secrete other cytokines, such as IL-1β and TNF-α and free radicals, such as nitric oxide (NO). These cytokines promote the migration of CD8+ cytotoxic T cells into the islets and stimulate the β cell to release chemokines and IL-15 which further augment attraction and activation of immune cells (**Cardozo AK et al., 2003**).

On recognizing specific autoantigen on β-cells in association with class I molecules, these CD8+ cytotoxic T cells cause β-cell damage by releasing perforin and granzyme and by Fas-mediated apoptosis of the β cells.

Recent evidence points to apoptosis as the main form of β cell death in animal models of T1D. The initiation phase of the β cell apoptosis is mediated by contact-dependent mechanisms or by contact-independent cytokine action via induction of proapoptotic signalling in β cells. Previous adoptive transfer experiments indicated that both $CD4^+$ and $CD8^+$ T lymphocytes are important final effectors in β cells destruction. T lymphocytes exert contact-dependent cytotoxicity upon target cells using mainly two separate arms: the perforin-granzyme ($CD8^+$ cells) and the Fas-mediated pathways ($CD4^+$ cells) (**Kagi D et al., 1994**; **Hahn S et al., 1995**). However Fas-Fas ligand (FasL) system cannot fully explain β cell death by $CD4^+$ T cells, the most important death effector cells in T1D (**Kim S et al., 2000**). Besides contact-dependent cytotoxicity, $CD4^+$ T cells induce contact-independent target cell death via soluble mediators such as TNF-α, IL-1 and IFN-γ. Previous reports showed that combinations of proinflammatory cytokines exert much stronger or synergic effects on β cells viability in vitro, since most single cytokines have negligible effect on islet cell viability. IL-1/IFN-γ combination has been considered as a strong candidate for death effectors in T1D (**Pukel C et al., 1990**), while recent in vivo results do not fully support its role (**Schott W et al., 2004; Thomas E et al., 2004**). A combination of IFN-γ and TNF-α, but not either cytokine alone, induced a caspase-dependent apoptosis in murine insulinoma and pancreatic islet cells (**Suck K et al., 2001**).

The important role that autoreactive T lymphocytes T play in the insulitis process was also confirmed in human studies. In a study of 30 patients Himagawa and coworkers have shown that 15 out of 29 had insulitis, which in most cases was composed of CD3+ lymphocytes. Characterization of the inflammatory infiltrate in these patients revealed that it was caused by CD4+ and CD8+ lymphocytes with a predominant of CD8+ cells. A predominant CD8+ lymphocytic infiltrate has also been reported by Sozoma et al. in 18% of the islets of newly diagnosed T1D patients (**Sozoma N et al., 1994**). Moreover, autoreactive T cells specific for β cell proteins have been isolated from peripheral blood of newly diagnosed individuals with diabetes (**Hawkes CJ et al., 2000**).

One way by which the pathogenic potential of autoreactive T cell clones against insulin-producing β cells is kept under control in the periphery is through regulatory T cells (Tregs). Naturally occurring Tregs (nTregs) represents a minor population of CD4+ T cells (~4-10%), which emerge from the thymus and are characterized by the ability to suppress proliferation of responder T cells through a cell-cell contact-dependent mechanism. nTregs are characterized by the constitutive expression of CD25 (IL-2 receptor), the transcription factor forkhead box protein 3 (Foxp3) (**Sakaguchi S et al., 2005**), as well as CTLA-4, glucocorticoid-induced tumor necrosis factor receptor (GITR), OX40 (CD134) and L- selectin (CD62L). Although the expression of CD25 has been used to specifically identify nTreg in naïve mice, the value of CD25 as a marker of human nTreg is limited because CD25 is highly expressed (as are CTLA-4 and GITR) on activated CD4+ T cells. Based on the level of expression of CD25, freshly isolated human T cells can be split into suppressive

($CD25^{high}$) and non-suppressive ($CD25^{low}$) cells. However, analysis at the clonal level revealed that even the small fraction of $CD25^{high}$ cells is not a homogeneous population of suppressor cells. In contrast to CD25, the expression of Foxp3 is highly restricted to a subset of αβ TCR T cells. Studies on mice suggest that Foxp3, which binds to DNA, localizes to the nucleus, acts as transcriptional repressor (**Shubert L et al., 2001**) and functions as a nTreg lineage specification factor (**Fontenot JD et al., 2005; Fontenot JD et al., 2003; Hori S et al., 2003**). It has been shown that Foxp3 expression correlates with suppressor activity (**Fontenot JD et al., 2005**), primarily in mice and less clearly in humans. It has been demonstrated that ectopic expression of Foxp3 can endow murine T cells with regulatory capacities in vitro and in vivo (**Ramsdell F. et al., 2003**). However, in contrast to results described in murine cells, in humans it is still controversial whether Foxp3 overexpression in naïve CD4+ T cells is sufficient to confer a regulatory function (**Yagi H et al., 2004; Allan SE et al., 2005**). Of note is that in human cells, Foxp3 is also expressed by activated non-suppressive CD4+CD25- T cells (**Allan SE et al., 2007; Wang J et al., 2007**) suggesting that in addition to this transcriptor factor other components may be required for optimal suppressor activity (**Schofield L et al., 1999**). Recently, the low expression of CD127, α-chain of interleukin 7 receptor, was described as another specific marker of nTregs (**Liu W et al., 2006**). Previous observations described the highest suppressive ability in the subsets of CD127- and CD+CD25+Foxp3+ T cells and that the level of Foxp3 expression positively correlates with the suppression rate (**Walker MR et al., 2003; Yagi H et al., 2004; Liu W et al., 2006; Seddiki N et al., 2006**). Microarray analysis of mRNA, flow

cytometry and functional assays from individual T-cells subsets showed that CD127 was expressed at significantly lower levels in CD4+CD25^{high} versus CD4+CD25- T cells and inversely correlates with Foxp3, suggesting that Foxp3 interacts with a promoter of CD127 as a repressor (**Liu W et al., 2006)**. Genetic mutation of Foxp3 gene, and the resulting deficiency or dysfunction of nTreg, is the primary cause of IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X–linked syndrome), a monogenic human disease characterized by an autoimmune phenotype with enteropathy, T1D, thyroiditis, haemolytic anemia and thrombocytopenia, and results from mutations of Foxp3 (**Chatila TA et al, 2000; Wildin R et al., 2001; Bennett CL et al., 2001**). nTregs do not produce IL-2 and are anergic in vitro (**Sakaguchi S et al., 2005**). This hypo-responsiveness can be reversed by potent stimulation via TCR and high concentrations of IL-2 (**Beacher A et al., 2004**). Importantly, IL-2 is not required for thymic development of nTreg cells, but it plays a central role in their peripheral homeostasis and function. Indeed, the presence of IL-2 is required for nTreg cells to exert their suppressive function (**Fontenot JD et al., 2005**). Recently, IL-2RA was tested as a T1D candidate gene based on the previous association of IL2 in the NOD mouse model. In particular, the region on 10p15.1 that harbors the genes encoding IL-2Rα was found to be associated with T1D (**Vella A et al., 2005; Qu HQ et al., 2007; Lowe C al., 2007**), suggesting an important role of IL-2 signalling in human T1D pathogenesis.

Many studies have implicated Treg cells in the control of diabetes onset and progression, and that reduced CD4+ Treg cell frequencies or function in NOD mice, might represent a primary

predisposing factor to diabetes. Surprisingly, the NOD background proved superior in generating Treg cells in the thymus relative to non-autoimmune prone strain C57/BL6, suggesting that central tolerance mechanisms are intact (**Feuerer Me t al., 2007**). Furthermore, the frequency and function of single-positive CD4+ Foxp3+ Treg cells in the thymus of NOD was comparable to diabetes-resistant C57/BL6 mice (**Tritt M et al., 2008**). Depletion of CD25-expressing T cells or disruption of the B7/CD28 pathway results in a marked acceleration of T1D (**Tang Q et a., 2003**). Chen et al.reported that Foxp3-/- NOD mice display an increased incidence and earlier onset of T1D compared to WT NOD mice, although it did not address whether the infusion of Treg cells, which rescues from T1D, compensates for the primary deficit in Treg cells believed to underlie T1D pathogenesis in these mice, or whether such injection was actually suppressing the global inflammation/pathology that likely arose as a secondary consequence of Foxp3 deficiency (**Chen Z et al., 2005**). Interestingly, BDC2.5 CD4+ TCR transgenicNOD mice, whose T cells express a TCR specific for an unknown pancreatic islet antigen, are characterized by a low incidence of diabetes (10 – 20% by 20 weeks of age), while recombination-activating gene (RAG)-/- BDC2.5 animals that are completely devoid of Treg cells, exhibit fulminant diabetes and no lag time between insulitis and overt diabetes (**Gonzalez A et al., 2008**). Infusion of CD4+ splenic T cells to Treg cell-deficient BDC2.5 protects against T1D by suppressing inflammation in the islets (**Gonzalez A et al., 2008**). Collectively, this data supports the notion that Treg cells exert suppression on diabetes progression in NOD mice.

Several lines of evidence show that Treg cells are functionally operative in prediabetic NOD mice. Many studies have reported an expansion of CD25-expressing CD4+ T cells with apparent regulatory activity in inflamed tissues of adult, prediabetic, and insulitic NOD mice. It is unclear from these studies whether these CD4+ CD25+ T cells are induced Treg cells from CD4+ Foxp3− progenitors during pancreatic inflammation, or whether they emerge from the thymus-derived Foxp3+ Treg cell pool. Diabetes transfer is inhibited by CD4+ CD25+ Treg cells, with the highest regulatory activity attributed to the CD62Lhi fraction (**You S et al., 2004**). Transfer of such cells after long-term in vitro expansion has been shown to be protective, although it is unknown whether in vitro conditioning of Treg cells may have adversely affected their physiological role. NOD mice do eventually develop diabetes, despite the presence of Treg cells, suggesting a Treg cell-dependent breakdown in self-tolerance.

Different reports have been published analyzing peripheral blood Tregs frequency and function in T1D patients. While some found reduced Tregs frequency (**Kukreja A et al., 2002**) and function (**Lindley S et al., 2005; Brusko TM et al., 2005**), others did not find any change compared with healthy controls (**Putnam AL et al., 2005**). Much of this muddle is probably due to methodological differences in various laboratories, given that the precise definition of human Tregs has changed several times in recent years.

As a result of these preclinical studies, nTregs are considered a promising therapeutic tool for reestablishing self-tolerance in T1D, either to adoptively transfer nTregs previously expanded *ex vivo* or to

directly expand nTreg *in vivo* with selected immunomodulatory compounds. For example, the therapy involving treatment with the α-CD3 antibody has recently shown some efficacy in preserving β-cell function in T1D patients, partly by increased immune regulation via Tregs (**Herold KC et al., 2005**).

Moreover, administration of rapamycin (a non-calcineurin inhibitor currently used to prevent acute graft rejection after allogenic transplants) prevents T1D in NOD mice and reestablished long-term tolerance to self-antigens through expansion of nTreg (**Battaglia M et al., 2006**). Interestingly, various protocol to expand CD4+CD25+Foxp3+ cells have been successful both *in vitro* and *in vivo* using rapamycin, which, in addition to its therapeutic implications, unequivocally demonstrates the presence of these cells in T1D patients (**Battaglia M et al., 2006, Monti et al., 2008, Putnam L et al., 2009)**.

The appearance of autoantibodies is the first detectable sign of emerging β cell autoimmunity. The circulating antibodies, specific for β cell proteins, include anti-insulin antibodies (IAA), autoantibodies against the 65 kD isoform of glutamic acid decarboxylase (GAD) and against the protein tyrosine phosphatase (IA2). These autoantibodies are detectable in 85-90% of subjects with diabetes at the time of diagnosis (**Lesile RD et al., 1999; Palmer JP et al., 1983**). It is unclear whether they participate directly to β cell destruction or arise secondarily to the release of autoantigens from the islets damaged by other components of the immune system. They are, however, a good marker of the ongoing

development of the insulitis. The appearance of autoantibodies anticipates the clinical onset of disease, often by several years. Anti-insulin autoantibodies are often the first expressed autoantibodies, especially in young children (**Yu L et al., 1996; Ziegler A et al., 1999**). Family members of T1D patients who express autoantibodies to insulin, GAD 65, and IA-2 have a 75% 5-yr risk of Diabetes compared with a 25% 5-yr risk in relative who express only one of those antibodies (**Verge CF et al., 1996**). Therefore, the presence of multiple autoantibodies can be used as a sensitive marker to predict the risk of developing T1D (**Bingley PJ et al., 1997; LaGasse JM et al., 2002**), although there are some autoantibody positive individuals who do not progress to the disease.

Insulin: Insulin is the only known β cell specific antigen related to type 1 Diabetes. Anti-insulin antibodies (IAA) were first described in 1983 by Palmer in a group of new-onset diabetic patients before treatment with insulin (**Palmer JP et al., 1983**). IAA were subsequently discovered in first-degree relatives of T1D patients and can appear years before diabetes onset (**Schenker M et al., 1999**). Higher levels of IAA are found almost always in patients that develop diabetes before 5 years of age, and inless than 50% of patients developing diabetes after the age of 15 and are also strongly associated with the HLA DR4-DQ8 haplotype (**Knip M et al., 2008**). Numerous studies have reported that insulin is the most specific autoantigen of anti-islet autoimmunity (**Bonifacio E et al., 2002**). CD4+ T-cells clones specific for insulin could be derived from islet-infiltrating T cells in the NOD mouse and insulin specific T cells are a predominant component of the islet-infiltrate of pre-diabetic NOD mice (**Wegmann DR et al., 1994**). Wegmann et al.

further extended the examination of insulin-specific T cells to lines and clones established from mice ranging in age from 4 to 12 weeks and found that all the clones examined reacted to a region of the insulin molecule encompassing residues 9-23 of the insulin B chain (**Nakayama M et al., 2005**). Moreover, a diabetogenic CD8+ T cell clone, which causes diabetes in neonatal NOD mice, was found to recognize insulin β chain amino acid 15-23 (**Wong FS et al., 1999**). Successive studies on knockout NOD mice have shown that B9-23 insulin peptide is a primary target of the autoimmunity in NOD mice; mice lacking insulin gene fail to produce insulin autoantibodies and insulitis in contrast with mice containing at least one copy of the native insulin gene **(Nakayama M et al., 2005**).

GAD: GAD autoantigen was first identified in the pancreatic β cells in 1990 by Baekkeskow and co-workers (**Baekkeskow S et al., 1990**). It is a 64-kD antigen localized in synaptic-like microvescicle in β cells and represent the biosynthetic enzyme of the inhibitory neurotransmitter gamma-amino-butyric acid (GABA). Two distinct isoforms of GAD, GAD67 and GAD65, encoded by two different genes, have been identified. They have an amino acid sequence homology of approximately 70%, human islets predominantly express GAD65, in contrast, the isoform predominantly expressed in mouse islets is GAD67. The results of different studies on the role of GAD as triggering autoantigen in the development of T1D are controversial. Geng et al. in 1998 demonstrated that widespread expression of GAD transgene does not tolerize NOD mice but accelerate the onset of T1D (**Geng L et al., 1998**). To further investigate the role of GAD, different transgenic strategies have been used and transgenic NOD mice that express

GAD65 in the β cells were established. Results of these studies do not clarify the role of GAD. Strain of transgenic NOD mice with high expression of the autoantigen GAD65 in the islets exhibited a markedly lowered incidence of diabetes, another line, instead, developed diabetes at a similar rate and incidence as control NOD mice (**Bridgett M et al., 1998**). More recently Yoon et al. demonstrated that cell-specific suppression of GAD expression in two lines of antisense GAD transgenic NOD mice prevents autoimmune diabetes, in contrast to other four strains of antisense GAD transgenic NOD mice with persistent GAD expression in the β cells that develop diabetes (**Yoon JW et al., 1999**). In conclusion the role played by GAD in the pathogenesis of T1D remains uncertain.

IA2: IA- 2 protein is a member of the protein tyrosine phosphatase family and autoantibodies directed against this autoantigen have been detected in 70% of patients with autoimmune diabetes, while they are no detectable in NOD mice. Autoantibodies anti-IA-2 seems to identify patients with rapid progression of the disease, moreover the frequency of these autoantibodies varies with the age and the genotype of the subjects studied. Indeed, autoantibodies anti-IA-2 are a sensitive marker of T1D onset in childhood and adolescence (**Savola K et al., 1998**) and are detectable above all in patients with DR4 e DQA1*03-DQB1*0302 alleles.

1.3 Genetic predisposition

It has been suggest that multiple genes play an important role in the disease development, in particular HLA gene, which dramatically influences both triggering of islet autoimmunity and progression to T1D.

The results of several genome-wide searches have consistently reported significant evidence of linkage between the HLA region on chromosome 6p21 and T1D with a predicted odd ratio of approximately 6.8. DR3- DQA1*0501-DQB1*0201 (DR3) and DR4- DQA1*0301-DQB1*0302 (DR4) are the DR/DQ haplotypes which confers highest risk for T1D and 30-40 % of T1D patients have the heterozygous genotype DR3/4. (**She J et al., 1996**). Sequencing of HLA molecules has shown that the DQ locus has a stronger association with T1D (**Baisch JM et al., 1990**). In Caucasians, the highest risk to develop Diabetes is associated with DQA1*0501-DQB1*0201 (in linkage with DRB1*03) and DQA1*0301-DQB1*0302 (in linkage with DRB1*0401) haplotypes (encoding the DQ2 and DQ8 molecules respectively), both of which are in linkage disequilibrium with the DR3 and DR4 alleles. In contrast to such a high-risk genotype, the DQ6 molecules, DQA1*0102- DQB1*0602, provide dominant and almost complete protection from T1D (**Pugliese A et al., 1995**).

Genes outside the HLA region also contribute to the risk of T1D, although their individual contributions are smaller.

A variable Number Tandem Repeat (VNTR) microsatellite at the 5' end of the insuline gene (INS) on chromosome 11 has alleles that are

functionally associated with T1D, with an allelic odd ratio of 1.9 (**Concannon P et al., 2005; Spielman RS et al., 1993**). Alleles of the VNTR region are divided into two classes, class I alleles (26-63 repeats) and class III alleles (140-210 repeats). Class I alleles have been positively associated with T1D whereas class III alleles are considered protective (**Bennett ST et al., 1995**). Different studies have suggested that VNTR region affects the genetic regulation of insulin expression in the thymus thus modulating tolerance to insulin. The expression level of insulin mRNA in the thymus are 2.5 fold higher in class I/III heterozygotes compared with class I homozygotes; the increased expression associated with class III alleles might contribute to a more efficient deletion of autoreactive T-cells (**Pugliese A et al., 1997**). The genetic linkage analysis has suggested the presence of other susceptibility loci such as IDDM4 (FGF3/11q13), (**Davies JL et al., 1994; Hashimoto L et al., 1994**) IDDM5 (ESR/6q22) (**Davies JL et al., 1994**), and IDDM12 on chromosome 2q33 (**Nisticò L et al., 1996**), the last encoding CD28 and the cytotoxic T-lymphocyte antigen (CTLA-4), molecules involved in T cell activation and in the modulation of the immune response, respectively.

A recent addition to the list of identified candidate-gene associated to T1D is *PTPN22* gene (*fig. 1*).

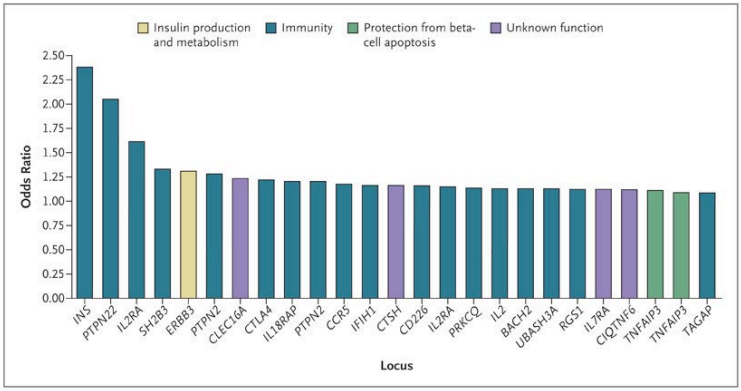


Figure 1. **Putative Functions of Non–HLA-Associated Loci in Type 1 Diabetes.**

The y axis indicates the best estimate of the odds ratio for risk alleles at each of the indicated loci on the basis of currently published data. Although not shown, the HLA region has a predicted odds ratio of approximately 6.8. On the x axis are indicated possible candidate genes within genomic regions in which convincing associations with T1D have been reported. On the basis of the known functions of these candidate genes, the corresponding bars in the graph depicting odds ratios have been color-coded to suggest possible roles of these loci in susceptibility to T1D. (**Concannon P et al., 2009**)

II *PTPN22* AND TYPE 1 DIABETES

2.1 *PTPN22* polymorphism as a risk factor for autoimmune diseases

Genetic variability at the *PTPN22* gene locus recently emerged as an important risk factor in the development of autoimmune diseases. The association between a single nucleotide polymorphism of *PTPN22* gene and T1D was first reported in a case-control study on two samples from North American and Sardinian populations (**Bottini N et al., 2004**). This polymorphism causes the substitution of a cytosine at position 1858 by a thymidine (C1858T), thereby yielding the replacement of arginine 620 of Lymphoid thyrosine phospathase (LYP) by a tryptophan (R620W substitution) (*Fig 2*).

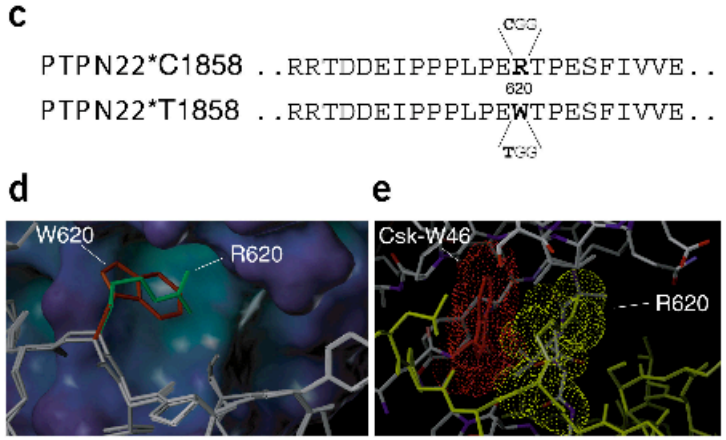


Figure 2. Single nucleotide polymorphism in the first proline rich motif (P1) in human LYP. (c) Amino acid sequence of the two alleles, with the P1 motif indicated. (d) Structure of the P1 motif peptide bound to the SH3 domain of Csk. The ligand peptide was shown in pale gray with both arginine (green) and tryptophan (red) at the position corresponding to residue 620 of LYP. The surface of the SH3 domain is shown in MODELLER with dark blue for basic and greenish for acid surface topology. (e) Interaction of R620 of LYP (yellow) with the SH3 domain of Csk (red). (**Bottini N et al., 2005).**

Linkage of this polymorphism with T1D was confirmed by several groups word wide, in different populations (**Smyth D et al., 2004; Ladner MB et al., 2005; Qu H et al., 2005, Zheng W et al., 2005**). Furthermore, the same association was found for rheumatoid arthritis (RA) (**Begovich AB et al., 2004; Van Oene et al., 2005; Hinks A et al., 2005; Simkins HM et al., 2005, Lee AT et al., 2005**), juvenile idiopatic arthritis (JIA) **(Viken MK et al., 2005; Hinks A et al., 2005**), systemic lupus eritematosus (SLE) (**Kyogoku C et al., 2004;**

Wu H et al., 2005; Reddy MV et al., 2005), Grave's disease (**Smyth D et al., 2004; Velaga MR et al., 2004; Skorka A et al., 2005**), myasthenia gravis, generalized vitiligo (**Canton I et al., 2005**) and Wegener's granulomatosis (**Jagiello P et al., 2005**). Association was seen also with autoimmune conditions such as Crohn's disease, ulcerative colitis, celiac disease, psoriasis, psoriatic arthritis, and multiple sclerosis **(Van Oene M et al., 2005; Santin I et al., 2008; Begovich AB et al., 2005**).

On the basis of genetics studies in different populations, there is a geographic gradient with regard to the frequency of the disease-associated T1858 allele in Europe. Even though this allele is relatively rare in Southern European populations (2% in Italy, 6% in Spain), the frequency increases northward through Europe (8% in the United Kindom, 12% in Sweden, 15,5% in Finland). Interestingly, in African American and Asian population is virtually absent, suggesting a Northern European origin and/or selective advantage for the T1858 allele in this region (**Vang T et al., 2007**).

2.2 *PTPN22* polymorphism: a predictor marker of T1D development and progression

Besides the association between *PTPN22* C1858T polymorphism and T1D, many groups investigated if the presence of C1858T allele is correlated to disease development and progression. In the DAISY cohort it has been shown that the *PTPN22* T/T allele genotype was associated with a higher incidence of persistent islet autoimmunity than the C/T or C/C genotype; interestingly this association is independent from the presence of the others genetic risk factor. These authors showed also that there is a higher, but not significant, incidence of progression to T1D in these children. These data suggest that PTPN22 T/T genotype is a strong independent predictor of the development of persistent islet autoimmunity in children at risk for T1D (**Steck AK et al., 2009**). Herman et al. showed that children at higher risk to develop T1D (DIPP cohort) carrying the T/T and C/T genotype had a strongly increased probability to develop IAA. They also observed that children with the T/T genotype developed IAA at an earlier age than others and also that affected boys with the T/T genotype had higher disease risk than girls. In the same study it has been reported that the effect of *PTPN22* appear to be stronger in individuals carrying non DR4-DQ8 genotypes (**Hermann R et al., 2006**). In contrast, in Type 1 diabetes Genetic Consortium Study there was no evidence for-sex specific and age-at-diagnosis effects of *PTPN22* polymorphism (**Howson J et al., 2009**).

The association between *PTPN22* C1858T polymorphism and the clinical course of T1D was also investigated by Petrone et al,

who showed that T1D patients carrying the *PTPN22* 1858T variant presented lower fasting C-peptide levels and higher A1C levels compared to T1D patients carrying the *PTPN22* 1858C variant. This difference was independent of age at diagnosis, sex and HLA and persisted from time of disease diagnosis through the 12-month follow up. (**Petrone A et al., 2008**).

All this data suggest that *PTPN22* 1858T allele may play an important role in both triggering islet autoimmunity and progression to T1D.

2.3 *PTPN22* is a critical regulator of TCR activation

TCR signal transduction is initiated following interaction of the TCR αβ chains with antigen bound to major histocompatibility complex (MHC) class I or II molecules. The signal is transmitted to a complex network of kinases, phosphatases and adaptors. The TCR αβ chains lack any ability to transmit signals on their own and depend on CD3 (ε, δ and γ) and ζ chain that contain numbers of immunoreceptor tyrosine-based activated motifs (ITAMs). The dual tyrosines of ITAMs are phosphorylated by members of the Src family kinases (SFKs), which, in T cells, are Lck and Fyn. Dually phosphorylated ITAMs, in turn, form docking sites for the tandem SH2 domains of Syk family kinases, ZAP-70 and Syk. The Syk kinases are activated upon binding to phospho-ITAMs and phosphorylated by the SFKs. Once activated, the Syk kinases phosphorylate the critical adaptors SLP-76 and LAT, which together form the scaffolds for assembly of further signalling molecules. LAT contains nine tyrosines that are phosphorylated upon TCR

engagement, which bind the C-terminal SH2 domain of phospholipase Cγ1 (PLC-γ1), the p85 subunit of phosphoinositide 3-kinase (PI3K), the adapters growth factor receptor-bound protein 2 (GRB2) and the adapter downstream of Shc (Gads). These interactions collectively are required to stabilize PLC-γ1 in the correct conformation within the complex to allow for its optimal activity. The proximal signalling complex results in the activation of PLC-γ1-dependent pathways including Ca2+ and Diacylglycerol (DAG)-induced responses, cytoskeletal rearrangements, and integrin activation pathways. Activated PLC-γ1 then hydrolyzes the membrane lipid Inositol (4,5)P_2, producing the second messengers Inositol(1,4,5)P_3 and DAG. The production of DAG results in the activation of two major pathways involving Ras and PKC. Ras is a guanine nucleotide-binding protein and is required for the activation of the Raf-1 kinase, which initiates a mitogen-associated protein kinase MAPK phosphorilation, which in turn phosphorylates Erk. Erk kinase activity results in the activation of Elk, which contributes to the activation of AP-1, Jun and Fos transcription complex. One critical pathway that PKC regulates involves the NF-κB transcription factor. In resting cells, NF-κB is found in the cytosol associated with inhibitor of NF-κB (IκB) family members that keep NF-κB from moving into the nucleus. Upon T cell activation, IκB is phosphorylated by the IκB kinase (IKK) complex, ubiquitinylated, and degraded, allowing NF-IκB to translocate into the nucleus, where it activates genes involved in the function, survival and homeostasis of T cells (Schulze-Luehrmann J. et al., Antigen-receptor signaling to nuclear factor κ*B*. Immunity 2006). TCR

recruitment and Vav activation induce Rac/cdc42/MKK and p38 activation. Rac-GTP activates Fos and JNK (c-Jun N-terminal kinase) which phosphorylate c-Jun. TCR-induced increases in intracellular Ca2+ levels result in the activation of Ca2+ and calmodulin-dependent transcription factors, including the phosphatase calcineurin and the Ca2+-calmodulin-dependent kinase that in turn activate a variety of transcription programs. Activated calcineurin dephosphorylates 14 members of the NFAT family, leading to their translocation to the nucleus. In the nucleus, NFAT isoforms can form cooperative complexes with a variety of other transcription factors, thereby integrating signaling pathways resulting in differential gene expression patterns and functional outcomes. The NFAT/AP-1 interaction integrates Ca2+ and Ras signals and results in the expression of genes important for T cell activation including IL-2. In contrast, NFAT activity in the absence of AP-1 activation induces a pattern of gene expression that results in T cell anergy and a characteristic lack of IL-2 production (*Fig. 3*).

There are also many levels of regulation in this signalling pathway. The SFKs themselves are tightly regulated by phosphorylation of their inhibitory C-terminal tyrosine residue. Reciprocal regulation of this phosphotyrosine by the receptor-like tyrosine phosphatase CD45 and the cytoplasmic kinase Csk can set thresholds for antigen receptor signal transduction (**Zikherman J et al., 2009**). Added complexity is presented by the tight regulation of the activating tyrosine of the SFKs. Negative regulators of TCR signalling, such as the phosphatases PEP and SHP-1, can dephosphorylate this critical residue, as described in mice (**Acuto O et al., 2008, Hermiston ML et al., 2002**).

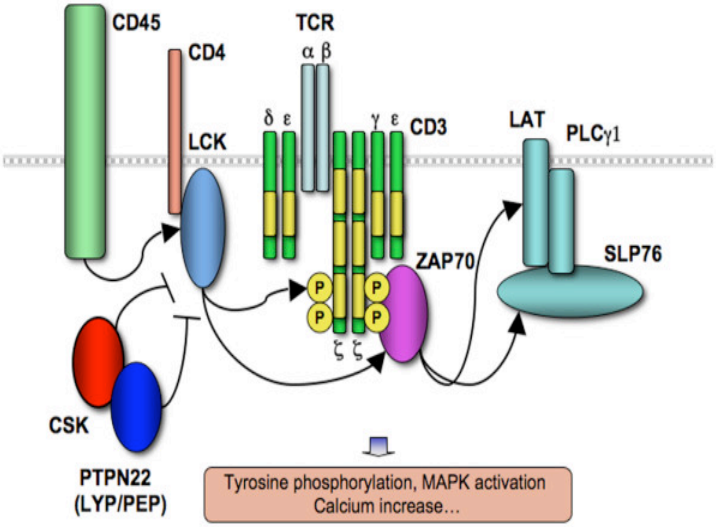


Figure 3. Schematic representation of T-cell receptor signal transduction. CD4-associated Lck is reciprocally regulated by CD45 and Csk/PTPN22 and in turn phosphorylates the CD3 ς chain ITAMs and ZAP-70. ZAP-70 phosphorylates additional downstream effectors, including the adaptors SLP-76 and LAT. Yellow bands represent CD3 chain ITAM domains. Phosphotyrosines are not depicted on all CD3 ς chain ITAMs. MAPK, mitogen-activated protein kinase; PLCγ1, phospholipase C γ1; TCR, T-cell antigen receptor (**Zikherman P et al., 2009**).

An important regulator of TCR signalling is the phosphatase LYP, an 807-amino acid residue protein encodes by the *PTPN22* gene, located on chromosome 1p13.3-13.1 (**Cohen S et al., blood 1999**). LYP belongs to the PEST group of non receptor I phospatases (PTPs), contains an N-terminal PTP domain, a central region and a C-terminal portion of approximately 200 amino acids containing four proline-rich motifs termed P1-P4. LYP is the human ortholog of PEP (**Matthews RJ et al., 1992**). Both LYP and PEP are expressed exclusively in hematopoietic cells and share 89% identity between their PTP domains and 61% identity for their non-catalytic portions (**Vang T et al., 2008**).

Both PEP and LYP inhibit TCR signalling by acting immediately downstream of the TCR. Specifically, PEP phosphorylates the positive regulatory tyrosine residue in the activation loop of the Src family kinases (SFKs) Fyn, Lck and Zap70. It has been reported that overexpression of wild type PEP in a mouse T-cell line (BI-141) caused a diminution of tyrosine phosphorylation of most TCR-regulated substrates, including ZAP-70 and the ITAMs of ζ chains (**Cloutier JF et al., 1999**). It has also been described that microRNA-181a targets and downregulates PEP expression, resulting in elevated Lck phosphorylation in resting T cells that became hyperresponsive to TCR stimulation (**Li QJ et al., 2007**). Hasegawa et al. found that PEP-deficient mice exhibited an age-dependent increase of the number of effector memory ($CD44^{hi}CD62L^{lo}$) T cells in peripheral lymphoid tissues. Both CD4+ and CD8+ T cells were affected. This phenotype was accompanied to lymphadenopathy, splenomegaly, spontaneous germinal center formation, and elevated serum antibody levels, although

autoantibodies and autoimmune diseases were not observed. The authors also observed an increase of the kinase activity of Lck, supporting the notion that PEP operates by inhibiting the Src family kinases. Intriguingly, this biochemical alteration was seen only in effector and memory T cells, not in naïve T cells, implying that PEP has a critical inhibitory role in previously activated T cells (**Hasegawa K et al., 2004**). In accordance with these observation for PEP, acute elimination of LYP in jurkat T cell using RNA interference resulted in increased TCR-mediated activation of NF-kB (**Begovich AB et al., 2004**). A recent study using a substrate trapping mutant version of LYP identified Lck (Y349), the ITAMs of CD3 ζ chains, Zap-70 (Y493) and Vav as LYP substrates (**Wu J et al., 2006**). A repressive effect of human LYP on T-cell activation was also documented using a specific LYP inhibitor (I-C11). Indeed jurkat cells treated with I-C11 and stimulated with αCD3 for 5 minutes showed an impaired down-regulation of TCR activation and an increased Lck and ERK1/2 phosphorylation **(Xiao Yu et al., 2007**).

The ability of LYP/PEP to bind Csk through the P1 motif may be the mechanism by which LYP downregulates TCR activation. In mouse and human T cells, approximately 20% of Csk is in complex with ~50% of PEP/LYP (**Cloutier JF et al., 1999**) and the inhibitory effect of PEP in mouse T cells was observed to require not only its PTP activity, but also its capacity to associate with Csk.

One hypothesis is that LYP-Csk complex may provide a cooperative mode of suppressing Src family kinase-dependent signalling. In fact, while PEP/LYP dephosphorylates the positive regulatory tyrosine in the activaction loop of Lck and Fyn, Csk phosphorylates the C-

terminal negative regulatory sites in Lck and Fyn (*Fig. 4*)

Moreover, the latter finding suggested that PEP/LYP might be recruited in the vicinity of the TCR signaling machinery by way of Csk (**Cloutier JF et al., 1999**).

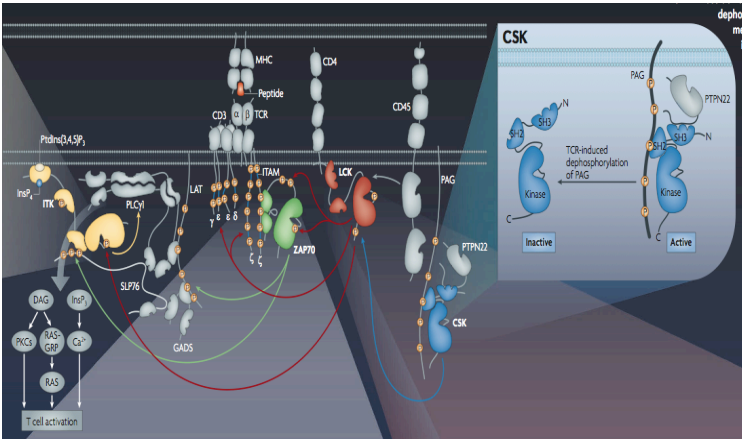


Figure 4. **Schematic representation of LYP-Csk interaction during TCR activation.**
LYP (*PTPN22*) contains a proline-rich sequence that interacts with the SH3 domain of Csk. LYP dephosphorylates the activation loop tyrosine of SRC family kinases. Thus, Csk and LYP act in tandem to negatively regulate SRC family kinases. The interaction of LYP with Csk is disrupted in the allele of the phosphatase associated with autoimmunity.
(**adapted from Lin J and Weiss A, 2010**).

2.4 *PTPN22* polymorphism is a gain of function variant

Given the tight linkage between the C1858T *PTPN22* polymorphism and autoimmunity, a significant emphasis was directed to elucidate the impact of this mutation on the function of LYP.

The R620W substitution, which is located in the LYP-Csk interaction motif, strongly reduces the affinity of LYP for Csk (**Vang T et al., 2005**). These authors showed that LYP-W620 is a gain-of-function variant of the enzyme. Indeed in transfected jurkat cells, overexpressed LYP620W dephosphorylates Lck, TCRς, ERK2 and LAT much more efficiently than LYPR620 and it was more potent in reducing calcium mobilization and interleukin-2 gene trans activation (**Vang T et al., 2005**). A diminution of TCR-mediated responses in T cells from humans carrying the R620W mutation was recently confirmed by Rieck et al. These authors observed a profound deficit in the ability of T cells from individuals homozygous for the 1858T variant to mobilize calcium in response to TCR activation. They also examined the expression of the early activation marker CD25 and they found a significant decrease in the percentage of CD4+T cell expressing CD25 from *PTPN22* T/T carriers when compared to C/C subjects after activation. (**Rieck M et al., 2007**). All this data suggest that the presence of 1858T is associated with an early inhibition of TCR activation. Moreover, no differences in T cell proliferation were found in T cell from healthy controls carrying the two LYP variants. In contrast, Aarnisalo et al. found that CD4+ cells from healthy controls carrying the C/T or T/T

genotype showed a defective proliferation in comparison of CD4+ cells from healthy controls carrying the C/C genotype, upon specific TCR activation. These authors also described that T1D patients carrying the C/T or T/T polymorphism, showed both a lower calcium-flux peak upon PHA stimulus (**Aarnisalo J et al., 2008**).

The cytokine profile after TCR activation was also reported to be altered in the presence of 1858T allele. CD4+ cells from T1D patients carrying the C1858T genotype produced less IL-2 than CD4+ cells from T1D patients carrying the C1858C genotype in response to TCR stimulation, but the same amounts of IL-2 in response to PMA/Ionomycin, which bypasses early tyrosine phosphorylation events (**Vang T et al., 2005**). The defects in IL-2 production on CD4+ cells from T1D patients carrying the C/T or T/T genotype was also confirmed by Aarnisalo et al. Interestingly, this defect was absent in CD4+ cells from healthy controls carrying the C/T or T/T genotype (**Aarnisalo J et al., 2008; Rieck M et al., 2007**). Conversely, CD4+ cells from healthy controls carrying the C/T or T/T genotype showed a significant defect of IL-10 production compared to CD4+ cells from healthy controls carrying the C/C genotype, upon specific TCR activation. No differences were described for the other analyzed cytokines like IL-4, IFN-γ and TNFα. (**Rieck M et al., 2007**).

In contrast with the described defect of T cells on TCR stimulation response, responsiveness to stimuli by naturally presented and processed antigens was similar between subjects with different *PTPN22* genotypes. Indeed PBMC form T1D patients carrying the two LYP variants, stimulated with tuberculin or tetanus-toxoid antigens did not show differences of proliferation and

cytokine production (**Aarnisalo J et al., 2008**).

Following the observation that PEP-deficient mice exhibited an age-dependent increase in the number of effector memory ($CD44^{hi}CD62L^{lo}$) T cells (Hasegawa K, science 2004), the composition of the T cell pool was also analyzed in healthy controls carrying the two LYP variants. A significant increase in the mean percentage of memory T cells in C/T subject compared to C/C subject was observed; this difference is not correlated to the age of the subjects (**Rieck M et al., 2007**).

The strong association between *PTPN22 C1858T* polymorphism and the development of autoimmune diseases is also supported by the fact that the same polymorphism confers a protective effect towards tuberculosis (TB), presumably through its ability to reduce the cell-mediated immune response in the granulomatous reaction. Indeed it has been described that TB patients present a significant lower frequency of *PTPN22 C1858T* polymorphism compared to healthy controls (**Lamsyah H et al., 2009; Gomez M et al., 2006**).

Interestingly, it has also been reported that another polymorphism of this gene, that confers a loss-of function to the protein, predisposes to infectious disease (**Lamsyah H et al., 2009**) but is a protective factor for the development of LES (**Orru' V et al., 2008**).

BACKGROUND AND AIMS

PTPN22 is the third gene predisposing to T1D and in the past few years numerous genome wide association studies have confirmed the strong association of a *PTPN22* polymorphism with this disease.

This polymorphism consists in a Cytosine to Tymidine substitution at position 1858 in the coding region of the gene that changes an Arginine (R) to a Triptofan (W) in the P1 motif of the protein.

PTPN22 encodes for LYP, a phosphatase that negatively regulates TCR activaction and this single nucleotide polymorphism (*SNP)* confers to the protein a gain-of-function. Indeed, *In functional studies on T cells from individuals carrying the C1858T polymorphism it has been described a defect of TCR-mediated response* (**Aarnisalo J et al., 2008; Rieck M et al., 2007; Vang T et al., 2005)**.

Given the involvement of LYP in TCR signalling and the important role that T cells play in the autoimmune process leading to T1D, it is reasonable to hypothesize that the link between PTPN22 and human T1D is mediated by the effects of the phosphatase on TCR signalling, which may affects T cell activation and function in both central and peripheral tolerance.

For instance, in non obese diabetic mouse model peripheral T cells exhibit hyporesponsiveness to TCR engagment in the early phase of the autoimmune disease (**Salojin KV et al., 1998; Rapoport MJ, J et al., 1993**) and peripheral T cells from T1D patients are

hyporesponsive rather than hyperesponsive to TCR stimulation (**Salojin KV et al., 1998**). Furthermore, thymocytes from NOD mice are also hyporesponsive with regard to TCR-mediated activation and proliferation (**Salojin KV et al., 1998; Rapoport MJ, J et al., 1993**).

In The **part I** of the study we investigated the association between *PTPN22* polymorphism and T1D in a cohort of patients followed in the Pediatric Diabetology Unit of the University of Rome "Tor Vergata". We also tested whether the T1858 allele presence is correlated with stage of the disease, gender, family history of autoimmune diseases and the concurrent autoimmunity in our T1D patients.

Since LYP is phosphatase involved in the early events of TCR signalling and the *PTPN22* polymorphism confers to this protein a gain-of-function, the **part II** of the study was meant to examine the effect of this polymorphism on proliferation and cytokine production by lymphocytes from T1D patients carrying the two LYP variants.

In the **part III** of the study we examined if there are molecular mechanisms that might contribute to the gain-of-function phenotype of LYP-W620 in T cells. In particular we investigated if there are differences in the phosphorylation status of the two LYP variants and if the C1858T polymorphism affects protein expression.

RESULTS

Study I. Association of *PTPN22* polymorphism and T1D in a cohort of children from continental Italy

1.1 Subjects

We examined 216 T1D patients recruited from the Pediatric Diabetology Unit of the University of Rome "Tor Vergata" with a mean age at sample collection of 15.6 years (±6 years): 46.3 % were males and 53.7 % were female. The mean age at diagnosis was 8.6 years (±4 years); 66.1% children were positive for anti-islet antigen-2A Ab (IA-2A), 47.1% were positive for anti-insulin antibodies (IAA) and 64.1% were positive for anti-glutamic acid decarboxylase Ab (GADA). More than 20% of T1D patients were affected by at least one concurrent autoimmune disease, including Hashimoto's thyroditis (AT), celiac disease (CD) and other less frequent ones (sclerodermia, RA and psoriasis).

As controls, we examined 271 healthy subjects from Rome with a mean age of 36.8 years (±12 years); 70.1% were males and 29.9% were females.

1.2 Results

We found a higher frequency of 1858T allele in T1D patients than in healthy controls. The frequency of 1858T allele was 11.6% in T1D patients and 5.9% in healthy controls ($p < 0.03$) (*Tab.1*).

Table 1. Frequencies of *PTPN22* genotypes in T1D patients and controls

	%Carriers of the 1858Tallele %(n)	Total Number
T1D patients	11.6 (25)	216
Healthy controls	5.9 (15)	271

There were no differences in 1858T allele distribution between male and female both in patients and controls.

No significant association was found between the 1858T allele and the presence or the titer of autoantibodies at diagnosis.

We found a significantly higher frequency of autoimmune thyroiditis (20% in 1858T carriers vs 12% in non carriers, $p < 0.05$) but not of celiac disease in carriers of the 1858T allele.

We also observed a significant correlation between the 1858T allele and the DQ2 (A1*0501) HLA genotype (*Tab.2*).

Of note, T1D patients carrying the 1858T allele presented a higher presence of autoimmune diseases in the family history.

Table 2. Association between PTPN22 1858T allele and co-occurrence of other autoimmune diseases in T1D patints carrying the C/C or C/T genotype. Different distribution of HLA DQ2 (A1*0501) in T1D patients carrying C/C or C/T genotype.

	Subjects with associated autoimmune diseases % (n)		**Carriers of HLA DQ2 (A1*0501) % (n)**
	AT*	**CD****	
T1D patient C/C	12.04 (23)	6.8 (13)	50.4 (123)
T1D patients C/T	20 (5)	8 (2)	80 (12)

*AT= Autoimmune Thyroiditis, **CD= Celiac Disease

Study II. Effect of *PTPN22* polymorphism on proliferation and cytokine production by lymphocytes from T1D patients and healthy controls carrying the two LYP variants

2.1 Subjects

The study population comprises children with type 1 diabetes (T1D) recruited from the Department of Pediatrics of Tor Vergata University, previously genotyped for *PTPN22* C1858T polymorphism.

We selected 16 T1D patients carrying the C/C polymorphism, 10 T1D patients carrying the C/T polymorphism and 1 T1D patient carrying the T/T polymorphism, age and sex matched. The two groups of patients presented a different distribution of the HLA highrisk haplotypes, with a greater percentage of HLA DQ2 (A1*0501) in T1D patients carrying the *PTPN22* polymorphism, according with our previous observation (*Tab 3*).

As control we recruited 3 C/C healthy children (2 males and 1 female) age and sex matched with T1D patients; all controls had a negative family history for autoimmune diseases.

Table 3. Correlation between *PTPN22* polymorphism and clinical characteristics of the patients recruited for the study

T1D Patients	*Age at diagnosis (mean*	*Years of disease (mean ±*	*% of co-occurrence autoimmune*		*% of positive familiar history for autoimmune*		*HLA distribution*		
			CD**	**AT***	**T1D**	**AT**	**DQ2/DQ8 neg**	**DQ2 (A1*0501)**	**DQ2 (A1*0501)/ DQ8**
C/C (n=16)	**12.5 ± 6**	**5.7 ± 4.3**	**18%(3)**	**5% (1)**	**23% (4)**	**5% (1)**	**23% (4)**	**29% (5)**	**23% (4)**
C/T-T/T (n= 11)	**12.7 ± 4**	**3 ± 2.9**	**9% (1)**	**27% (3**	**18% (2)**	**36% (4)**	**9% (1)**	**55% (6)**	**18% (2)**

*AT= Autoimmune Thyroidytis, **CD= Celiac Disease

2.2 Results

We have previously made the observation that PBMC from T1D patients showed a defective proliferation after OKT3/αCD28 stimulation compared to PBMC from healthy controls (**Piccinini S et al., unpublished observation**) (*Fig. 1*).

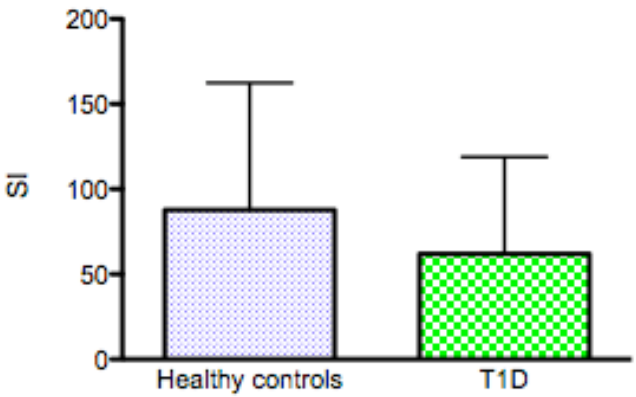


Figure 1. **Proliferative response of PBMC from T1D patients and healthy controls.** Freshly isolated PBMC were obtained from T1D patients (n=11) and healthy controls (n=8) and stimulated with or without OKT3/αCD28 for 72 hours. Proliferation was measured by [3H] thymidine incorporation for the last 6 hours of culture. The proliferative response was expressed as ratio between stimulated and unstimulated cells stimulation index (SI).

We therefore asked if this difference is correlated with the presence of *PTPN22* C1858T polymorphism. To answer this question we examined the proliferative capacity of T cells from T1D patients and

healthy controls, previous genotyped for this polymorphism, after stimulation with OKT3/αCD28, a specific TCR activator. We observed a lower proliferative response in T cells from T1D patients, carrying both the C/C and C/T genotype, compared to healthy controls carrying the C/C genotype. Nevertheless, we found no significant differences in T cell of patients carrying the two genotypes. When we expressed the proliferation response as ratio between stimulated and unstimulated cells (SI) we observed a slightly higher T cell response in T1D patients carrying the C/T genotype, due to the fact that cmp values of unstimulated T cell from C/T subjects were lower than cpm values of unstimulated T cell in C/C subjects (*Fig 2 A-B*).

Moreover T cells from T1D patients and healthy controls were stimulated with PMA/Ionomycin, which activates T cells through a pathway independent from TCR. We observed a lower proliferative response in T cells from T1D patients carrying the C/C genotype compared to healthy controls carrying the C/C genotype. Moreover there was a slightly decreased T cell proliferation upon PMA/ionomycin in C/T compared to C/C patients; this trend was not present if we expressed the proliferation assay as stimulation index (*Fig. 2 C-D*). Finally, we observed a decrease of proliferative response by T cells from the T1D patient carrying the T/T genotype. Interestingly this impaired response is present only after specific TCR activation.

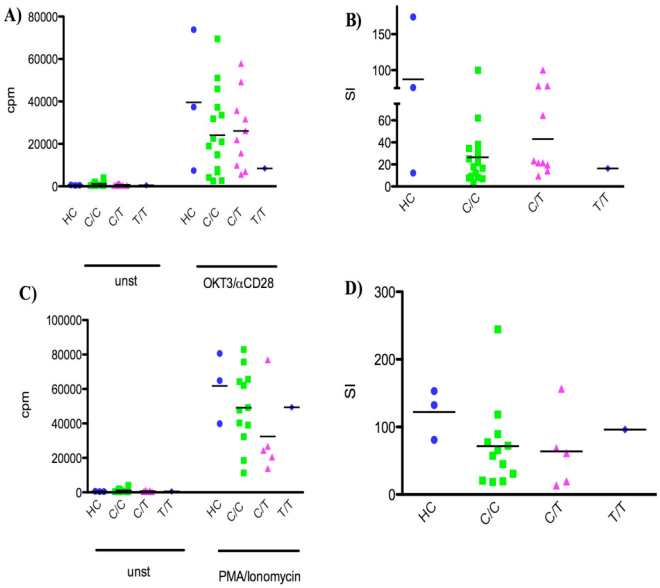


Figure 2. **Proliferative response of T cells from T1D patients carrying the C1858C and C1858T *PTPN22* variants.** Freshly isolated CD14- cells were obtained from C/C (n=16), C/T (n=10) and T/T (n=1) T1D patients and C/C healthy controls (n=3) and stimulated with or without OKT3/αCD28 for 72 hours. Proliferation was misurated by [3H] thymidine incorporation for the last 6 hours of culture. The proliferative response was expressed as count per minute (cpm, **panel A**) or as ratio between stimulated and unstimulated cells (SI, **panel B**). We also performed a proliferation assay in CD14- cells from C/C (n =12), C/T (n=5) and T/T (n=1) T1D patients and C/C healthy controls (n=3) after stimulation with PMA/ionomycin for 72 hours. The proliferative response was expressed as count per minute (cpm, **panel C**) or as ratio between stimulated and unstimulated cells (SI, **panel D**). Averages was indicated by horizontal bars.

To address the effect of LYP620W on T cell function, we examined cytokines production of T cells from T1D patients carrying the C/C, C/T and T/T genotypes. Supernatants were collected after 24 hours of stimulation with OKT3/αCD28 for IL-2 detection and after 72 hours of stimulation with OKT3/αCD28 for IL-10, IFN-γ and IL-17 detection.

Subjects carrying the C/T genotype had significantly lower IL-2 production compared to those with the wild type genotype (p= 0.002). Interestingly, in T cells from the T1D patient carrying the T/T genotype the production of IL-2 was totally absent, suggesting that this defect is correlated to the quantity of mutated LYP (*Fig. 3A*).

We found no difference of IL-10 production among T cells from T1D patients carrying C/C and C/T or T/T genotype (*Fig.3C*). We also observed no differences of IL-17 and IFN-γ production by T cells from patients carrying the C/C or C/T genotype. Nevertheless we observed a marked decrease of IL-17 and IFN-γ production by T cells from the T1D patient carrying the T/T genotype (*Fig. 3B* and *D*).

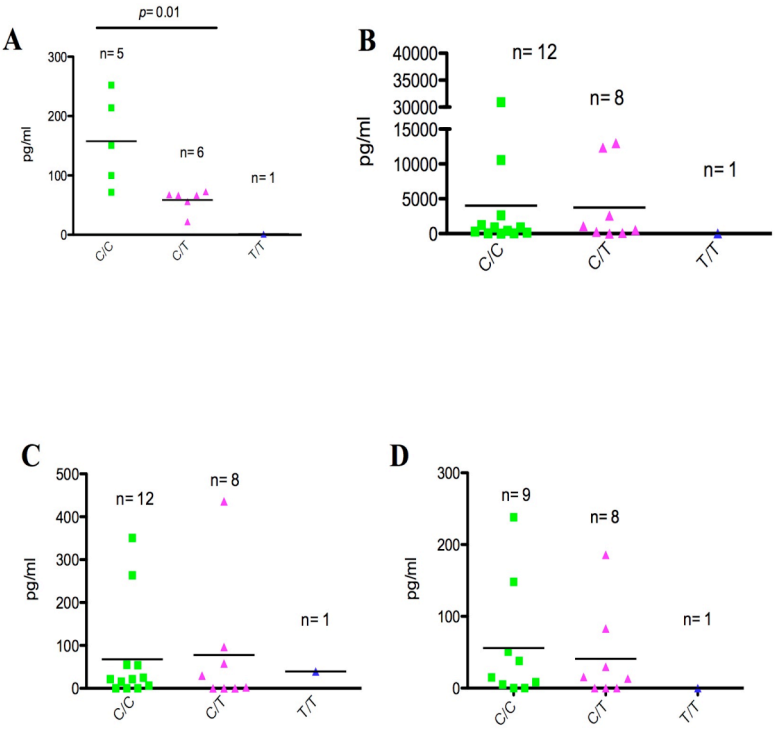


Figure 3. **Cytokine production of T cells from T1D patients carriyng the 1858C, C1858T or 1858T genotype.**
Freshly isolated CD14- cells from C/C, C/T and T/T T1D patients were stimulated with OKT3/αCD28mAb. Culture supernatants were harvested after 24 hours for IL-2 (**panel A**) detection and after 72 hours for IFN-γ (**panel B**), IL-10 (**panel C**) and IL-17 (**panel D**) detection; cytokine concentration was determined by Enzyme-Linked Immunosorbent Assay.
Cytokine secretion is expressed as net individual production (production by stimulated-unstimulated cells); the average is indicated by horizontal black bars.

Study III. Effect of *PTPN22* polymorphism on LYP protein expression

3.1 Subjects

We selected 1 C/C and 1 C/T adult healthy subject from the blood donor Centre of Tor Vergata University Hospital of Rome. Subjects were genotyped for *PTPN22* polymorphism as described in the Methods section.

We recruited 3 C/C and 3 C/T patients previously genotyped for *PTPN22* polymorphism

3.2 Results

To determine whether the expression of LYP protein may be regulated by activation, T cells from a healthy subject carrying the C/C genotype were incubated with either OKT3/αCD28 or PMA/Ionomycin and harvested after 12, 24, 48 and 72 hours. An increase of the level of LYP expression was observed after 24 hours of either stimulus, with a further increase after 72 hours with OKT3/αCD28.

As expected, LYP was up regulated upon TCR engagement in a time-dependent manner. This up-regulation was quicker and time-independent if cells were activated with a TCR-independent stimulus, suggesting that the protein expression is strictly correlated with TCR activation. LYP expression in unstimulated T cells at different time points was low (*Fig. 4*).

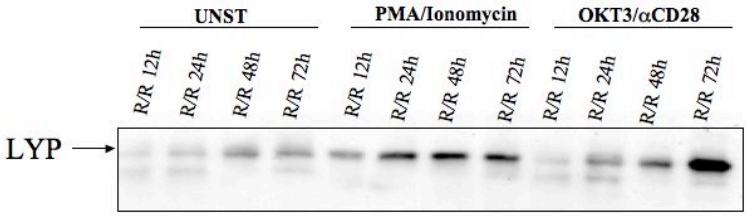


Figure 4. **Expression of LYP protein in T cells from C/C subject upon activation**
Freshly isolated CD14- cells from a C/C healthy donors were incubated with or without OKT3/αCD28 or PMA/IONO for 12, 24, 48 or 72 hours.

Besides the observation that LYP expression was correlated with TCR activation, we investigated whether LYP-R620 and LYP-W620 display any difference of TCR-induced phosphorylation. To address this question, since unstimulated T cells express low levels of LYP protein, we first stimulated T cells from healthy controls carrying the two LYP variants with PMA/Ionomycin for 24 hours, then, to assess LYP phosphorylation status, we further stimulated T cells with OKT3/αCD28 for 5 minutes and immunoprecipitated endogenous LYP. We observed that T cells isolated from healthy subjects of C/C genotypes have greater LYP phosphorylation than T cells of C/T genotype. (*Fig.5*). The lower phosphorylation status on negative regolatory site suggests that mutated LYP is more active than wild-type protein, providing an explanation of its increased function.

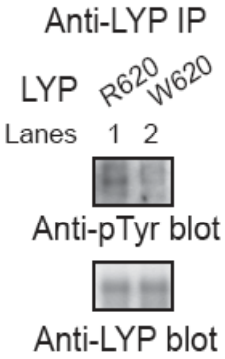


Figure 5 Immunoprecipitation of endogenous LYP in primary human T cells from healthy subjects of C/C (lane 1) or C/T (lane 2) genotype. Upper panel shows anti-pTyr blot and lower panel shows control anti-LYP blot of anti-LYP IPs.

Next, to compare the expression of the two LYP variants, T cells from healthy donors carrying the C/C or C/T genotype were treated with or without OKT3/αCD28 or PMA/IONO for 24 or 72 hours.

Interestingly we observed that upon specific TCR activation LYP is up-regulated only in C/C subjects. In particular, the expression of LYP protein is completely absent in T cells of the C/T subject only upon stimulation with OKT3/αCD28 at any time point. On the other hand LYP is up-regulation is conserved upon stimulation with PMA/Ionomycin, although later and to a lesser extent than in C/C subjects (*Fig. 6*).

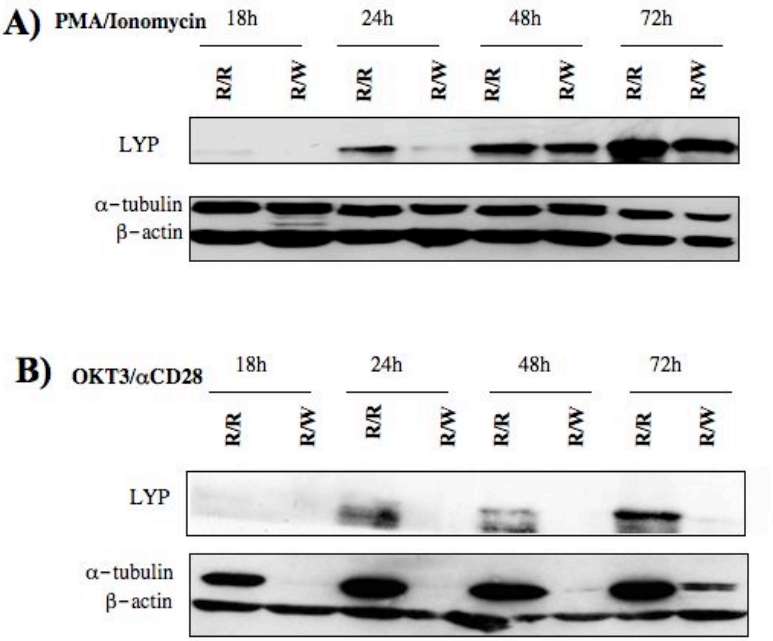


Figure 6. **Expression of LYP protein in T cells from C/C or C/T subjects upon activation**

Freshly isolated CD14- cells from a C/C (R/R) and a C/T (R/W) healthy control were stimulated with OKT3/CD28 or PMA/ionomycin for 18, 24, 48 and 72 hours.

Panel A shows Lyp expression after stimulation with PMA/Ionomycin and **Panel B** shows Lyp expression after stimulation with OKT3/αCD28 at different time points. The lower panels show the expression of α-tubilin and β-actin, as loading controls.

We performed the same experiments also in T1D patients carrying the two LYP variants and we confirmed the impaired expression of LYP in T cells from heterozygous only upon specific TCR activation. Moreover, like in healthy controls, LYP is expressed very little in unstimulated cells from T1D patients carrying both C/C and C/T genotype (*Fig.7*).

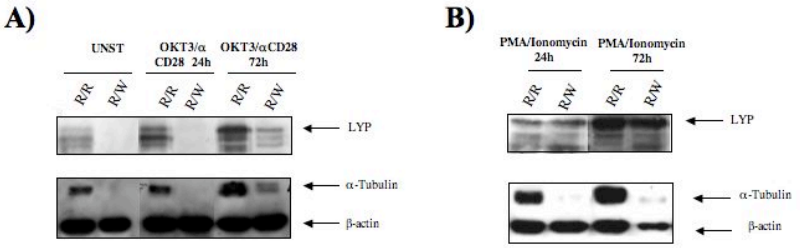


Figure 7. **Expression of LYP protein in T cells from C/C or C/T T1D patients upon activation**
Freshly isolated CD14- cells from a C/C and a C/T T1D patients were stimulated with OKT3/CD28 or PMA/ionomycin for 24 and 72 hours
Panel A shows Lyp expression after stimulation with PMA/Ionomycin and **Panel B** shows Lyp expression after stimulation with OKT3/αCD28 at different time points.The lower shows the expression of α-tubilin and β-actin. The lower panels show the expression of α-tubilin and β-actin, as loading controls.

All this data showed that *PTPN22* C1858T polymorphism is correlated with a defect of LYP expression, suggesting that the gain-of-function might be caused an alteration of the protein expression. This alteration might affect either the pre or post-translational processing of the protein, leading to a different mRNA transcription

and mRNA stability, or an alteration in the protein stability, respectively.

To understand which mechanism leads to a defect of LYP W620 expression, we firstly investigated LYP mRNA levels on unstimulated and stimulated T cells from C/C and C/T T1D patients by RT-PCR.

We observed no differences in the presence of mRNA in both ustimulated and stimulated cells from C/C and C/T T1D patients (*Fig. 8*). This data is in agreement with a previous report in which it has been shown that LYP-R620 and W620 mRNAs were expressed at similar level in T cells from genotyped individuals (**Nielsen C et al., 2007**).

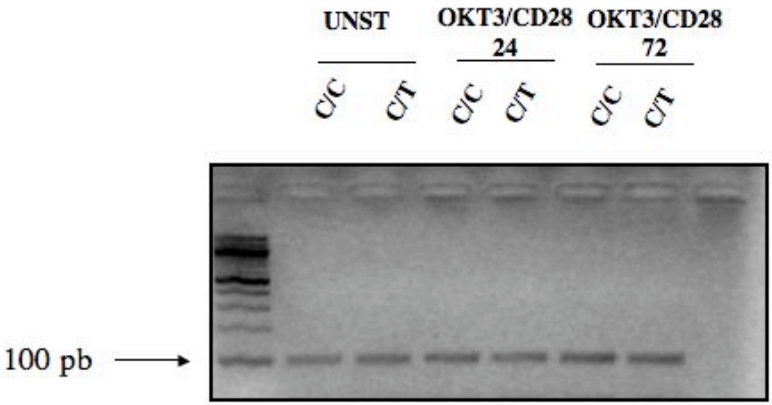


Figure 8. **LYP mRNA levels.** Freshly isolated CD14- cells from one C/C and one C/T T1D patients were stimulated with or without OKT3/αCD28 for 24 and 72 hours. After stimulation cells were harvested and total mRNA was extracted.

Then we investigated LYP protein half-life to establish what is the timeframe in which the protein gets degraded. We performed this experiment in PBMC from a C/C healthy control.

Because the amount of LYP in unstimulated cells is very little, we firstly stimulated PBMC with OKT3/αCD28 for 48 hours and then we treated cells with cyclohexamide at different time point. We observed that the half amount of protein is between 4 and 8 hours after treatment (*Fig. 9*).

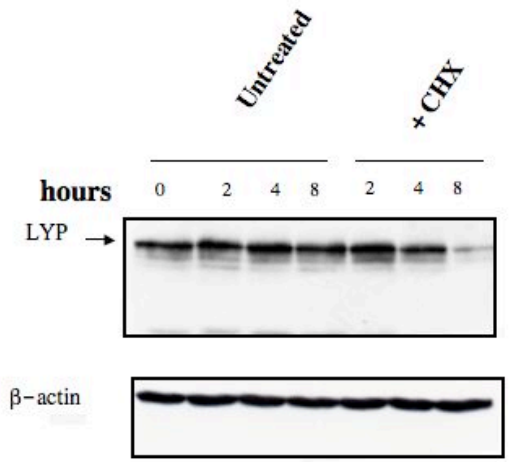


Figure 9. **LYP half life** Freshly isolated PBMC cells from a C/C healthy donor were stimulated with OKT3/αCD28 for 48 hours, then treated with cyclohexamide
Upper pannel shows LYP expression with or without cyclohexamide treatment at different time point.

Once established LYP half-life, we investigated if LYP degradation is regulated *via* ubiquitin-proteasome pathway, which is the more powerful system to regulate the homeostasis of the cytoplasmatic proteins in mammilian cells. We treated PBMC from a C/C healthy control with cyclohexamide with or without MG-132, which is a specific, potent, reversible, and cell-permeable proteasome inhibitor. We observed that upon MG-132 the amount of the protein was restored compared to the amount of protein upon cyclohexamide treatment (*Fig. 10*). We concluded that LYP is degraded *via* ubiquitin-proteasome pathway.

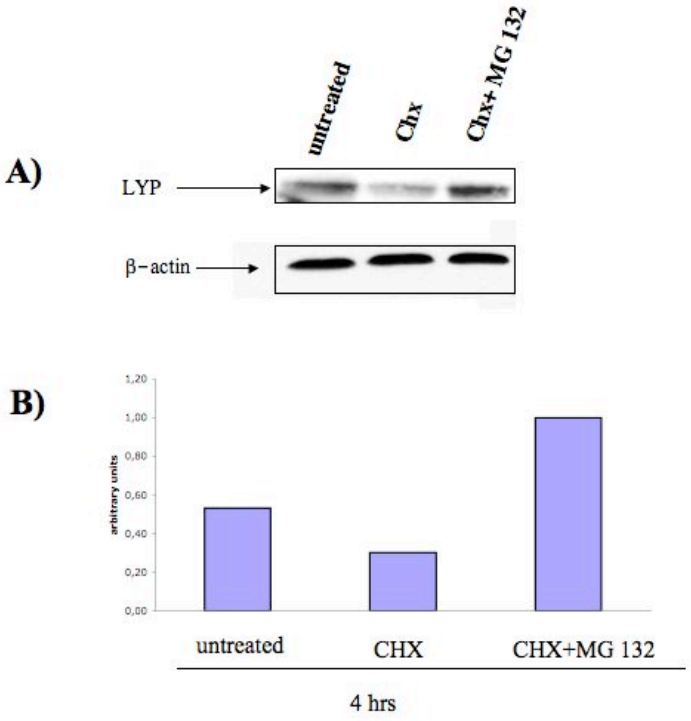
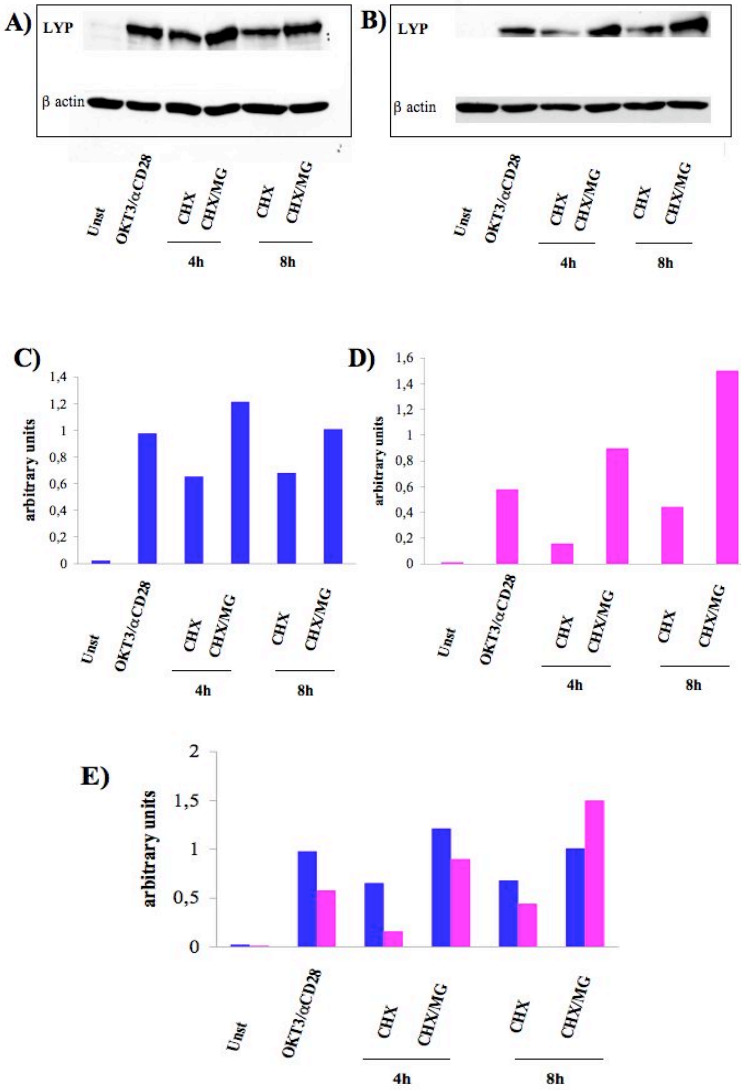


Figure 10. **LYP is degradated *via* ubiquitin-proteasome pathway**
Freshly isolated PBMC cells from a C/C healthy control were stimulated with OKT3/αCD28 for 48 hours, then treated with cyclohexamide alone or plus MG132 for 4 hours.
Panel A shows LYP expression after cyclohexamide alone or plus MG132 treatment. Lower pannel shows the expression of β-actin. This result is representative of two independent experiments
Panel B shows relative LYP expression obtained by densitometric analysis, normalizing LYP for its loading control

Then we tested the effect of the proteasome inhibitor in PBMC from C/C and C/T T1D patients and we observed that upon 8 hours of treatment LYP R620 quantity is the same than before

treatment. Interestingly, the amount of LYP W620 is greater than that we observed before treatment, suggesting that the blocking of the degradation is able to restore the same protein level than we observed in C/C patients. We also observed that both LYP R620 and W620 showed the same half-life (*Fig. 11*), suggesting that the stability of the protein is not affected by the presence of the polymorphism. All this data suggest that the different expression of LYP R620 and LYP W620 might be due to a different level of degradation of the protein.


A)
LYP
β actin
Unst
OKT3/αCD28
CHX
CHX/MG
CHX
CHX/MG
4h
8h
B)
LYP
β actin
Unst
OKT3/αCD28
CHX
CHX/MG
CHX
CHX/MG
4h
8h
C)
arbitrary units
1,4
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1
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CHX/MG
4h
8h

Figure 11. **LYP W620 is more degradated than LYP R620 after TCR activation.** Freshly isolated PBMC cells from one C/C (**panel A**) and one C/T (**panel B**) T1D patients were stimulated with OKT3/CD28 for 48 hours, then treated with cyclohexamide, with or without plus MG1 for 4 hours.
Lower pannel shows the expression of β-actin.
Panels C, D and E show relative LYP expression obtained by densitometric analysis normalizing LYP for its loading control. This result is representative of two independent experiments

DISCUSSION

In the first part of this study we characterized, for the first time, the association between *PTPN22* C1858T polymorphism and T1D patients from continental Italy. According with the literature we found an association between *PTPN22* C1858T polymorphism and T1D in the Italian population. An effect of gender on the association between *PTPN22* and T1D has been reported in the German and Danish populations (**Kahles H et al., 2005; Nielsen C et al., 2007**). In our population the association was independent of gender, although the sample size might be too small to detect a modest effect.

We also investigated the correlation between 1858T allele and the presence of autoantibodies (IAA, IA2 and GAD) specific for T1D in our population. Antibodies are important markers of development and progression of T1D and it has been described in some population that there is a correlation between the presence of 1858T allele and the presence of antibodies, nevertheless, we did not find this correlation in our population, probably due to the fact that the sample is too small to detect some differences.

PTPN22 is a shared autoimmunity gene, responsible for familial aggregation of different diseases, thus is possible that carriers of T1858 are also at increased risk for co-occurrence of multiple autoimmune diseases. In our T1D patients, the 1858T allele confers an increased risk for comorbid autoimmune thyroiditis but not for celiac disease. This is in line with published observations: while autoimmune thyroiditis was reported to be associated with the

C1858T polymorphism, several studies could find no association between *PTPN22* and celiac disease in multiple populations.

In Caucasian populations the contribution of *PTPN22* to the genetic risk of autoimmunity is substantial: *PTPN22* currently ranks in third place (after the HLA and the Insulin genes) and in second place (after the HLA) in terms of single-gene contribution to the aetiology of T1D. On this basis we investigated the correlation between *PTPN22* C1858T polymorphism and high-risk HLA genotypes in our cohort of patients. Interestingly we found that in patients carrying the C/T polymorphism there is a higher frequency of HLA DR3/DQ2 in comparison whit patients carrying the C/C polymorphism who showed a higher frequency of HLA DR3/DR4, which is the high-risk HLA genotype. This data confirmed the previous finding by Hermann at al, that the relative risk of *PTPN22* is higher in low-risk HLA genotype compared to the high-risk HLA genotype, suggesting that there is a strong interaction between HLA class II genotypes and non-HLA susceptibility locus in the genetic predisposition of T1D.

Besides the confirmed association between *PTPN22* polymorphism and T1D, the biological effect that this mutation exerts on the function of the cell of the immune system has not been completely understood. Therefore, in the second part of the study we investigated the contribution of *PTPN22* polymorphism on T cells proliferation and cytokines production.

Given our previous observation that T1D patients showed a defect in PBMC proliferation in comparison with healthy controls, we firstly investigated if this difference is LYP dependent. We observed that T cells from C/C T1D patients showed a defect in the

proliferative response compared to C/C healthy controls. We also observed a lower proliferative response in T1D patients carrying the C/T polymorphism compared to healthy controls, but this difference is not observed in comparison to T1D patients carrying the C/C polymorphism, confirming previous observations that this defect is "intrinsic" to the disease (**Nervi S et al., 2000**). However, the T/T T1D patient showed a dramatic defect on T cell proliferation only upon TCR activation, and not upon PMA/Ionomycin activation. This data confirms the pivotal role that LYP exerts on TCR signalling and suggests that the effect of the gain-of-function on proliferative response probably might appear only if both of *PTPN22* alleles are mutated.

Interestingly, we observed a difference in the pre-activated status of T cells, suggesting that LYP might be involved in the regulation of the normal balance between inactivation and activation in resting T lymphocytes. This is in agreement with the data reported in the literature in which it has been described a substantial defect of T cells from subjects carrying the mutated form of LYP in the early events that follow TCR activation, as intracellular calcium flux and CD25 expression (**Rieck M et al., 2007**).

To further characterise the effect of the polymorphism on lymphocyte function, we then analysed T cells cytokine production upon TCR activation. According with previous reports we observed a significant lower IL-2 production in T cells from T1D patients carrying the C/T polymorphism compared to patients carrying the C/C polymorphism. Moreover, the IL-2 production of T cells from T1D patient carrying the T/T polymorphism is totally absent, suggesting that this defect is correlated to the quantity of mutated

protein. We observed a discrepancy between reduced IL-2 production and normal proliferation when we analized C/T patients compared to T/T patient, in which there is a perfect concordance of the tests. This difference could be explained by the conserved activity of the protein in the first case, wich allows the production of an amount of IL-2 reduced compared to C/C subjects, but nevertheless sufficient to sustain T cell proliferation in C/T subjects. IL-2 plays a central role in Tregs peripheral homeostasis and function (**Jailwala P et al., 2009**). Indeed, Treg must derive IL-2 from effector T cells in a paracrine manner in order to sustain their functions. A tightly regulated feedback loop seems to be in place whereby Tregs, requiring IL-2 producing by activated T cells for their mantainance and activation, limit the expansion of effector T cells by shutting down their production of IL-2. Furthermore, neutralization studies have shown that IL-2 deficiency precipitates the breakdown of tolerance and the progression of T1D by selectively depleting the Treg cell subsets, and suggest that IL-2 may be essential for controlling diabetogenic T cells by promoting Treg cell functions **(Setoguchi R et al., 2005**). Consistently, low dose administration of IL-2 in prediabetic female mice resulted in enhanced frequency of Treg, and this regimen resulted in T1D protection (**Tang Q et al., 2008**). Since T cells from T1D patients carrying the mutated LYP showed a defect on IL-2 production, we might postulate that LYPW620 predisposes to T1D because the lack of IL-2 invalidate Treg suppressive function in the periphery, allowing an enhanced activity of autoreactive T cells. This hypothesis is also supported by the observation that T cells from T1D patients carrying the LYP variant did not show a defect in

proliferative response, suggesting that some compensative mechanisms might intervene in the late phase of TCR activation and mask the gain-of-function effect of LYPW in bulk T cell population. On the other hand, an inpairement of IL-2 might preferantially affect Treg compartment, wich is the most critically dependent on the integrity of the IL-2/CD25 patway. This hypothesis also is supported by the observation that Tregs from T1D patients showed a marked defect in their ability to suppress the proliferation of autologous effector-T cells in vitro. Interestingly this defect was associated with reduced production of IL-2 and IFN-γ (**Brusko M et al., 2005**). The hypothesis that *PTPN22* might be involved in Treg function is also suggested by the finding that *PTPN22* is one of the major target of Foxp3, the signatur transcriptor factor of naturally arising Treg, it is upregulated upon stimulation in $Foxp3^-$ cells, and this upregulation is inhibited in $Foxp3^+$ hybridomas and *ex-vivo* isolated Treg cells (**Marson A et al., 2007**).

Both IFN-γ and IL-17 are involved in pro-inflammatory response but their role in T1D is still not clear. We observed a marked decrease of IL-17 and IFN-γ production by T cells from the T1D patient carrying the T/T genotype. This defect is in line with the finding that this variant is a gain-of-function that causes enhanced suppression of TCR transduction and hyporesponsiveness of T cells.
It has been suggested that IFN-γ plays an important role in the early stage of the insulitis, contributing on β-cell apoptosis and death (**Sarvetnick N et al., 1991**). In contrast, many studies suggest that IFN-γ response is not to be essential for insulitis and diabetes development in NOD mice (**Wang B et al., 1997; Kanagawa O et**

al., 2000). More recently, according with our data, it has been described an impairment of IFN-γ and IL4 in newly diagnosed diabetic patients (**Halminen M et al., 2001**). The defect of IFN-γ signaling pathway in T1D patients might lead to a reduced numbers of CD4+CD25+ Foxp3+ T-cells or to a decreased apoptosis of beta-cell targeting effector CD4+ T-cells. This hypothesis is in agreement with our observation that T1D patients carrying the T/T genotype showed a slight defect in IFN-γ pathway that might contribute to disease development.

There is little information, as yet, regarding the role of IL-17 and Th17 cells in T1D. Studies in NOD mice shown a possible involvement of Th17 cells in pancreatic β cell destruction in the early stages of disease. Conversely it has been shown that the production of IL-17 by peripheral blood lymphocytes from patients with T1D is reduced after stimulation with both GAD and GAD-derived peptide ligand, suggesting that IL-17 exerts a protective role in the development of T1D. We observed a marked defect of IL-17 production in T cells from the T/T patient. Nevertheless, what is the contribution of this impairment remains to be addressed.

We also investigated the production of IL-10, which is a immunomodulatory cytokine, after TCR stimulation in T cells from T1D patients carrying the C/T or T/T genotype compared to T cells from T1D patients carrying the C/C genotype. In this case the trend is not very clear in correlation with the genotype, due to the fact that there is a slight increase of IL-10 production in T cells from C/T T1D patients but a decrease in T cells from the T/T T1D patient. To better understand the correlation between this cytokine and PTPN22 polymorphism, in consideration of the pivotal role of IL-10 in

immune tolerance, we will test more patients with the C/T or T/T genotype.

Since the observations that T cells from T1D patients carrying the *PTPN22* polymorphism showed a normal proliferative response but a defect of IL-2 production and a substantially unchanged production of IFN-γ and IL-17, we speculated that some compensative mechanisms might act in the late events after TCR activation. Indeed previous report failed also to produce definitive results about the immunological effect of *PTPN22* C1858T polymorphism on T cell from T1D patients, while both human and mouse studies clearly showed that mutated LYP confers a defect in the early stages of TCR activation.

It is now established that the polymorphism affects the binding of LYP with Csk, therefore we asked if the lack of protein-protein interaction might lead to some defect in the protein expression. Interestingly, we observed that in T cells from T1D patients and healthy controls carrying the C/T polymorphism stimulated *via* TCR there is a dramatic defect of LYP expression. This defect is also present if the cells were stimulated with PMA/Ionomicyn, known to bypass TCR. This suggests that the lack of expression of LYP W640 is due to a defect in the mutated protein and not secondary to an impaired activation status of the cells, that would cause, in turn, a reduction of the phosphatase up-regulation. This last possibility was also excluded in our study by the observation that T cells proliferation is conserved in patients carrying LYP620 variant. We therefore hypothesized that the lack of binding of LYP to Csk leads to a greater amount of free protein in the cytosol, which is more susceptible to degradation. To test this

hypothesis we compared the expression of LYP in T cells from C/C and C/T T1D patients after treatment with a proteasome inhibitor and, as expected, we observed a complete restoration of the level of the mutated protein. This data provides an explanation for the lack of expression of LYP in T cells from C/T T1D patients, during TCR activation LYPW620 is more degraded than the wild-type variant, and the reduction of its activity could probably masking the gain-of function effect in late events of T cells activity, such as the proliferative response, differently from what happens with IL-2 production. In other words, the discrepancy observed among the *in vitro* test performed, could be due to the different levels of protein at different time points point during TCR activation which might cause a full display of the gain-of-function effect only during the early events and, on the other hand, diminishing this effect during the late events of TCR activation. The reason why we do not appreciate this hypothetical compensative effect in T/T patients, can be due to the complete lack of the wild-type protein.

Over the last few years, genome-wide association studies have reported an extraordinary harvest of new genetic associations in the area of T1D, but the exact mechanisms underlying these associations are unknown. Identifying the functional consequence of the *PTPN22* 1858T variant has the potential to elucidate the immune phenotype linking the presence of the polymorphism with the pathogenesis of T1D.

METHODS

Subjects

For the Study I we examined 216 T1D patients recruited from the Pediatric Diabetology Unit of the University of Rome "Tor Vergata" with a mean age at sample collection of 15.6 years (±6 years). The diagnosis of T1D was established according to the definition of the American Diabetes Association 1997 (The Export Committee on the diagnosis and Classification of T1D, 1997).

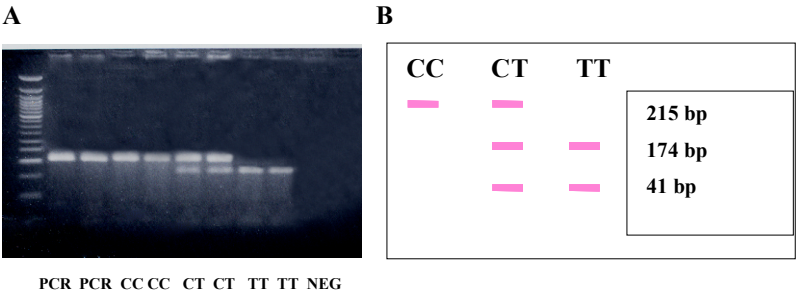
As controls, we examined 271 healthy subjects from Rome with a mean age of 36.8 years (±12 years).

For the **studies II** and **III** we selected 16 T1D patients carrying the C/C polymorphism, 12 T1D patients carrying the C/T polymorphism and 1 T1D patient carrying the T/T polymorphism, age and sex matched. The healthy children carrying the C/C genotype (2 boys and 1 girl) were recruited in the Pediatric Endocrinology Unit of the University of Rome "Tor Vergata". They had a mean age of 8 years (range 5-11) and had negative family history for autoimmune diseases. We also selected C/C and C/T adult healthy controls from the Blood Centre of the University of Rome "Tor Vergata" to perform western blotting experiments.

The study was approved by the Ethical Committee of the Hospital and informed consent was obtained from every study participant after the nature of the study was explained.

Genotyping

Blood was obtained by forearm venipuncture and genomic DNA was extracted using the Quiagen kit (Invitrogen, Alameda, USA). The *PTPN22* C1858T polymorphism was evaluated using a restriction fragment length polymorphism-polymerase chain reaction (PCR) method. The following primer sequences were used as follows: sense oligo 5' –TCA CCA GCT TCC TCA ACC ACA-3' and antisense oligo 5'-GAT AAT GTT GCT TCA ACG GAA TTT A-3'. The PCR reaction was carried out in a total volume of 25 μl containing 100 ng of genomic DNA, 10 pM of each primer, 2mM $MgCl_2$, 0.2 mM deoxy-nucleotides (dNTPs), 1X buffer and 2U of Taq polymerase. Amplification was performed for 35 cycles with an annealing temperature of 60° C. The C→T transition at codon 620 (NCBI refSNP) creates in the 1858T allele a restriction site for *X*cmI. The polymorphism was identified by *X*cmI restriction endonuclease digestion of PCR-amplified fragment. DNA fragments were resolved by electrophoresis on a 3% agarose gel.



RFLP-PCR based genotyping assay.

(A) Two representative individuals of each of the three genotype C/C, C/T or T/T. The polymorphism was identified by XCm1 restriction endonuclease digestion of the PCR amplified fragment. Each digestion was resolved on 3% agarose gel. Folowing electrophoresis, the gel was stained with ethidium bromide and the fragments were visualized by U.V.
(B) Schematic representation of DNA fragments resolution obtained upon digestion with Xcm1 enzyme.

T cell isolation

We separated PBMC from heparinized blood by Ficoll-Hypaque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). Lymphocytes were obtained from PBMCs by negative selection, using anti-CD14 conjugated magnetic Microbeads (MACS; Miltenyi Biotec, Belgish, Gladbach, Germany).

Proliferation and cytokine production

T cells were plated on 96 well at the cell concentration of

$2x10^6$/ml in medium containing 10% FCS, streptomycin (50 ng/ml, GIBCO-Invitrogen) and penicillin (100 μg/ml, GIBCO-Invitrogen). Cells were cultured in triplicate with medium alone or incubated with OKT3 (3 μl/well, supernatant of hybridoma ATCC, Sigma, Milan, Italy)/α-CD28mAb (0.15 μg/ml, BD Pharmigen, San Diego, California, U.S.A), or PMA (25 ng/ml, Sigma, Missouri, USA)/Ionomycin (0.5 μM, Sigma Aldrich, USA) for 24 or 72 hours. Proliferation was assessed on day 3 after overnight pulse with [^{3}H]TdR (0,5 μCi/well, Amersham International, Amersham U.K) and was expressed as count per minute (cpm) or stimulation index (SI= cpm stimulated/cpm unstimulated).

Culture supernatants were harvested at 24 and 72 hours after stimulation with OKT3/α-CD28mAb and IFNγ, IL-10, IL-17 (Endogen, Woburn, MA, USA) and IL-2 (Thermo Scientific, Waltham, MA, USA) concentrations were determined by Enzyme-Linked Immunosorbent Assay. The detection limit for IFN-γ, IL-10, IL-17 and IL-2 were 26.5, 15.4, 20 and 38 pg/ml respectively.

Cycloexhamide and MG132 treatment

In order to induce expression of LYP, PBMC were cultured in medium alone or stimulated with OKT3/α-CD28mAb for 48 hours; then, to estabilish the half-life of LYP, PBMC were further cultured in presence of Cyclohexamide (100 μg/ml, Sigma Aldrich, USA), a potent inhibitor of proteic synthesis. Cells were harvested at 0-2-4-6-8 and 12 hours after treatment, washed and lysed as described below to perform Western blotting. Based on LYP half-life cells were treated with Cyclohexamide alone or with MG-132 (1μM Sigma Aldrich), a potent proteasome inhibitor and were harvested at 0-4- and 8 hours after treatment, washed and lysed as described below to perform Western blotting.

LYP phosphorylation and mmunoprecipitation assay

In order to induce expression of LYP, T cells were cultured in the presence of 10 ng/μl PMA (SIGMA) for 24 hours. To induce LYP phosphorylation T cells were than stimulated for 5 minutes with OKT3/αCD28. Cells were lysed in 20 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM EDTA (TNE), with 1 mM PMSF, 10 mg/ml aprotinin/leupeptin and 10 mg/ml soybean trypsin inhibitor. 10 mM Na3VO4 was added to preserve the phosphorylation of LYP in TCR-stimulated cells. Total cellular extracts were subjected to immunoprecipitation with anti-LYP. Equal amounts of total extracts (50-80μg) were incubated with 1 μg of polyclonal goat anti-LYP antibody (R&D, Minneapolis, MN) 2h on ice. To immunoprecipitate LYP protein was added 10% glutathione-sepharose beads according

to the manufacturer's instructions (Amersham Pharmacia Biotech) and incubated with gentle rotation for 1 h at 4°C. The beads were then collected by centrifugation at 400 x g for 5 min at 4°C followed by supernatant removal, and washed three times at room temperature by addition of 800 µl of JS with inhibitors, inversion three times, and centrifugation.

The supernatant was collected, separated by 10% SDS-PAGE and analyzed by immunoblotting with anti-pTyr Antibody, and anti-LYP antibodies respectively, as described below.

Western blotting

Unstimulated and stimulated cells were washed two times in PBS 1X and lysed for 20 min on ice in JS buffer (50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 1.5 mM MgCl2, 150 mM NaCl, 5 mM EGTA, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 1µg of pepstatin/ml, 5 ug of aprotinin/ml and 1µg of leupeptin/ml). After centrifugation at 12000 x g for 15 min at 4°C, protein concentration was calculated by Bradford assay (Thermo Scientific, Pierce , USA). Equal amounts of total cellular extracts (20-30µg) were separated by 10% SDS-PAGE, transferred on nitrocellulose and analyzed by immunoblotting.

In order to evaluate the level of LYP expression, the membrane was blocked with TBS-0.05% Tween, 5% milk for 1h and ibridizated with goat anti-LYP antibody diluted 1:500 (R&D) and followed by anti-goat IgG-HRP diluted 1:3,000 (Santa Cruz Biotechnology, CA, U.S.A.)

In order to verify the correct protein loading the membranes were

ibridizated with mouse anti-β actin antibody diluted 1:10,000 (Santa Cruz Biotechnology) or with mouse anti-α tubulin antibody diluited 1:10,000 (Sigma-Aldrich) and secondary anti-mouse IgG-HRP diluted 1:10,000 (Santa Cruz Biotechnology) The signals were detected with the ECL system (Amersham Pharmacia Biotech) and proteins' specific signals were quantified by densitometric analysis (Image J Software).

In order to evaluate the level of LYP phosphorylation, the membranes were blocked with TBS-0.05% Tween, 5% BSA for 1h and ibridizated with anti-pTyr antibody clone 4G10 (Chemicon International, Temecula, CA) diluted 1:1,000, followed by anti-mouse IgG-HRP antibody diluted 1:3,000 in TBS-0.05% Tween, 5% BSA. Next the membrane was stripped with stripping buffer (2% SDS, 62.5% TRIS HCl pH 6,7, 100 mM β-Mercaptoetanol) for 20 min 50°C with gentle agitation to remove the antibodies of first ibridization. After three washing the membrane was re-probed with goat anti-LYP antibody diluted 1:500 (R&D) followed by anti-goat IgG-HRP diluted 1:3,000 (Santa Cruz) in TBS 1X, 0,1% Tween, 5% milk .

RNA extraction and RT-PCR

Total RNA was isolated from PBMCs and MNC cells using TRIZOL Reagent procedure (Invitrogen-Life Technologies, Milan - Italy). Isolated total RNA was analyzed by spectrophotometer (OD 260) and 500 ng was used for cDNA synthesis carried out with random primers and SuperScript TM III First-Strand Synthesis SuperMix (Invitrogen-Life Technologies, Milan - Italy) in a total

volume of 20 ul. Reverse transcription was in agreement with the manufacturer's instructions.

Statistic analysis

For the first study the SPSS 13.0 analysis software was used. For the second study variables were tested for normality by the Kolmogorov-Smirnov method. Since the variables analysed in each group were normally distributed the differences between the mean±SD were compared with Student's *t*-test. When found non normally distribuited, they were further analysed with the Mann-Whitney's non-parametric test. Values of $p<0.05$ were considered statistically significant.

Graphs and statistical analysis were obtained using the Graphpad Prism software package.

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