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XX CYCLE

Involvement of Gut Immune System in the Pathogenesis of Type 1 Diabetes. Detection of T Cell Reactivity to Gliadin

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A mio figlio
il dono più grande della mia vita

Una cosa ho imparato nel corso della mia lunga vita:
che tutta la nostra scienza, se paragonata alla realtà,
è primitiva, infantile….
eppure è il bene più prezioso di cui disponiamo.

"Albert Einstein"

Table of Contents

Abbreviations

APCs: Professional antigen presenting cells

BB: Diabetes-prone BioBreeding rat

CD: Celiac Disease

CLA: Cutaneous leukocyte antigen

CM: Cow Milk

CTL: Cytotoxic T-lymphocyte

CTLA-4: **C**ytotoxic T-lymphocyte antigen

DC: Dendritic Cells

ELISA: Enzyme-Linked Immunosorbent Assay

FAE: Follicle-associated epithelium

GAD: Glutamic acid decarboxylase

GALT: Gut-associated lymphoid tissue

HC: Healthy Control

HLA: Human leucocity antigen

IA-2: Protein tyrosine phosphatase

IAA: Anti-insulin antibodies

MAdCAM-1: Mucosal addressin cell-adhesion molecule 1

MFI: Mean fluorescence intensity

MLNs: Mesenteric Lymph Nodes

NOD: Non-Obese Diabetic mouse

PBMC: Peripheral Blood Mononuclear Cells

PTG: Peptic-tryptic digest of gliadin

SD: Standard Deviation

SED: Subepithelial dome

SI: Stimulation Index

T1D: Type 1 Diabetes

TCR: T cell receptor

TECK: Thymus-expressed chemokine

TGase: Transglutaminase

TG-PTG: Transgluatminase-treated peptic-tryptic digest of gliadin

TJs: Tight junctions

TT: Tetanus toxoide

VLA-4: Very late antigen 4

ΔMFI: Differential mean fluorescence intensity

Abstract

Accumulating data indicate that a dysregulation of the gut immune system may play a role in the development of β cell autoimmunity and type 1 diabetes (T1D).

The aim of this study was to determine a possible link between the gut immune system and T1D, in particular, a possible role of gliadin as a T cell antigen in human T1D. Peripheral blood mononuclear cells (PBMC) were isolated from 25 children with T1D (aged from 3.4 to 19.6 yrs), 22 healthy controls (HC) (aged from 3.5 to 17 yrs), both negative for both anti-endomysial and anti-human tissue transglutaminase antibodies and 15 children with celiac disease (CD) (aged from 2.2 to 13.3 yrs). In the first part of the study, in 6 children with T1D, 5 with CD and in 6 HC, the PBMC were cultured with or without OKT3 plus anti-CD28mAb. After 18 hours the expression of β_7 integrin was determined by flow cytometry.

In patients with T1D we observed a higher expression of β_7 integrin on memory $CD4^+$ T cells. After polyclonal stimulation we found a significant reduction of β_7 expression on memory $CD4^+$ T cell in T1D patients compared with healthy controls.

In the second part of the study, in 19 children with T1D, in 10 with CD and in 17 HC, PBMC were cultured with the peptic-tryptic digest of gliadin (PTG) and/or transgluatminase-treated (TG)-PTG at increasing concentrations, or left unstimulated. PBMC

proliferation was assessed on day 5 by [^{3}H]-thymidine incorporation assay.

In 11 T1D patients, 2 with CD and 5 HC, we also assessed IFN-γ and IL-4 production in culture supernatants by ELISA.

In 6 T1D patients and 2 HC we studied the expression of β_7 integrin by flow cytometry.

In T1D patients we detected a dose-response PBMC proliferation to both PTG and TG-PTG with the maximal proliferation at the concentration of 100 μg/ml. PBMC from 7 out of 11 T1D patients (64%) responded to 100 μg /ml PTG. Mean stimulation index (SI) in T1D patients was higher than in HC (2.95±2 *vs*.1.3±0.6 respectively, p=0.02).

When PBMC were stimulated with TG-PTG we found that 6 out of 13 T1D (54%) and 2 out of 3 CD (67%) showed a proliferative response (T1D 2.4±2 *vs.* HC 1.5±0.7).

In 3 of 7 T1D responders (43%) but not in HC, TG-PTG induced IFN-γ production. In 1 patient with T1D we identified, after the stimulation with TG-PTG a discrete population of $CD4^+$ β_7^{hi+} cells.

Our data show that T cells expressing high levels of β_7 integrin are detectable in peripheral blood of CD and T1D patients and we found an enhanced T cell-mediated gluten-specific immunity in T1D patients.

This supports the hypothesis that immunity to oral proteins is altered in T1D patients. Long term follow-up is necessary to

establish whether these subjects are at increased risk for developing celiac disease.

***Key words**:* celiac disease, gut Immunity, $\alpha_4\beta_7$ integrin, T cell, type 1 diabetes.

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Type 1 Diabetes

Type 1 diabetes (T1D) is a multifactorial autoimmune disease, which is 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. The rate of β cell destruction varies from patient to patient, but tends to be more aggressive in infants and young children (**Couper JJ et al., 1991**). Hence, T1D usually presents during childhood or adolescence, although it may develop much later in life. The variation in age at onset could be indicative of disease heterogeneity, with different mechanisms leading to β cell destruction in childhood onset *versus* adult onset diabetes. This might reflect the involvement of different genetic and/or environmental susceptibility determinants.

The early stages of disease process leading to T1D are characterized by insulitis, the infiltration of pancreatic islets by mononuclear immune cells, including dendritic cells (DC), macrophages, and T cells (**Gepts W et al., 1978**). Although this could reflect a normal inflammatory response to tissue damage, perhaps induced by exogenous factors such as viral infections, the lymphocytic infiltrate is thought to contribute directly to β cell destruction. In support to this hypothesis, 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**). Some of these T cells have been shown to be capable of destroying β cells in vitro (**Boitard C et al., 1981**). Animal studies

have also shown that T cells play an important role in the disease pathogenesis. The non-obese diabetic (NOD) mouse spontaneously develops insulin deficient diabetes that shares many immunological and pathological features with T1D (**Todd JA et al., 1991**). The pathogenesis of T1D has been extensively studied, but the exact mechanism involved in the initiation and progression of β cell destruction is still unclear.

The presentation of β cell-specific autoantigens by antigenpresenting cells (APCs) (macrophages or DC) to $CD4^+$ helper T cells in association with MHC class II molecules is considered to be the first step in the initiation of the disease process. Macrophages secrete IL-12 stimulating $CD4^+$ T cells, to secrete IFN-γ and IL-2. IFN-γ stimulates other resting macrophages to release, in turn, other cytokines such as IL-1β, TNF-α, and free radicals, which are toxic to pancreatic β cells. During this process, cytokines induce the migration of β-cell autoantigen specific $CD8^+$ cytotoxic T cells. 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. Continued destruction of β cells eventually results in the onset of diabetes (**Kukreja A, 1999**).

The autoimmune aetiology of T1D is also reflected by presence of circulating autoantibodies, specific for β cell proteins such as insulin, glutamic acid decarboxylase (GAD), and the protein tyrosine phosphatase (IA2). These autoantibodies are detectable in 85-90% of subjects with diabetes at the time of diagnosis (**Leslie**

RD et al., 1999; Palmer JP et al., 1983). It is unclear whether they participate directly in 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 precedes the clinical onset of disease, often by several years. 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.

Clinical diabetes occurs when approximately 80-90% of the islet β cell mass has been destroyed, but islet autoantibodies continue to be detectable for some time after the onset of the disease.

Insulin: Insulin is the only known β cell specific antigen related to type 1 Diabetes. Anti-insulin antibodies (IAA) were first identified in 1983 by Palmer in a group of new-onset diabetic patients before treatment with insulin (**Palmer JP et al., 1983**). IAA appear years before diabetes onset and their level correlate inversely with the time from diabetes onset and are usually the first anti-islet autoantibody to be produced (**Schenker M et al., 1999**). Results of several studies indicate that insulin represent a primary autoantigen in type 1 Diabetes. Insulin specific T cells are a predominant component of the infiltrate in the pancreatic islet of pre-diabetic NOD mice (**Wegmann DR et al., 1994)** and insulin-specific T cells have been found to be present in high frequency among

nominally islet-cell-specific T cells in the islet infiltrates that accumulate in 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 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 B 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 both native insulin genes 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**). The positivity of anti-insulin antibodies usually is associated to the presence of autoantibodies to other autoantigens in subjects who will develop diabetes, the only presence of IAA is not a predictor of the development of the disease. Recent studies indicate that immunity to insulin is a common phenomenon in healthy children due to their dietary exposure to bovine insulin in cow milk (CM) (**Vaarala O, 2006**). It is possible that the early immunization to CM insulin explains the findings of CM as a risk factor of T1D. This route of the primary immunization to insulin in humans implies a key role for the gut immune system in the regulation of insulin-specific immunity. The factors which break the suppression

of the insulin-specific immunity in the gut immune cells may thus be responsible for the autoimmune process leading to T1D.

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 microvescicles 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. 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**). These results indicate that the role played by GAD in the pathogenesis of T1D remains uncertain.

IA-2: The role of IA-2 in the pathogenesis of T1D is unknown. 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.

Genetic Factors

The genetic of human T1D is complex (**Gianani R, 2005**). Linkage analysis and genetic association studies have identified more than 20 loci believed to be associated with increase disease risk. However, the task has proven difficult due to variable and often low penetrance of risk alleles.

The application of genome-wide scans for T1D in families with two or more affected sib pairs has revealed that, in both humans and animal models, the major determinants of the disease are genes

within the major histocompatibility complex on chromosome 6p21, accounting for approximately 50% of the genetic susceptibility. Most of patients with T1D express HLA-DR3 or DR4 class II alleles and approximately 30-40% are DR3/DR4 heterozygous (**Thomson G et al., 1989**). HLA DQ locus appears to confer the strongest susceptibility to T1D **(Baisch JM et al., 1990; She JX, 1996; Thorsby E, 1997)**, 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. The haplotype DRB1*1501-DQA1*0102-DQB1*0602, which encodes the DQ6 molecule, is rarely found among patients with T1D so it may play a protective role (**Pugliese A et al., 1995**). Another major risk gene is IDDM2 corresponding to the variable number of tandem repeats region (VNTR) located upstream of insulin gene *INS* (chromosome 11p15.5), which accounts for 10% of genetic susceptibility. 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**). The highest risk of T1D is conferred by class I homozygosity. 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**), encoding the cytotoxic T-lymphocyte antigen (CTLA-4) and CD28, molecules involved in T cell activation and in the modulation of the immune response. Another diabetes susceptibility locus recently identified (**Bottini N et al., 2004; Steck AK, 2006, Saccucci P et al., 2008**), is represented by a single-nucleotide polymorphism (SNP) in the gene *PTPN22* encoding the lymphoid protein tyrosine phosphatase (LYP), a suppressor of T-cell activation. The variants encoded by the two alleles, 1858C and 1858T, differ in a crucial amino acid residue (arginine to tryptophan at amino acid 620), involved in association of LYP with the negative regulatory kinase Csk. Unlike the variant encoded by the more common allele 1858C, the variant associated with T1D does not bind Csk and cannot interrupt LYP inhibition of TCR signaling. All together these results suggest that susceptibility to T1D is multigenic and that the overall penetrance of the genetic susceptibility is dependent on the influence of the environmental factors.

Environmental Factors

While there is considerable evidence that the environment has an influence on the pathogenesis of T1D the factors triggering anti-islet autoimmunity in man remain unknown.

The identity of T1D-related environmental factors in human has been sought using epidemiological analyses, natural studies, and animal experiments.

The incidence of T1D has increased dramatically, 2-4 fold over the past 50 years at rate of 3% per years in children of 0-14 years (**Gale EA, 2002; Gillispie KM et al., 2004**). There was also an increase in disease incidence of almost 5% per year in European children of age 0-4 years. Changes of this magnitude over such a short time cannot be due to fluctuations in genetic risk alone and must involve factors in the environment.

There is a wide geographic variation in T1D incidence, ranging from 0,1/100.000 in China and Venezuela to 37/100.000 in Finland, Sardinia and Newfoundland (**Newhook LA et al., 2004**). T1D incidence can also differ between genetically similar populations, and immigrants often assume the risk of their new region or country (**Kondrashova A et al., 2005**).

A number of studies have examined the concordance rate of T1D in twin pairs to determine to what extent common genetics and environmental factors modulate disease development. The concordance of T1D among monozygotic twins depends on age at initial diagnosis and is less than 40% overall. The concordance rate of monozygotic twin is higher than dizygotic twins or siblings. The

rate of diabetes among sibling (6%) is approximately 15 greater than (0,4%-0,6%) in the general population. These findings indicate incomplete penetrance of diabetes risk genes and are consistent with a requirement for genetic susceptibility that is influenced by environment.

For more than a century it has been suspected that viruses cause human T1D. The multiplicity of viruses and the problem of relating early infection with later appearance of diabetes have made it difficult to link viral infections and diabetes. Mumps, rubella, cytomegalovirus, enterovirus and retrovirus have been implicated in the pathogenesis of the disease. Maternal rubella virus infection is associated to T1D. Approximately 20% of children born with congenital rubella syndrome in New York City in the 1960s developed T1D. Fetal exposure to enterovirus has also been linked to T1D (**Hyoty H et al., 1995**).

Circulating T cells specific for these viruses were found more prevalent among patients with T1D than healthy controls (**Ou D et al., 2000; Varela-Calvino R et al., 2002**). However, the mechanism by which the viruses lead to β cell destruction is yet to be determined.

The mechanism of molecular mimicry have been suggested to mediate the putative diabetogenic effect of enteroviruses. A non-structural protein, 2-C, of enterovirus Coxsackie B4 includes an area with homology to GAD (**Åtkinson MA et al., 1994**). On the other hand, experimental studies have suggested that enterovirus could contribute to development of autoimmune diabetes by

indirect mechanism (**Horwitz MS et al., 1998**). The interference with the gut immune system could provide a link between the enterovirus infections and T1D. Viruses that replicate in the gut cause changes in the cytokine environment of the gut and thus may activate or suppress the gut associated lymphocytes via so-called by-stander mechanisms. Viral infections may also change the permeability of the gut and thus cause alterations in the mucosal immunity to dietary proteins (**Jalonen T et al., 1991**).

However, a recent systematic review of 26 case-control studies showed no serological evidence to support a causal role for the major candidate enterovirus, coxsackie B (**Green J et al., 2004**), and no relationship with diabetes autoimmunity was found in two prospective studies, DASY in Denver (**Graves PM et al., 2003**) and BABYDIAB in Munich (**Fuchtenbusch M et al., 2001**).

Recent data suggest there is an association between vitamin D_3 deficiency and T1D (**Mathieu C, 2005**). It has been demonstrated that early dietary supplementation with vitamin D may decrease risk of T1D in humans (**Hypponen E et al., 2001**). Administration of high levels of vitamin D to NOD mice inhibited insulitis and prevented diabetes. Giulietti et al. (**Giulietti A et al., 2004**) confirmed their original finding in NOD mice, showing that vitamin D deficiency in early life increased diabetes cases.

The Effect of the Diet on the Development of Autoimmune Diabetes

The effect of diet on the development of autoimmune diabetes has been studied in the two animal models of T1D, BB rats and NOD mice. The classical study by Elliott and Martin demonstrated that the incidence of diabetes in BB rats was decreased when diet containing aminoacids, instead of whole proteins, was introduced after weaning (**Elliott RB et al., 1984**). Later, it was demonstrated that use of hydrolyzed proteins did not prevent the insulitis in BB rats, but the lymphocyte infiltrating the islets showed a functional profile with lowered cytotoxic activity demonstrated as increased expression of IL-4, IL-5 and IL-10 and decreased expression of IFN-γ (**Scott FW et al., 1997**). Also the exposure to dietary soy proteins and wheat gluten seems to modify the incidence of diabetes in both BB rats and NOD mice (**Hoorfar J et al., 1993; Funda DP et al., 1999**). These study clearly demonstrate that the development of autoimmune diabetes is modified by environmental factors, such as dietary factors, which cause detectable immunological changes in the pancreatic islets.

In humans, the exposure to cow milk (CM) formulas in early infancy has been associated with the risk of T1D in several epidemiological studies (**Åkerblom HK, 1998**). Epidemiological data suggest that CM exposure in early life (less than 3 month of age) may increase about twofold the risk of T1D later in life.

However, the interpretation of the epidemiological data is difficult because the consumption of CM is such a common use and the

genetic factors, as well as other environmental factors, may modify the possible diabetogenic effect of dietary factors. The association of early exposure to CM formula and risk of T1D has stimulated pilot study on the dietary prevention of T1D. The TRIGR study (Trail to Reduce IDDM in the Genetically at Risk) have been performed in infants at genetic risk of diabetes with elimination of CM proteins during the first 6-8 months of life. The result of this study were promising, because by the age of 2 yr the appearance of islet cell autoantibodies was lower in children who received casein hydrolysate formula than in children who received CM-based formula during the first 6-8 months of life (**Åkerblom HK et al., 1993**).

Some studies did not find any relationship (**Bodington MJ, 1994; Karisson MG, 2001**). The variability may be due to variation in milk composition or the existence of a subset of individuals susceptible to milk-related T1D.

Refined wheat flour is a major component of the diet in developed countries where T1D incidence is highest.

Wheat flour is composed mainly of gluten, a complex mixture of several hundred polypeptides, mainly glutenins and gliadin storage proteins, which reside in the water-insoluble fraction of the wheat endosperm. Gliadins are monomers, whereas glutenins form large polymeric structures. On the basis of their amino-acid sequences, gliadins can be subdivided into α, γ and ω gliadins (~250–300 residues long), and glutenins can be subdivided into high-molecular-weight glutenins (~650–800 residues long) and low-

molecular-weight glutenins (~270–330 residues long). Many variants exist of each polypeptide type, with the greatest variation being of the gliadins. However, complete separation of proteins based on differential solubility is difficult and there is contamination among the fractions, with albumins and globulins still present in gluten.

Moreover, current understanding indicates that different gluten peptides are involved in the disease process in a different manner, same fragment being "toxic" and other "immunogenic". Specifically, a fragment is defined "toxic" if is able to induce mucosal damage either when added in culture to duodenal mucosal biopsy, or when administreded in vivo on proximal and distal intestine, whereas a fragment is defined "immunogenic" if it is able to specifically stimulate HLA-DQ2 or DQ8 restricted T cell lines and T cell clones derived from jejunal mucosa or peripheral blood of celiac disease (**Ciccocioppo R, 2005**). Nevertheless, it is not excluded that the same peptide may have both capacities simultaneously.

A wheat-T1D link is suggested by the high prevalence of celiac disease (CD) in patients with T1D (1%-16%) **(Collin P et al., 2002)** compared with the general population (0,4%-1%).

CD is a chronic immune mediated disorder of the gut driven by T cells reacting locally to gliadin (**Sollid LM, 2002**).

In some studies, the risk of T1D seems to be correlated with the duration of gluten exposure (**Ventura A et al., 1999**).

In patients with CD, immune responses to gliadin fractions promote

an inflammatory reaction, primarily in the upper small intestine, characterized by infiltration of the lamina propria and the epithelium with chronic inflammatory cells and villous atrophy. This response is mediated by both the innate and the adaptive immune system. The adaptive response is mediated by gliadin-reactive $CD4^+$ T cells in the lamina propria that recognize gliadin peptides which are bound to HLA class II molecules DQ2 or DQ8 on antigen-presenting cells; the T cells subsequently produce proinflammatory cytochines, particulary IFN-γ. Tissue transglutaminase (TGase) is an enzyme in the intestine that deamidates gliadin peptides, increasing their immunogenicity. The level of expression of TGase is increased in active coeliac disease.

The ensuing inflammatory cascade releases metallo-proteinases and other tissue-damaging mediators that induce crypt hyperplasia and villous injury (**Green PHR, 2007**).

A key observation is that the T cells invariably recognize gluten peptides that are presented by DQ2 or DQ8 molecules. The peptide-binding motifs of DQ2 and DQ8 predict a preference for negative charges at anchor positions of the bound peptides.

DQ2 has a preference for negatively charged residues at the P4, P6 and P7 anchor positions, whereas DQ8 has a preference for negatively charged residues at anchor positions P1, P4 and P9 (**Tollefsen S et al., 2006**). However, generally, gluten proteins contain few negatively charged residues. A clue to help explain this paradox was provided by the findings that lesion-derived T cells predominantly recognize deamidation gluten peptides and that this

deamidated, that increase the negatively charged residues, can be mediated in situ by the enzyme transglutaminase. Interestingly, the ratio of deamidation to transamidation in the presence of primary amines is markedly increased when the pH is below 7.3. This indicates that the deamidation of gluten peptides in the small intestine might occur in a slightly acidic environment. Immunohistochemical staining has shown that in the active coeliac lesion, TGase is expressed at the epithelial brush border, as well as being expressed extracellularly in the subepithelial region. The pH in the proximal small intestine is ~6.6, which should allow marked TGase-mediated deamidation of peptides in the brush border. Another possibility is that TGase is endocytosed; the enzyme could be active during the first stage of endocytosis when the pH is low (**Sollid LM, 2002**).

In NOD mice, wheat-based diets have been reported to be diabetes promoting in some (**Hoorfar J et al., 1993**), but not all studies (**Coleman DL et al., 1990; Elliot RB et al., 1988**). In three studies, wheat-free diet protected NOD mice from developing diabetes (**Funda DP et al., 1999; Maurano F et al., 2005; Schmid S et al., 2004**). In the report by Maurano et al. (**Maurano F et al., 2005**), adding back wheat gluten to a wheat-free diet in which amino acids were supplied from milk, peanuts and supplemental amino acids, significantly increased diabetes incidence.

Evidence supporting the role of wheat proteins in the pathogenesis of diabetes is accumulating also in humans. Reports from the BABYDIAB prospective study indicated an increase risk for

development of islet autoantibodies associated with early gluten exposure (<3 months) in offspring of T1D parents whereas introduction of gluten after the age of 6 months did not alter the risk (**Ziegler AG et al., 2003)**. The DAISY prospective study reported that children exposed to cereals from 0-3 months or after 7 months were at increased risk for islet autoantibody development (**Norris JM et al., 2003**). This suggests that wheat proteins could be linked to diabetes autoimmunity and that the timing of early exposure is important.

The goal of the BABYDIET study, which is on going, is to clarify whether delayed introduction of gluten decrease the risk of developing islet autoimmunity in susceptible children (**Schmid S et al., 2004**).

Moreover, the study of Ventura et al. showed that diabetes-related antibodies, present in adolescent with untreated celiac disease, disappear when patients are put on gluten-free diet (**Ventura et al., 2000**).

Nevertheless, a recent study (**Pastore MR et al., 2003**) demonstrated that when patients newly diagnosed with T1D were placed on a gluten-free diet, neither the level nor frequency of anti-GAD, IA-2, or insulin autoantibodies decreased, this is only apparently in contrast with the other studies since this result might be expected in patients already diagnosed with T1D.

On the other hand, Klemetti et al. demonstrated that there is an increased T cell proliferation in response to wheat gluten in newly diagnosed T1D patients (**Klemetti P et al., 1998**).

Moreover, sign of mucosal inflammation were present in jejunal biopsies from T1D patients, and organ culture studies indicate a deranged mucosa immune response to gliadin.

Troncone et al. used the rectal gluten challenge to investigate the local mucosal specific immune response and after a local instillation of gliadin, a significant percentage increment of lamina propria epithelium $\gamma\delta^+$ lymphocytes was observed 5 out of 19 children with T1D (**Troncone R et al., 2003**). Auricchio et al. have reported an increased numbers of intraepithelial $CD3^+$ and $CD25^+$ mononuclear cells in lamina propria together with increased expression of ICAM-1 in intestinal biopsy samples from patients with T1D. This study demonstrated that stimulation of intestinal biopsy samples with wheat gliadin results in an expansion of $CD3^+$ and $CD25^+$ cells and an upregulation of ICAM-1 indicating that patients with T1D respond to wheat gliadin with intestinal inflammation though do not develop CD (**Auricchio R et al., 2004**).

The organ culture data indicate a prevalence of potential CD higher then suspected. Some diabetic patients who are negative for CD-associated auto-antibodies will go on to become sieropositive and will eventually develop frank enteropaty (**Maki M, 1995**). The conditions influencing this process are poorly understood and, in this perspective, type 1 diabetes may represent a model with which to clarify the pathogenesis of CD.

Using a strategy similar to the approach used to identify all the autoantigens in T1D, a first candidate diabetes-related wheat

protein was identified by screening a wheat cDNA expression library using serum from diabetic BB rats (**MacFarlane AJ et al., 2003)**. Clone WP5212 shared 90% identity at the gene level and 80% identity at the protein level with the *Triticum aestivum* wheat storage globulin, Glb1. Antibody reactivity to Glb1 was significantly higher in diabetic rats than in asymptomatic and control rats and correlated with pancreatic inflammation and damage. This suggest either that Glb1 is antigenic and somehow participates in diabetes pathogenesis or alternatively that immune reactivity to Glb1 is an indicator of abnormal gut function, lack of oral tolerance, or cross-reactivity with self antigens. It was concluded that Glb1 is a normal contaminant of wheat gluten and contains peptides that are highly antigenic in a majority of wheat-fed BB rats.

There are preliminary indications that Glb1 could also be antigenic in humans with T1D (**MacFarlane AJ et al., 2003**). One case was reported of a highly wheat-sensitive patient with both T1D and CD who displayed strong immune reactivity to Glb1 (**Mojibian M et al., 2006**).

Gut Immune System in Type 1 Diabetes

Gut-Associated Lymphoid Tissue (GALT)

The gut immune system is a major T-cell organ. It has been estimated that the number of small intestinal intraepithelial lymphocytes is more than half of the T-cell number estimated for peripheral lymphoid organs. The gut–associated lymphoid tissue (GALT) can be divided into effector sites, which consist of lymphocytes scattered throughout the epithelium and lamina propria of the mucosa, and organized tissues, that are responsible for induction phase of immune response. These are the Peyer's patches and mesenteric lymph nodes (MLNs), as well as smaller, isolated lymphoid follicoles, which have the appearance of microscopic Peyer's patches and are distribuited throughout the wall of the small and large intestines (**Hamada H et al., 2002**) (**Fig. 1**).

The Peyer's patches are macroscopic lymphoid aggregates that are found in the submucosa along the length of the small intestine. Mature Peyer's patches consist of collections of large B-cell follicles and intervening T-cell areas. The lymphoid areas are separated from the intestinal lumen by a single layer of columnar epithelium cells, known as the follicle-associated epithelium (FAE), and a more diffuse area immediately below the epithelium, known as the subepithelial dome (SED).

The FAE differs from the epithelium that covers the villous mucosa, as it has lower levels of digestive enzymes and a less pronounced Brush Border, also it is infiltrated by a large numbers of B cells, T cells, macrophages, and dendritic cells (DCs).

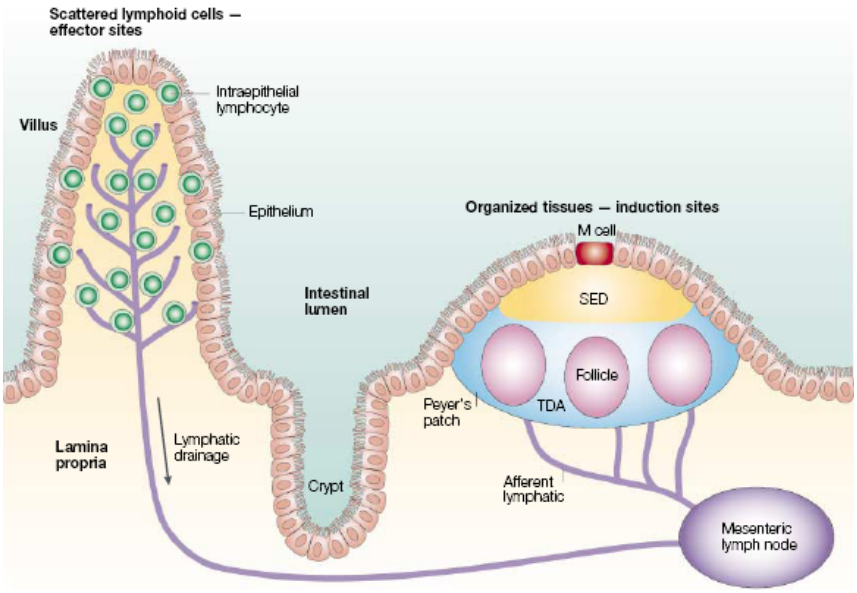


Figure 1. Schematic representation of the lymphoid elements of the intestinal immune system. The organized tissue of the Peyer's patches and mesenteric lymph nodes (MLNs) are involved in the induction of immunity and tolerance, whereas the effector sites are scattered throughout the lamina propria and epithelium of the mucosa. Both the Peyer's patches and villus lamina propria are drained by afferent lymphatics that go to the MLNs. SED, subepithelial dome; TDA, thymus-dependent area (**Mowat AM, 2003**).

The most notable feature of the FAE is the presence of microfold (M) cells, which are specialized enterocytes that lack surface microvilli and the normal thick layer of mucus. M cells differentiate from enterocytes under the influence of membrane-

bound LT $\alpha_1\beta_2$ that is present on local lymphoid cells, mainly B cells (**Debard N et al., 2001; Golovkina TV et al., 1999**).

The MLNs are the largest lymph nodes in the body. Accumulation of lymphocytes in the MLNs also requires both L-selectin and $\alpha_4\beta_7$ integrin adhesion molecules, which normally direct lymphocytes to enter peripheral and mucosa tissues, respectively (**Wagner N et al., 1998**).

It has been assumed for many years that M cells provide the main, if not only, way in which complex antigens can gain access to the intestinal immune system. M cells probably do not process antigens themselves; they do not express MHC class II molecules; and instead, they are believed to pass on intact antigen to professional antigen presenting cells (APCs), either in the epithelium or in the underlying dome region. From there, the APCs move to the T-cell areas and/or B-cell follicles, where they can interact with naïve lymphocytes. DCs are probably the APC involved in this process, and several DC subsets have been described recently in Peyer's patches. In Peyer's patches, B cells undergo immunoglobulin class switching from expression of IgM to IgA under the influence of several local factors, including transforming growth factor-β (TGF-β), IL-10 and cellular signals that are delivered by DCs and T cells.

The lymphocytes that are primed in the Peyer's patches exit through the draining lymphatics to the MLNs, where they reside for an undefined period of further differentiation, before they migrate into the blood-stream through the thoracic duct and finally accumulate in the mucosa. The exit of lymphocytes into the

mucosa occurs because lymphocytes that are primed by antigen in the GALT lose expression of L-selectin and selectively up-regulate expression of $\alpha_4\beta_7$ integrin. This directs the emigration of lymphocyte from the bloodstream by interacting with the ligand for $\alpha_4\beta_7$ integrin, the mucosal addressin cell-adhesion molecule 1 (MAdCAM-1), which is expressed at high levels by the vasculature of mucosal surface (**Berlin C et al., 1993; Butcher EC et al., 1999**). In parallel, the expression of the chemokine receptor CCR9 is induced by gut-derived T cells, allowing them to respond to the chemokine CCL25, also know as TECK (thymus-expressed chemokine), which is expressed selectively by small-bowel epithelial cells (**Yoshida H et al., 2002; Bowman EP et al., 2002; Campbell DJ et al., 2002**).

Although this local imprinting of homing specificity on activated lymphocytes has been appreciated for a long time, only in recent years the mechanism of this phenomenon have became clearer. An important role for local DCs was inferred from experiments in which naïve T cells were incubated with DCs of either peripheral or mucosal origin. When naïve $CD8^+$ T cells were incubated with DCs from spleen, from the peripheral lymph nodes, and Peyer's patches, the T cells could be equally activated by three cell types. But only DCs from Peyer's patches induced prominent expression of $\alpha_4\beta_7$ and reactivity to CCL25, the ligand of CCR9 (**Mora JR et al., 2003**) (**Fig. 2**).

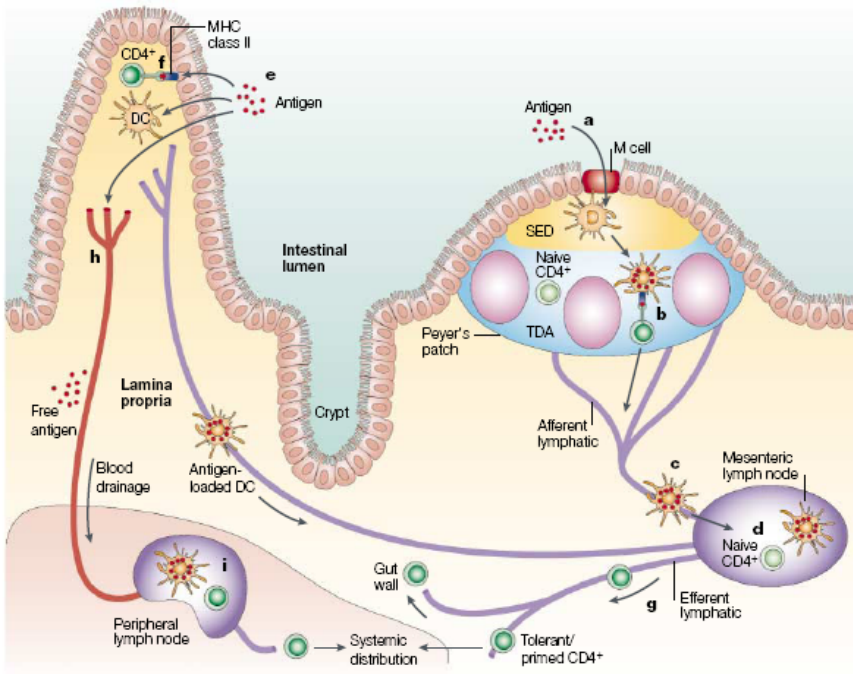


Figure 2. Antigen uptake and recognition by CD4$^+$ T cells in the intestine. Antigen might enter through the microfold (M) cells in the follicle-associated epithelium (FAE) (**a**), and after transfer to local dendritic cells (DCs), might then be presented directly to T cells in the Peyer's patch (**b**). Alternatively, antigen or antigen-loaded DCs from Peyer's patch might gain access to draining lymph (**c**), with subsequent T-cell recognition in the mesenteric lymph nodes (MLNs) (**d**). A similar process of antigen or antigen-presenting cell (APC) dissemination to MLNs might occur if antigen enters through the epithelium covering the villus lamina propria (**e**), but in this case, there is the further possibility that MHC class II $^+$ enterocytes might act as local APCs (**f**). In all cases, the antigen-responsive CD4$^+$ T cells acquire expression of the $\alpha_4\beta_7$ integrin and the chemokine receptor CCR9, leave the MLN in the efferent lymph (**g**) and after entering the bloodstream through the thoracic duct, exit into the mucosa through vessels in the lamina propria. T cells which have recognized antigen first in the MLN might also disseminate from the bloodstream throughout the peripheral immune system. Antigen might also gain direct access to the bloodstream from the gut (**h**) and interact with T cells in peripheral lymphoid tissues (**i**) (**Mowat AM, 2003**).

This pattern of adhesion-molecule and chemokine-receptor expression is distinct from that of T cells that are primed in peripheral lymphoid organs, which acquire the $\alpha_4\beta_1$ integrin VLA-4 (very late antigen 4) and the chemokine receptor CCR4 and so cannot migrate to mucosal surfaces (**Campbell DJ et al., 2002**).

The lymphocytes that enter the mucosa redistribute into distinct compartments. B-cell blasts mature into IgA-producing plasma cells and remain in the lamina propria. $CD4^+$ T cells also remain in the lamina propria, but are distributed more evenly throughout the villus-crypt unit. $CD8^+$ T cells migrate preferentially to the epithelium, although ~40% of the cells in the lamina propria are also $CD8^+$.

The functions of mucosal T cells are still largely uncertain, but cells with a "memory" phenotype predominate in both the epithelium and the lamina propria, indicating that they have been exposed to antigen. $CD4^+$ T cells in the lamina propria are of particular importance to local immune regulation. They are generally unresponsive to T-cell receptor (TCR)-mediated proliferate signals, but in humans, they can be induced to proliferate when CD2 is used as an accessory molecule. They produce large amounts of cytokine, particularly IFN-γ, but also IL-4 and IL-10 (**Braunstein J et al., 1997; Hurst SD et al., 1999**). Lamina propria $CD8^+$ T cells can also have potent cytotoxic T-lymphocyte (CTL) activity (**Lefrançois L et al., 1999**). Alternatively, they might be "effector memory" cells (**Sallusto F et al., 1999**), as indicated by the findings that antigen specific

memory $CD4^+$ and $CD8^+$ T cells accumulate preferentially in non-lymphoid tissues, in particular the intestinal mucosa (**Reinhardt RL et al., 2001; Masopust D et al., 2001**).

Lymphocyte Homing to the Gut

The initiation and maintenance of an effective immune response and the establishment of immunological memory are crucially dependent on the orchestrated migration of T cell subsets to distinct tissue locations. T cell migration to lymphoid and extralymphoid tissue is a multistep process that is regulated by the coordinated interaction of various cell-surface molecules on the T cell with their respective ligands on the surface of vascular endothelial cells.

Extravasation is directed by selective leukocyte-endothelial cell recognition, a process that involves four consecutive steps (**Fig. 3**).

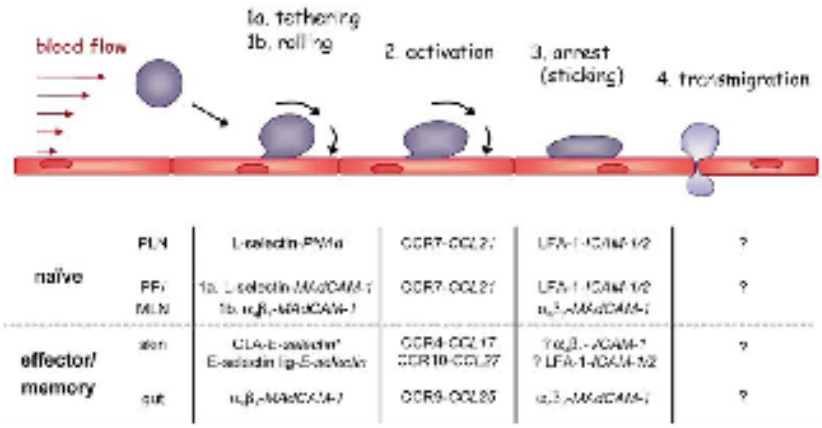


Figure 3. Organ-specific molecular steps involved in the extravasation of naïve and antigen-experienced T cells (**Ipparaguirre A, 2003**).

In the first step, the leukocyte establishes a reversible rolling interaction along the vessel wall, which is mediated by constitutively active molecules, primarily selectins and their ligands. During rolling, leukocytes screen the endothelium for an activating signal (step 2), which is necessary to convert surface-expressed integrins from an inactive to an active state. The conformational change of integrins triggered by chemoattractants facilitates binding to their ligands and, consequently, firm attachment (sticking) of the rolling leukocyte (step 3). In the absence of such a signal, the rolling cell eventually detaches from the vessel wall and returns to the bloodstream. Finally, firmly adhering cells transmigrate through the endothelial layer into the surrounding tissue (step 4) (**Ipparaguirre A, 2003**).

Putative naïve T and B cells in peripheral blood uniformly express low levels of $\alpha_4\beta_7$ and enter secondary lymphoid tissue in a process involving L-selectin and LFA-1 (**Bargatze RF et al., 1995**). In contrast, memory T and B cells can be divided into $\alpha_4\beta_7^{hi}$ and $\alpha_4\beta_7^{-}$ subsets. Only the $\alpha_4\beta_7^{hi}$ subsets is able to bind MAdCAM-1 and traffic to intestinal tissues (**Mackay CR et al., 1996**). Expression of CCR9, a receptor for chemiokine TECK that is present only in the thymus and small intestine, is found only in this memory T cell subset (**Yoshida H et al., 2002**).

The concept that antigen-experienced lymphocyte subsets show tropism for distinct extra-lymphoid tissues arose from early studies in which adoptively transferred lymphocytes were found to migrate preferentially to those tissues from which they were isolated

(**Cahill RN et al., 1977; Hall JG et al., 1977**). Subsequent studies defined two non-overlapping antigen-experienced T-cell populations in human blood: one subset expressing the $\alpha_4\beta_7$ integrin, and the other expressing cutaneous leukocyte antigen (CLA), which is a carbohydrate epitope recognized by the antibody HECA-452. These two T-cell subsets have preferential homing capacity for intestinal and cutaneous tissues, respectively (**Butcher EC et al., 1999**). T cells expressing the skin homing molecules CLA are $CCR9^-$ and found within the $\alpha_4\beta_7^-$ population. These CLA^+ $\alpha_4\beta_7^-$ memory T cells alone express CCR4 and respond to its ligand TARC that is found in skin (**Campbell JJ et al., 2002**).

Studies using neutralizing antibodies specific for $\alpha_4\beta_7$ integrin or MAdCAM-1, and studies using knockout mice, have show a role for $\alpha_4\beta_7$ integrin and MAdCAM-1 in mediating the entry of $CD4^+$ and $CD8^+$ effector T cells to these sites (**Hamann A et al., 1994; Lefrançois L et al., 1999, Wagner N et al., 1996**).

In the same way, an antigen-specific T-cell response was found in the $\alpha_4\beta_7$ positive T-cell population in rotavirus infection, indicating that $\alpha_4\beta_7^+$ memory T cells show reactivity for intestinal antigens (**Rott LS et al., 1997**).

The importance of $\alpha_4\beta_7$ integrin as an intestine-specific "area code" seems to be maintained during intestinal inflammation, as antibodies specific for either $\alpha_4\beta_7$ integrin or MAdCAM-1 attenuate inflammation in animal models of colonic inflammation (**Picarella D et al., 1997; Kato S et al., 2000**), and a humanized

$\alpha_4\beta_7$ integrin-specific antibody was found to induce clinical and endoscopic remission in patients with active ulcerative colitis (**Feagan BG et al., 2005**).

Role of the Gut in the Pathogenesis of T1D

The main T1D-promoting environmental candidates are enteroviruses and dietary proteins, both of which enter the body via the gastrointestinal tract.

Some studies have indicated that mucosal lymphocytes may be involved in the pathogenesis of T1D. MAdCAM-1 is expressed in the inflamed islet of NOD mice, and the infiltrating T-cells express the same adhesion molecules as gut-associated lymphocytes the $\alpha_4\beta_7$ integrin (**Hänninen A et al., 1993; Yang XD et al., 1994**). The importance of MAdCAM-1/$\alpha_4\beta_7$ integrin interaction in the binding of the lymphoid cells to the pancreatic vascular endothelium has also been shown in the functional studies. In NOD mice, the treatment from age 7 to 29d or 8 to 12 weeks with monoclonal antibody against β_7 integrin or MAdCAM-1 resulted in long-standing protection from diabetes and insulitis (**Yang XD et al., 1997**). In a study by Hänninen et al. the blockade of the function of MAdCAM-1 by monoclonal antibodies reduced the incidence of diabetes when started at 3 weeks of age in NOD mice. The same treatment also inhibited diabetes and the homing of the lymphocytes into the pancreas in a adoptive transfer performed in NOD/severe combined immunodeficient (SCID) recipients. In the same adoptive transfer model, lymphocytes derived from the

mesenteric lymph nodes of young NOD donors were diabetogenic (**Hänninen A et al., 1998**). This study suggests that islet-cell reactive lymphocytes share the lymphocyte and endothelium adhesion molecules involved in the migration of lymphocytes into mucosal lymphoid tissues. This study also indicates a role for MadCAM-1 as a mucosal addressin involved in the initiation of diabetogenic autoimmunity in young NOD mice. Accordingly, β-cell autoreactive lymphocytes may belong to the compartment of gut-associated lymphocytes and may even originate from the gut mucosa. Supporting the latter hypothesis, the mesenterial lymphocytes derived from 3-week-old NOD mice have been shown to transfer diabetes.

Also, some studies in humans have indicated that mucosal lymphocytes may be involved in the pathogenesis of T1D.

When the endothelial cell-binding properties of a T-cell line derived from a diabetic pancreas were studied, a strong adherence to the endothelium of diabetic pancreas and mucosal lymphoid tissue but weak binding to endothelium of peripheral lymph nodes and normal pancreas was observed (**Hänninen A et al., 1993**).

The study by Paronen et al. have demonstrated that GAD-reactive lymphocytes in the patients with newly diagnosed T1D express the gut-specific homing receptor $\alpha_4\beta_7$ integrin, whereas tetanus toxoid reactive lymphocytes expresses low levels of $\alpha_4\beta_7$ (**Paronen J et al., 1997**). Accordingly, aberrant finding in gut immuno-histology have been reported in T1D by Savilahti et al. (**Savilahti E et al., 1999**). The result of their study suggest that the structurally normal

intestine of the patients with T1D shows a stage of immune activation. The densities of T-cell subtypes were similar in patients and controls, but the patients had increased intensity of $\alpha_4\beta_7$-expressing cells in the lamina propria. The finding were not restricted to the patients who carried the HLA DQB1*02 allele, suggesting that the activation of the gut immune system may be associated with T1D and does not associated only with genetic risk allele shared with celiac disease.

There are also several indications that increased gut permeability may contribute to the development of diabetes. Abnormally increased gut permeability was reported in newly diagnosed patients. The studies on intestinal permeability in T1D are mostly based on the oral lactulose/mannitol test in which the ingested sugars are measured in urine. As the high levels of lactulose/mannitol are not found in all patients with T1D, the increased permeability may not be directly associated with the hyperglycemia-induced alterations in the mucosa, but could reflect a true aberrancies in these patients (**Secondulfo M et al., 2004**). Meddings et al. (**Meddings JB et al., 1999**) reported similar finding in studies on BB rats.

The increase in permeability appears to occur via the paracellular route and could be related to changes in tight junction complexes (**Neu J et al., 2005**).

An important first direct demonstration that the gut leakiness is associated with the development of diabetes was reported recently. A novel protein, Zonulin, modulates intestinal permeability by

dissembling the intracellular tight junctions (TJs). This protein is most likely involved in the innate immunity of the gut (**El Asmar R et al., 2002**) and appears to play a key role in the pathogenesis of autoimmune diseases. Endogenous zonulin protein, increased by 6 to 35 fold in the BB rat intestine during the development of insulitis and blocking this response with an inhibitor of zonulin receptor, which closes tight junctions and decreases gut leakiness, prevented diabetes (**Watts T et al., 2005**). This result is consistent with the finding that gut damage and increased permeability predate insulitis in BB rats (**Graham S et al., 2004)**. Gut permeability could also have direct effects on islet metabolism as it was correlated with impaired glucoregulation in diabetes-prone BB rats (**Malaisse WJ et al., 2004**). Furthermore, gut permeability was decreased significantly in hydrolyzed casein fed BB rats (**Courtois P et al., 2005; Malaisse WJ et al., 2004**), a finding that suggests that gut leakiness could be modified by diet.

Moreover, Clemente et al. recently reported a link between enterocyte gliadin exposure and zonulin release. The result of this study indicate that gliadin activated the zonulin signaling pathway in normal intestinal epithelial cells in vitro. The cellular response observed only a few minutes after gliadin incubation was characterized by significant cytoskeleton reorganization with a redistribution of actin filaments mainly in the intracellular subcortical compartment (**Clemente MG et al., 2003**).

The study by Sapone et al. showed that a large subgroup of T1D has high serum zonulin level that correlated with increased

intestinal permeability (**Sapone A et al., 2006**). This study also provided preliminary evidence that, like in the BB rat model of the disease, zonulin upregulation precedes the diagnosis of the disease in T1D patients.

The increased permeability of the mucosal barrier leads to increased exposure of intestinal immune cells to antigenic load, which may change the functional stage of the immune system and break tolerance to oral antigens.

In healthy individuals, the gut immune system dampens the immune response to dietary antigens, inducing a state of immunological non-responsiveness, known as oral tolerance (**Mowat AM, 2003**). The colonization of gastrointestinal track with the normal microflora is a necessity for the development of oral tolerance (**Vaarala O, 2003**). The composition of intestinal microflora varies between individuals and some disease-related differences have been demonstrated (**Bjorksten B et al., 2001**). Vaarala et al. have started the PRODIA study in which the use of probiotics, health-promoting intestinal bacteria, are tested in the prevention of β cell autoimmunity in children at genetic risk of T1D (**Ljungberg M et al., 2006**). It is also evident that the composition of intestinal microflora differs between populations (**Bjorksten B et al., 1999**). The changes in the microflora of children with time may be associated with the changes in the incidence of T1D with time and the differences between the intestinal microflora between the populations may explain the

differences in the incidence of T1D between different populations with similar genetic background.

It seems evident that patients with T1D show aberrancies of the gut immune system, which could favour the deviated immune response to oral antigens.

Background and Aims

T1D is considered a T-cell mediated autoimmune disease in which autoreactive T lymphocytes infiltrate the islets of pancreas and destroy the insulin-producing β-cell population. The origin of autoimmunity leading to the destruction of β-cell is not known. Several studies suggest that exists a link between the gut immune system and the islets infiltrating lymphocytes. Inflamed pancreatic islets express the same adhesion molecules (MAdCAM-1) involved in the homing of lymphocytes to the gut (**Hanninen A et al., 1993**). The manifestation of autoimmune diabetes in the animal models can be modified by dietary factors, which cause changes in the cytokine production by islet infiltrating lymphocytes.

In the patients with newly diagnosed T1D was found anti-glutamate decarboxylase (GAD)–reactivity in the subpopulation of lymphocytes expressing the gut-associated homing receptor $\alpha_4\beta_7$ (**Paronen J et al., 1997**). The $\alpha_4\beta_7$-expression on lymphocytes reactive with islet cell antigen suggests that autoreactive lymphocytes show homing properties to the intestinal lymphoid tissues and emphasized the role of the gut immune system in the development of T1D.

Patients with T1D are at a high risk of celiac disease (CD) and the risk seems to be correlated with the duration of gluten exposure (**Ventura A et al., 1999**).

A wheat-T1D link is suggested by the high prevalence of celiac disease in patients with T1D (1%-16%) (**Collin P et al., 2002**) compared with the general population (0,4%-1%). In patients diagnosed with both diseases, the majority develop T1D first, suggesting that the standard treatment for celiac disease, a strict gluten-free diet, may decrease the risk of diabetes if implemented in the prediabetic period (**Ventura et al., 2000**). In addition, offspring of parents with T1D develop antibodies associated with celiac disease (**Hummel M et al., 2000**), all this findings could reflect shared genetic risk for T1D and CD or gut barrier dysfunction or wheat-specific immune activation.

The aim of the study was to identify a possible link between the gut immune system and T1D; to this end we compared the expression of the integrin β_7 on peripheral blood activated and memory T lymphocytes in children affected by T1D and CD and in healthy children (**Study n°1**).

In a second phase we compared the specific T cell immune response to gluten of T1D, CD patients and healthy children (HC), and we tested the expression of the integrin β_7 on activated and memory T cells specific to gliadin in all groups **(Study n°2**).

Methodology

Subjects

Twenty-five children with T1D, 15 with CD and 22 age-matched healthy controls, attending the Department of Pediatrics of Tor Vergata University of Rome were enrolled in the study. T1D patients (14 boys and 11 girls) were diagnosed according to the definition of the American Diabetes Association 1997 (**The Export Committee on the diagnosis and Classification of T1D, 1997**). The mean age was 10.6 years (range 3.4 to 19.6 yrs) with mean duration of T1D 1.8 years (range 0.1–7 yrs). All of them were negative for celiac disease (anti-endomysial and anti-human tissue transglutaminase antibodies), thyroid disorders and atrophic gastritis.

CD patients (3 boys and 12 girls) were diagnosed according to the definition of the ESPHGAN 1990 (**Walzer-Smith JA et al., 1990**). The mean age was 6.1 years (range 2.2 to 13.3 yrs) and all were on a gluten-containing diet and negative for other autoimmune diseases.

The healthy children (9 boys and 13 girls) had a mean age of 10.2 years (range 3.5 to 17 yrs) and were negative for anti-endomysial and anti-human tissue transglutaminase antibodies.

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.

None of the patients who were enrolled in the study showed gastrointestinal symptoms at the moment of the blood withdraw, or was affected by a concomitant autoimmune or allergic disease.

Gliadin Preparation

The alcohol-soluble protein fraction from whole cereal flour of bread wheat (*Triticum aestivum*, variety S. Pastore) was kindly provided by Dr. De Vincenzi of the Division of Human Nutrition and Health, Istituto Superiore di Sanità, Rome, Italy.

Extraction and peptic-tryptic digestion were performed as previously described (**De Ritis G et al.**, **1979**). Gliadin preparations were assayed for endotoxin by using the QCL-100 reagent kit (BioWhittaker, Walkersville, MD, USA) and found to have endotoxin levels of <0.5 EU/ml.

To deamidate gliadin peptides, the peptic-tryptic digest was incubated for 4 h at 37°C in a mix consisting of 100 µg/ml guinea pig liver tTG (Sigma chemical Co, St. Louis, MO) in PBS with 2nM CaCl at the concentration of 400 µl. These concentrations of tTG and $CaCl_2$ were found to give optimal T-cell stimulation and are comparable to that described in other system.

DQ Determination

The HLA-DQA and DQB genotype of all subjects was determinated in peripheral blood DNA using PCR with sequence-specific primer mixes.

Peripheral Blood Mononuclear Cell (PBMC)Cculture Conditions

PBMC were separated from heparinized blood by Ficoll-Hypaque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden**)** and were plated on 96 well at the cell concentration of $1x10^6$ with medium containing 10% FCS, streptomycin (50 ng/ml, GIBCO-Invitrogen) and penicillin (100 µg/ml, GIBCO-Invitrogen).

In study n°1: PBMC were cultured in triplicate with medium alone or with OKT3 (1:10, supernatant of hybridoma ATCC) (Sigma, Milan, Italy) and anti-CD28mAb (0.15 µg/ml**,** BD Pharmigen, San Diego, California, U.S.A) for 18 hours.

In study n°2: PBMC were cultured in triplicate with medium alone, with the peptic-tryptic digest of gliadin (PTG), with transgluatminase-treated (TG)-PTG at increasing concentrations (50 µg/ml, 100 µg/ml, 500 µg/ml, 1000 µg/ml) and with tetanus toxoide (TT) (1:50 of Lot 48 UBT 1348) (Biocine Chiron, Siena, Italy), as a control antigen, for 5 days.

In parallel, PBMC were incubated with OKT3 (1:10, supernatant of hybridoma) (ATCC) and anti-CD28mAb (0.15 µg/ml**,** BD Pharmigen) for 3 days.

Proliferation was assessed on day 5 after overnight pulse with 0.5 µCi/well [^{3}H]Tymidine (Amersham International, Amersham U.K.). Proliferation was expressed as a stimulation index (SI): mean counts per min (cpm) incorporated in the presence of antigen divided by mean cpm incorporated in the absence of antigen (medium value) and responses were considered positive when the stimulation index (SI) was higher than controls average + 1 SD.

Analysis of PBMC Surface Phenotype by Flow Cytometry

Cells were stained according to standard protocols with mouse anti-human monoclonal antibodies to β_7-PE, CD3-APC, CD4-FITC, CD8-FITC, CD69-CyChrome, CD45RO-FITC, CD4-Cy5, CD8-Cy5, CD4-APC and CD3-CyChrome (BD Pharmingen) and analysed by multiparametric flow cytometry. A minimum of $1X10^4$ events for each sample was acquired with a flow cytometer (FACSCalibur, Beckton Dickinson, San Jose, CA, USA) interfaced with Cellquest software (Beckton Dickinson). The expression of β_7 was measured as percent of positive cells and as mean of fluorescence intensity (MFI). To evaluate β_7 modification after OKT3 and anti-CD28mAb stimulation, surface protein expression levels were also expressed as differential mean fluorescence intensity (ΔMFI) calculated as: (mean fluorescence intensity of the untreated cells) – (mean fluorescence intensity of the cells treated with OKT3 and anti-CD28mAb).

Cytokine Production Assay

Culture supernatants were harvested and IFN-γ and IL-4 concentrations were determined by Enzyme-Linked Immunosorbent Assay (Human IFN-γ and IL-4 ELISA, Endogen, Woburn, MA, USA) according to manufacturer's instructions. The detection limit was 15 pg/ml.

Statistical Analysis

Data are expressed as mean ± SD or median (1^{st}-3^{rd} quartile), as appropriate.

Interval scale variables were tested for normality by the Kolmogorov-Smirnov method.

Since the variables analysed in each of the 3 groups were normally distributed the differences of MFI were compared with Student's *t* test. When found statistically significant they were further analysed with the Mann-Whitney's *U* non-parametric test.

The frequencies of responders and non responders between the study groups were compared with X^2 test.

The SPSS 13.0 analysis software was employed.

Values of $p<0.05$ were considered statistically significant.

Experimental studies

Study n°1: Expression of Integrin β_7 on T Cell Subsets of T1D and CD Patients and Healthy Children

Patients

Peripheral blood mononuclear cells (PBMC) were isolated from 6 children with T1D, 5 with CD and 6 healthy children (mean age 10.9, range 7.2 to 20 yrs). T1D patients were 3 boys and 3 girls, the mean age was 11.6 years (range 7.9 to 19.7 yrs) and the mean duration of T1D was 4.4 years (range 0.7-7 yrs).

CD patients were 1 boy and 4 girls and the mean age was 7.4 years (range 3.7 to 13.3 yrs).

Results

We investigated the expression of integrin β_7 on resting T cell subsets obtained from T1D and CD patients and from healthy controls. Since $\alpha_4\beta_7$ mediates the adhesion to MAdCAM-1, but $\alpha_4\beta_1$ does not, we used the expression of β_7 integrin as a marker for T cells with gut-trafficking potential. The β_7 integrin is expressed together with the α_4 integrin on > 99% of peripheral blood T cells (**Raki M et al.; 2007**) indicating that these cells home to the gut. We also examined β_7 expression on $CD69^+$ (a marker of early T cell activation) and $CD45RO^+$ (a marker of memory T cells) T cells. The expression of β_7 was measured as percent of positive

cells and as MFI. There were no differences in the percent of positive cells detected in the 3 groups (data not shown) and the values as MFI are reported in table 1.

TABLE 1. Expression of β_7 integrin on resting T lymphocytes in children with type 1 diabetes (T1D), celiac disease (CD) and healthy controls (HC).

Subsets of T lymphocytes	**T1D**	**CD**	**HC**	***p***
CD3+β_7+	91.8±20.5	108.4±17.7	98.4±17.3	n.s.
CD3+CD4+β_7+	68.9±13.1	86.1±16.8	76.5±8.1	*p*=0.048***
CD3+CD8+β_7+	103.2±15	118.8±36.2	117.9±19.6	n.s.
CD3+CD4+CD69+β_7+	104.3±17.6	151.5±15.2	97.8±24.2	*p*=0.001* *p*=0.004***
CD3+CD8+CD69+β_7+	110.4±9.7	162.4±35.5	112.3±26.7	*p*=0.017* *p*=0.014***
CD3+CD4+CD45RO+β_7+	142.2±25.4	165.3±6.7	119.9±13	*p*=0.005* *p*=0,047**
CD3+CD8+CD45RO+β_7+	103.1±14.2	132.9±7.8	103.1±17.2	*p*=0.01* *p*=0.01***

*CD *vs*. HC; **T1D *vs*. HC; ***CD *vs*. T1D. n.s.: non significant.

Resting T cells of patients with CD showed, compared with healthy controls, a significantly higher expression of β_7 on both memory and activated $CD4^+$ and $CD8^+$ T lymphocytes (**Tab. 1**).

Resting T cells of patients with T1D showed, compared with healthy controls, a higher expression of β_7 on memory $CD4^+$ T lymphocyte ($CD3^+CD4^+CD45RO^+\beta_7^+$, MFI: T1D 142.2±25.4 *vs*. HC 119.9±12.9 p=0.047) (Fig. 4), but not on $CD8^+$ T cells. No differences were observed for $CD69^+$ T cells in T1D patients.

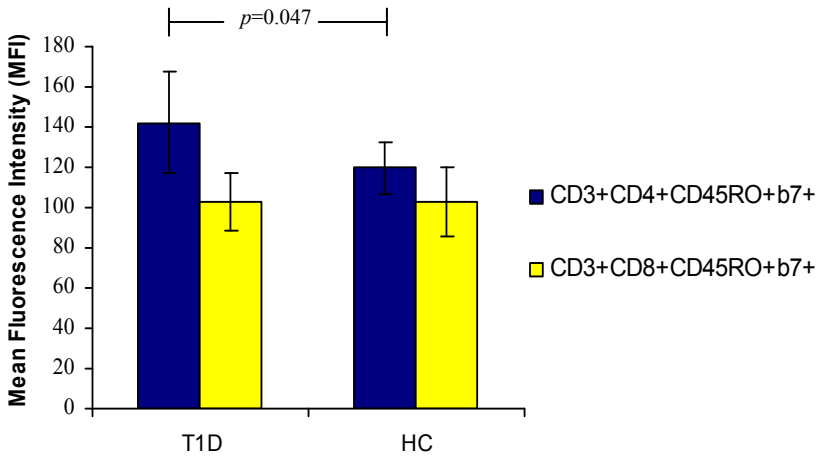


FIGURE 4. Increased expression of β_7 integrin on memory $CD4^+$ (blue bars) but not in $CD8^+$ (yellow bars) T lymphocytes in children with type 1 diabetes (T1D) compared with healthy controls (HC). Freshly isolated PBMC were cultured un-stimulated for 18 hours. At the end of culture, the cells were harvested and analyzed by flow cytometry. Average ± SD are reported.

When T lymphocyte were stimulated with OKT3 and anti-CD28mAb, they displayed a significant decrease of β_7 expression, compared to un-stimulated T cell, on all subset of T lymphocytes in patients with T1D, CD and in HC (**Fig. 5**).

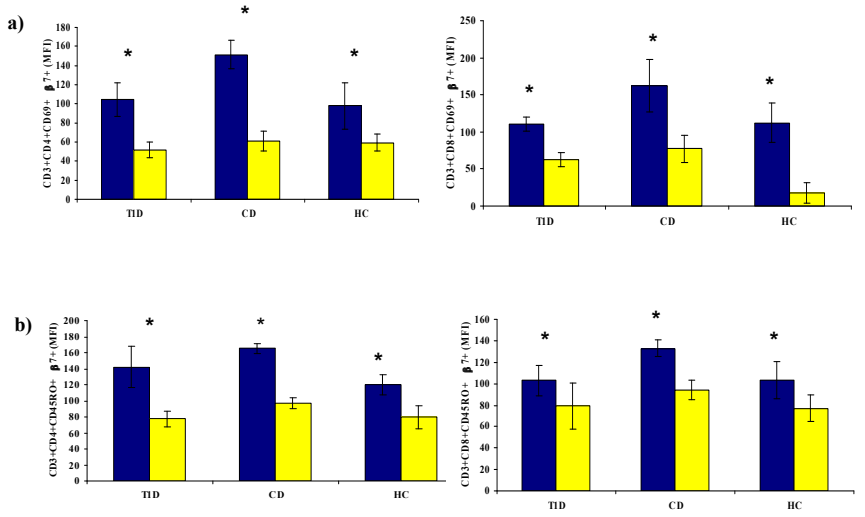


FIGURE 5. Decrease of β_7 integrin expression on activated (**a**) and memory (**b**) $CD4^+$ and $CD8^+$ T lymphocytes after stimulation with OKT3 and anti-CD28mAb in children with type 1 diabetes (T1D), celiac disease (CD) and healthy controls (HC). Freshly isolated PBMC were cultured un-stimulated (blue bars) and stimulated with OKT3 and anti-CD28mAb (yellows bars) for 18 hours. At the end of culture, the cells were harvested and analyzed by flow cytometry after staining with anti-CD3, anti-CD4, anti-CD8, anti-CD69, anti-CD45RO and anti-β_7 mAbs. Average MFI ± SD are reported. *p<0.05

Nevertheless, we did not observe significant differences in expression of β_7 in stimulated T cells between the 3 groups analyzed (**Tab. 2**).

Interestingly after stimulation, β_7 expression on activated CD4+ T lymphocyte was significantly reduced in patients with CD compared with both healthy controls and T1D patients (ΔMFI: CD 90.8±16.5 *vs.* HC 38.6±26.5, p=0.002; CD 90.8±16.5 *vs.* T1D 52.7±12.9, p=0.02) (**Fig. 6**).

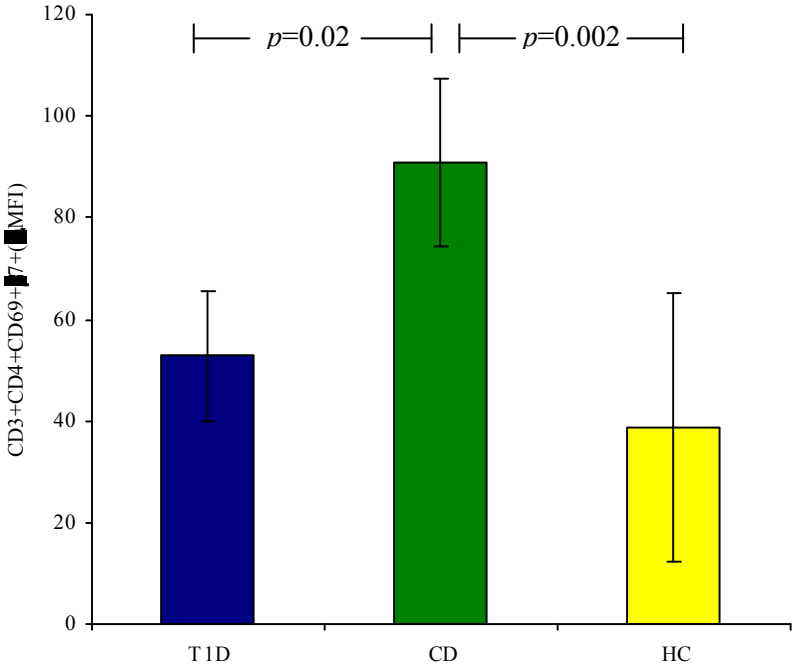


FIGURE 6. Reduction of β_7 integrin expression on $CD4^+$ activated T lymphocytes after stimulation with OKT3 and anti-CD28mAb in children with type 1 diabetes (T1D), celiac disease (CD) and healthy controls (HC). Freshly isolated PBMC were triggered by OKT3 and anti-CD28mAb and cultured for 18 hours. At the end of culture, the cells were harvested and analyzed by flow cytometry after staining with anti-CD3, anti-CD4, anti-CD69 and anti-β_7 mAbs. Average ΔMFI ± SD are reported. ΔMFI was calculated as following: mean fluorescence intensity of the untreated cells – mean fluorescence intensity of the cells treated with OKT3 and anti-CD28mAb.

With respect to activated $CD8^+$ T cells we also observed a significant reduction of β_7 expression in CD patients compared with HC [Median= 68.22 (55-105.2) *vs.* HC 44.2 (21.9-57.4), respectively p= 0.045].

TABLE 2. Expression of integrin β_7 on T lymphocytes stimulated with OKT3 and anti-CD28mAb in children with type 1 diabetes (T1D), celiac disease (CD) and healthy controls (HC).

Subsets of T lymphocytes	**T1D**	**CD**	**HC**	***P***
CD3+β_7+	67.7±10	75±15.1	73.9±13.2	n.s.
CD3+CD4+β_7+	53.9±7.3	60.1±14.7	62.5±4.3	n.s.
CD3+CD8+β_7+	68.9±10.7	76.5±30.5	75±12.6	n.s.
CD3+CD4+CD69+β_7+	51.6±8.5	60.7±10.3	59.2±8.9	n.s.
CD3+CD8+CD69+β_7+	62.5±9.8	77.2±18.1	71.8±13.6	n.s.
CD3+CD4+CD45RO+β_7+	77.3±10.1	97.4±6.6	79.8±14.2	n.s.
CD3+CD8+CD45RO+β_7+	79.2±21.2	94.2±9.1	77.1±12.1	n.s.

n.s.: non significant

Furthermore, we found that β_7 expression on memory $CD4^+$ T lymphocyte was significantly reduced in patients with T1D and

with CD compared with healthy controls (ΔMFI: T1D 56.5±9.6 *vs*. HC 40±8.5, *p*=0.041; CD 68±0.2 *vs*. HC 40±8.5, *p*=0.011) (**Fig. 7**).

On the other hand we did not find significant differences in the reduction of β_7 expression on memory $CD8^+$ T lymphocytes in all 3 groups.

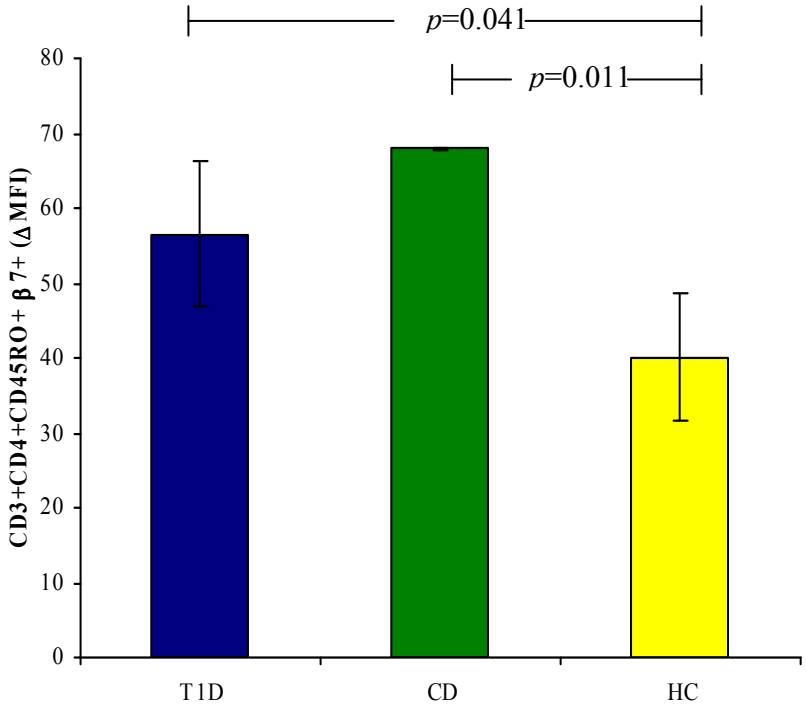


FIGURE 7. Reduction of β_7 integrin expression on $CD4^+$ memory T lymphocytes after stimulation with OKT3 and anti-CD28mAb in children with type 1 diabetes (T1D), celiac disease (CD) and healthy controls (HC). Freshly isolated PBMC were triggered by OKT3 and anti-CD28mAb and cultured for 18 hours. At the end of culture, the cells were harvested and analyzed by flow cytometry after staining with anti-CD3, anti-CD4, anti-CD45RO and anti-β_7 mAbs. Average ΔMFI ± SD are reported. ΔMFI was calculated as following: mean fluorescence intensity of the untreated cells – mean fluorescence intensity of the cells treated with OKT3 and anti-CD28mAb.

Study n°2: Immune reactivity to gliadin in children with type 1 diabetes.

Patients

PBMC were isolated from 19 children with T1D, 10 children with CD and 17 healthy controls (mean age 10.3, range 3,5 to 18 yrs). T1D patients (11 boys and 8 girls) had a mean age of 10.3 years (range 3.4 to 15.6 yrs) with mean duration of T1D 1 year (range 0.1-6.2 yrs).

CD patients (2 boys and 8 girls) had a mean age was 5.4 years (range 2.2 to 11.2 yrs).

Results

To set up conditions to promote gliadin specific T cell, we performed culture experiments using PBMC pulsed for 3, 5 and 7 days with different concentrations of PTG and TG-PTG (range from 50 to 1000 µg/ml). We observed a time course and a dose-response curve with the maximal proliferation at the concentration of 100 µg/ml on day 5. These culture conditions were used in all subsequent experiments. However, the gliadin preparation and concentration which gave the highest response varied from individual to individual.

Proliferation was expressed as a stimulation index (SI): mean counts per min (cpm) incorporated in the presence of antigen divided by mean cpm incorporated in the absence of antigen (medium value) and responses were considered positive when the

stimulation index (SI) was higher than controls average +1SD (Cut-off: 1.9 for the stimulation with PTG and 2.1 for the stimulation with TG-PTG).

When PBMC were pulsed with peptic-tryptic digest of gliadin (PTG) we found a positive proliferative response to 100 μg/ml PTG in 7 out of 11 T1D patients (64%), in 3 out of 14 healthy controls (21.5%) and in 6 out of 10 CD (60%) (T1D *vs.* HC p=0.03, CD *vs.* HC p= 0.05).

The mean SI in T1D patients was higher than in healthy controls (2.9±2 *vs*.1.3±0.6, respectively, p=0.02) (**Fig. 8**).

We did not observe significant differences in proliferative response to gliadin between patients with recent onset and patients with long duration of the disease (data not show).

The mean SI in the CD patients group was also higher than in healthy controls (3.3±3.9 *vs*. 1.3±0.6, respectively) but this difference did not reach statistical significance (**Fig. 8**).

In 5 patients we analyzed the proliferative response to both PTG and transgluatminase-treated peptic-tryptic digest of gliadin (TG-PTG) and we found that 3 out of 5 T1D patients (60%) responded to both antigens (data not show).

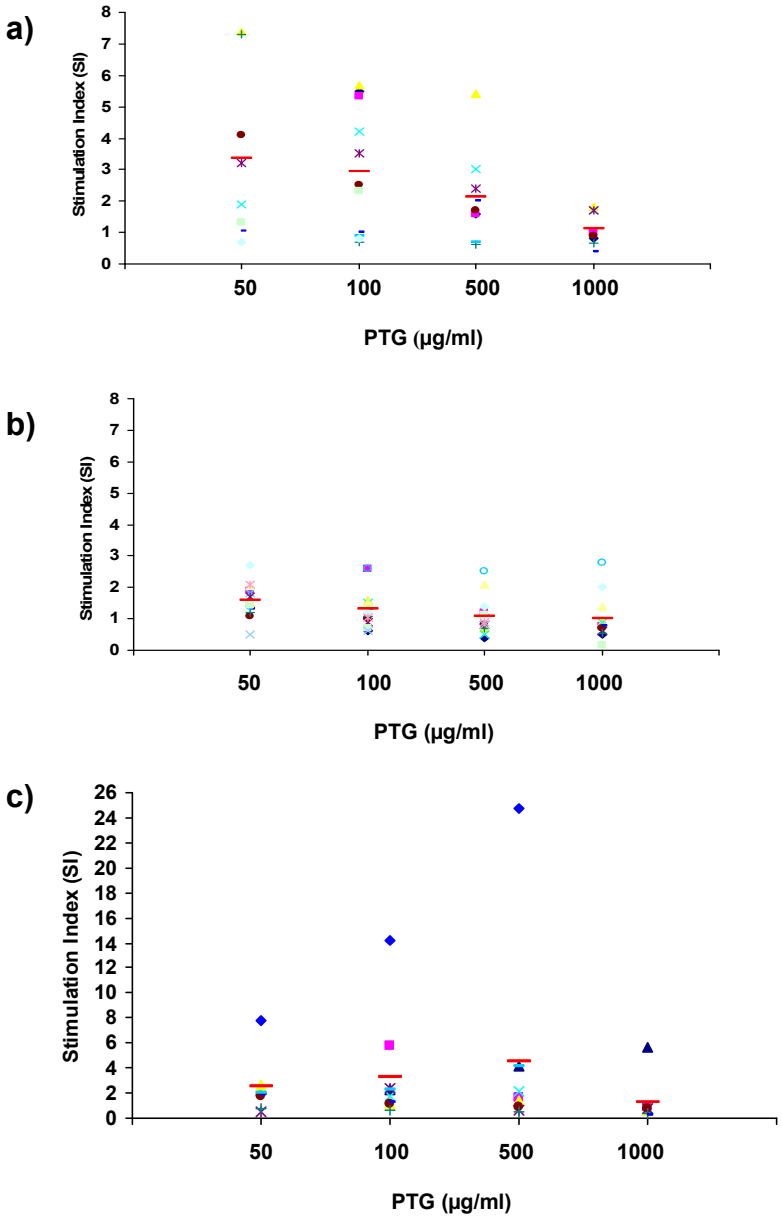


FIGURE 8. Proliferative response of PBMC in the presence of peptic-tryptic digest of gliadin (PTG) in patients with type 1 diabetes (T1D) (**a**), in healthy controls (HC) (**b**), and in patients with celiac disease (CD) (**c**). Freshly isolated

PBMC were cultured with medium alone, and with the PTG at increasing concentrations (50 µg/ml, 100 µg/ml, 500 µg/ml, 1000 µg/ml) for 5 days.
Proliferation was assessed on day 5 after overnight pulse with [^{3}H]Tymidine. Proliferation was expressed as a stimulation index (SI): median counts per min (cpm) in the presence of antigen divided by cpm in the absence of antigen (medium value) and the horizontal bars represent the means.

When PBMC were pulsed with 100µg/ml TG-PTG we found a positive proliferative response in 7 out of 13 T1D patients (54%), in 2 out of 7 healthy controls (28.6%), and in 2 out of 3 CD patients (67%) (T1D *vs*. HC, *p*=n.s.; CD *vs*. HC, *p*=n.s.).

The mean SI in the T1D patients and in CD patients group were higher than in healthy controls (T1D 2.4±2 *vs*. HC 1.5±0.7; CD 9.2±11.6 *vs*. HC 1.5±0.7), but this differences did not reach statistical significance.

Wherever possible, we used Tetanus Toxoide (TT) as a control antigen and when PBMC from T1D patients (n=5) were pulsed with TT the average SI was lower than that obtain with TG-PTG (SI: 1.8±1.4 *vs*. 2.4±2, respectively), and lower in T1D patients than for the healthy controls (n= 2) (SI: 1.8±1.4 *vs*. 9.9±12.7, respectively) (**Fig. 9.a**).

Interestingly, in PBMC incubated in parallel with OKT3 and anti-CD28mAb (n=8) for 3 days to check the vitality of the cells, we observed a lower proliferative response in patients with T1D than in HC (n=3) (SI: 78±58.2 *vs*. 168.9±53.6, respectively *p*=0.07) (**Fig. 9.b**). Therefore, T cell proliferation to a control antigen and to a polyclonal stimulys was found to be defective in T1D patients compared to healthy, age-matched, children.

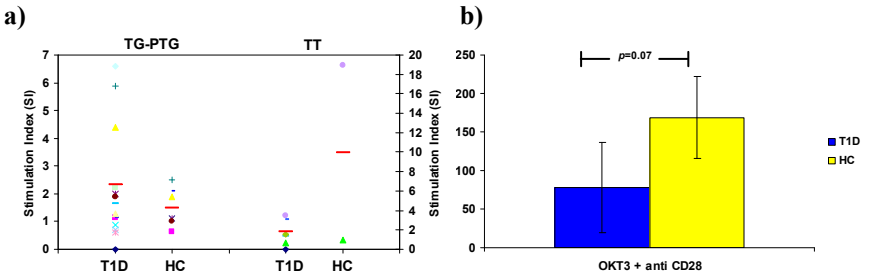


FIGURE 9. Proliferation response of PBMC in the presence of TG-PTG in patients with type 1 diabetes (T1D) and in healthy controls (HC). Freshly isolated PBMC were cultured with medium alone, with 100 µg/ml TG-PTG and with tetanus toxoide (TT) for 5 days (**a**). On day 2, part of PBMC left un-stimulated were incubated with OKT3 and anti-CD28mAb for the remaining 3 days (**b**).

Proliferation was assessed on day 5 after overnight pulse with [^{3}H]Tymidine. Proliferation was expressed as a stimulation index (SI): median counts per min (cpm) incorporated in the presence of antigen divided by cpm incorporated in the absence of antigen (medium value) and responses were considered positive when the stimulation index (SI) was higher than control average +1SD.
In (**a**) the horizontal bars represent the mean. In (**b**) average SI±SD are reported.

We performed HLA DQ typing in all patients with T1D. Distribution of SI upon gliadin stimulation in subjects with HLA-DQ2(A1*0501), DQ8(A1*0301), DQ2/DQ8 or with none of these risk allele is show in figure 10.

We observed that the mean SI to both type of gliadin was higher in individuals with HLA DQ8$^+$ or DQ8$^+$/DQ2$^+$ than in individuals with HLA DQ2$^+$ or with none of the risk HLA molecules, but this difference did not reach statistical significance (SI for TG-PTG: HLA DQ2$^+$ 2±1.9, DQ8$^+$/DQ2$^+$ 3.3±1.5, DQ8$^-$/DQ2$^-$ 1.1±0.7; SI for PTG: HLA DQ2$^+$ 1.25±0.7, DQ8$^+$ 4.6±1.6, DQ8$^+$/DQ2$^+$ 3.8±2.7).

We did not observe significant differences in the mean SI upon TG-PTG stimulation between individuals with HLA DQ2$^+$ and

individuals with none of the risk HLA alleles.

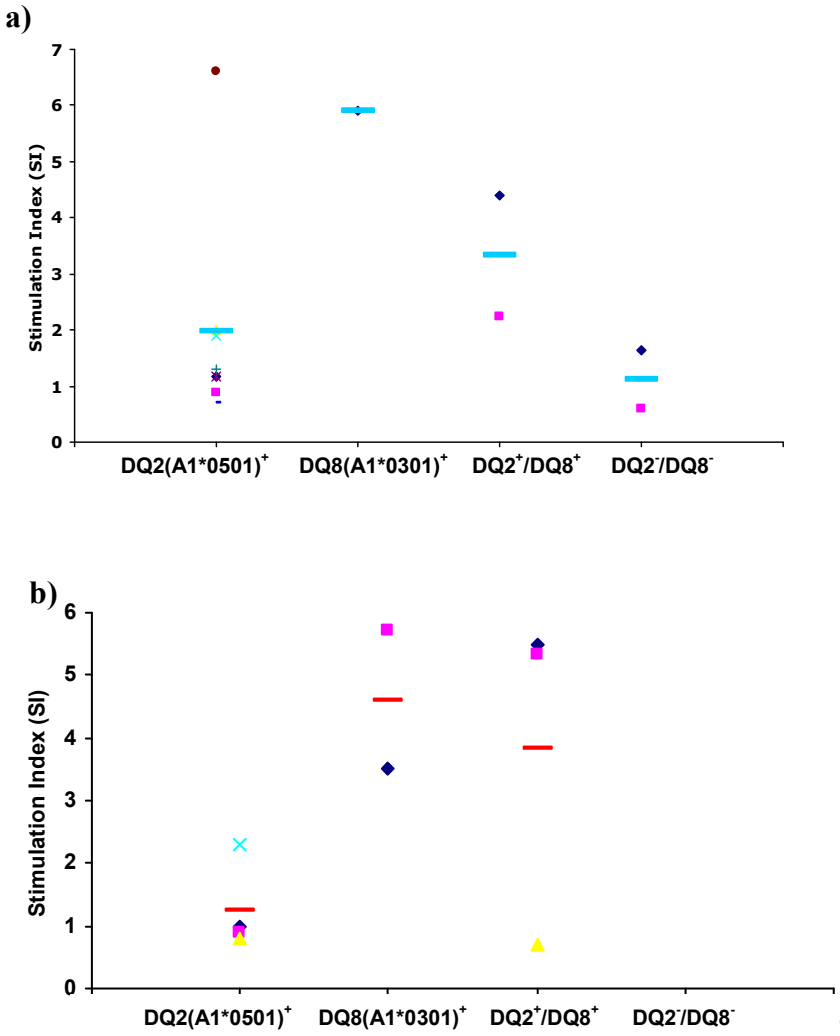


FIGURE 10. PBMC proliferation to gliadin distributed according to HLA DQ in patients with type 1 diabetes. Average SI of each group are marked with horizontal lines. PBMC cultured with 100 µg/ml TG-PTG (**a**) and with 100 µg/ml PTG (**b**).

We then analyzed the cytokine profile of T cells after 5 days of culture in presence of TG-PTG, IFN-γ and IL-4 were determined in culture supernatants by ELISA (**Tab. 3**). Detectable amounts of IFN-γ were observed in 3 out of 7 T1D patients with a positive response to gliadin (43%), in none of the non-responders patients (n=4), in 1 out of 2 CD patients (50%), but not in healthy controls (n=5).

Tabella 3. IFN-γ and IL-4 production in patients with type 1 diabetes (T1D), with celiac disease (CD) and in healthy controls (HC).

Subjects	IFN-γ (pg/ml)				IL-4 (pg/ml)			
	UNST	TG-PTG 50 µg/ml	TG-PTG 100 µg/ml	TG-PTG 250 µg/ml	UNST	TG-PTG 50 µg/ml	TG-PTG 100 µg/ml	TG-PTG 250 µg/ml
T1D14	0		0					
T1D15	0		0					
T1D16	0		26,2	55,5	0		0	0
T1D17	0	31,5	28,3	90	0	0	0	0
T1D18	0		0		0		0	
T1D19	0		0		0		0	
T1D20	51		0		0		0	
T1D21	0		73		0		0	
T1D22	0		0		0		0	
T1D23	0		0		0		0	
T1D25	0		0					
HC16	0		0					
HC17	0		0					
HC19	0		0		0		0	
HC20	0		0		0		0	
HC21	0		0		0		0	
CD14	0		0					
CD15	0		46,2					

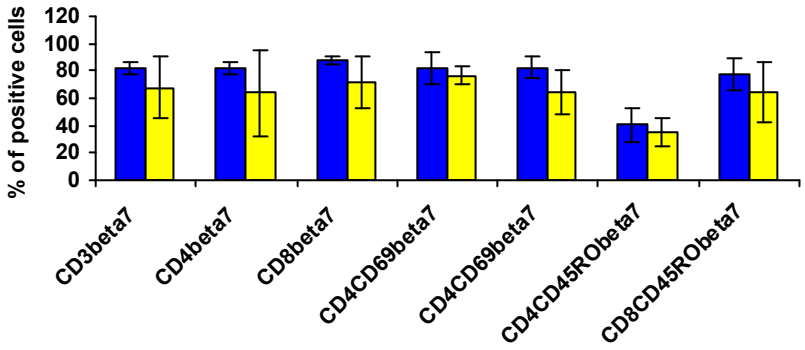
In addition none of 7 T1D patients with a proliferative response to gliadin produced IL-4. These data indicate that gliadin-specific T cell response is Th1 like.

We investigated the expression of integrin β_7 on different subsets of resting T cell and of T cells stimulated with TG-PTG, obtained from 6 T1D patients and from 2 healthy controls.

In particular, we examined integrin β_7 expression on $CD69^+$ and $CD45RO^+$ T cells.

There were not differences in the percent of β_7^+ cells detected in the 2 groups (**Fig.11.a**). Nevertheless, resting T cells of patients with T1D showed, compared with healthy controls, a higher expression of β_7 on memory $CD4^+$ T lymphocyte ($CD3^+CD4^+CD45RO^+\beta_7^+$, MFI: T1D 123.1±35.6 *vs*. HC 87.6±36.3) (**Fig. 11.b**), although this difference did not reach statistical significance. No differences were observed for other subsets of T cells analyzed in term of percent of β_7^+ cells as well as MFI.

a)



b)

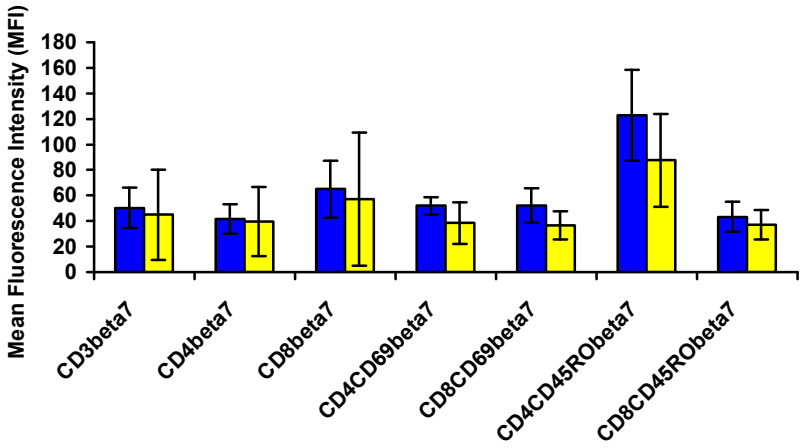


FIGURE 11. Expression of β_7 integrin on memory and activated $CD4^+$ and $CD8^+$ T lymphocytes at baseline in 6 children with type 1 diabetes (T1D, blue bars), and in 3 healthy controls (yellows bars). Freshly isolated PBMC were cultured with medium alone and with TG-PTG for 5 days. At the end of culture, the cells were harvested and analyzed by flow cytometry after staining with anti-CD3, anti-CD4, anti-CD8, anti-CD69, anti-CD45RO and anti-β_7 mAbs. The expression of β_7 was measured as percent of positive cells (**a**) as well as mean of fluorescence intensity (MFI) (**b**). Average ± SD are reported.

According to same reports indicating that a high expression of β_7 correlates strongly with T cell gut homing properties, we also

analyzed whether there is a population of cells expressing high levels of β_7 integrin within resting $CD3^+$ and $CD4^+$ T cell subsets obtained from T1D patients.

Indeed, resting T cells of patients with T1D (n=6) showed, compared with healthy controls, the presence of population β_7^{hi} on $CD3^+$ and $CD4^+$ T lymphocyte with higher MFI ($CD3^+\beta_7^{hi}$, MFI: T1D 263.5±123.4 *vs.* controls 175.3±42.4; $CD4^+\beta_7^{hi}$, MFI: T1D 250.3±130.1 *vs.* controls 148.9±47.8) (**Fig. 12**), although this difference did not reach statistical significance.

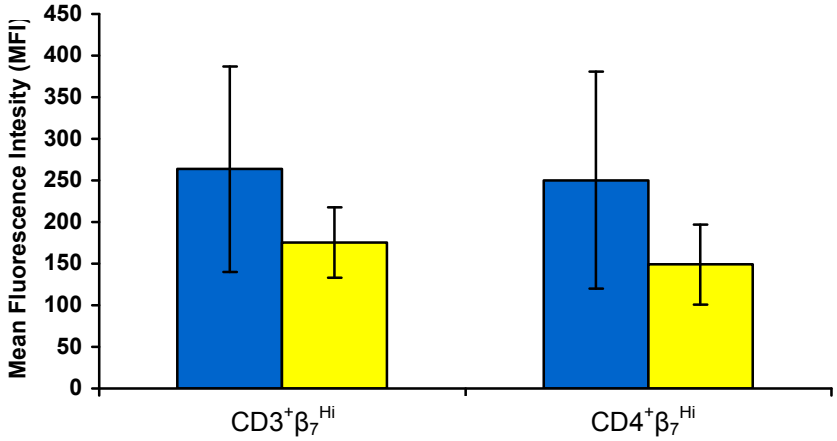


FIGURE 12. Expression of β_7^{hi} integrin on resting $CD3^+$ and $CD4^+$ in children with type 1 diabetes (blue bars, n=6), and healthy controls (yellows bars, n=3). Freshly isolated PBMC were cultured with medium alone and with TG-PTG for 5 days. At the end of culture, the cells were harvested and analyzed by flow cytometry after staining with anti-CD3, anti-CD4, and anti-β_7 mAbs. The expression of β_7 was measured as mean of fluorescence intensity (MFI). Average MFI ± SD are reported.

There were not differences in percent of β_7^{hi+} cells detected within the $CD3^+$ and $CD4^+$ population in the 2 groups (data not shown).

Moreover, we compared the expression of β_7^{hi} integrin on resting $CD3^+$ and $CD4^+$ cells in children with T1D with a positive response to gliadin (n=3) in T1D patients who did not respond to gliadin (n=3) and in healthy controls (n=2).

Resting T cells of T1D patients with a positive response to gliadin showed, compared with T1D patients who did not respond to gliadin and with healthy controls, an increase of β_7^{hi} on $CD3^+$ and $CD4^+$ T lymphocyte ($CD3^+\beta_7^{hi}$ MFI: T1D responders 318.9±197, T1D non responders 230.3±59.6 and HC 175.3±42.4; $CD4^+\beta_7^{hi}$, MFI: T1D responder 334.8±200, T1D non responder 199.6±32 and HC 148.9±47.8) (**Fig. 13**), althoug these differences did not statistical significance.

There were no differences in the percent of β_7^{hi+} cells detected in the 3 groups (data not shown).

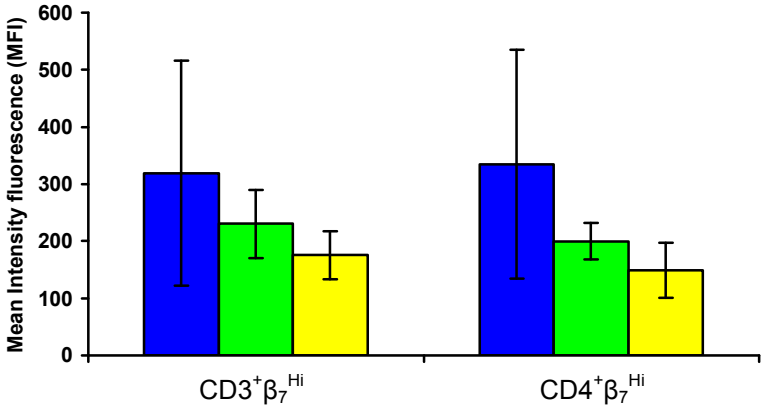
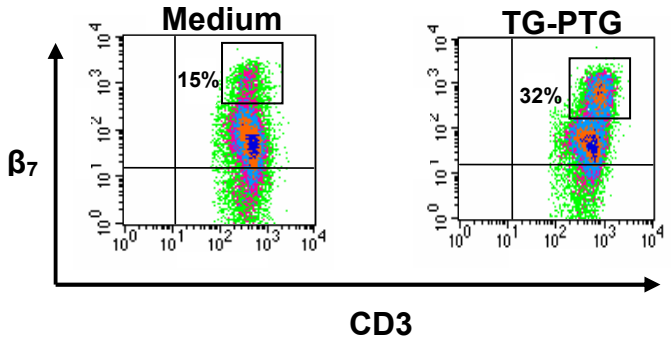


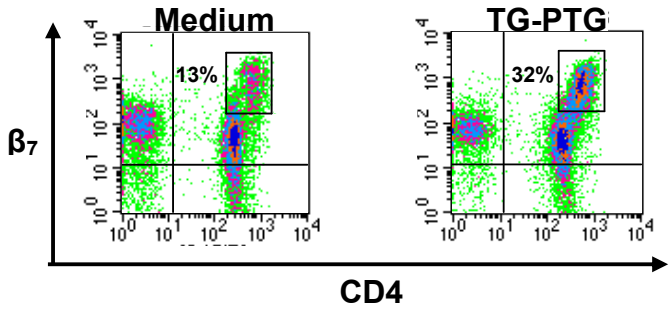
FIGURE 13. Expression of β_7^{hi} integrin on resting $CD3^+$ and $CD4^+$ in children with T1D responders to gliadin (blue bars, n=3), T1D non responders (green bars, n=3) and healthy controls (yellows bars, n=3). Freshly isolated PBMC were cultured with medium alone and with TG-PTG for 5 days. At the end of the culture, the cells were harvested and analyzed by flow cytometry after staining with anti-CD3, anti-CD4, and anti-β_7mAbs. The expression of β_7 was measured as mean of fluorescence intensity (MFI). Average MFI ± SD are reported.

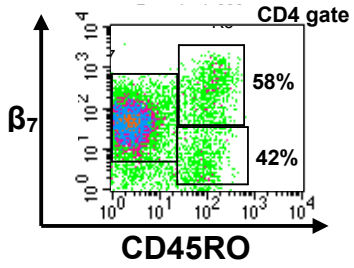
We also analyzed the expression of β_7^{hi} integrin on $CD3^+$ and $CD4^+$ cells after stimulation with TG-PTG. In 1 out of 3 patients tested, who responded to gliadin, we could identify a discrete population of $CD4^+\beta_7^{hi+}$ cells whose percent increased upon TG-PTG stimulation from 15% to 32% (**Fig. 14.a**), no increase was observed upon stimulation with TT (data not shown).

The fact that we detected a marked up-regulation of β_7 on $CD4^+$ T cells in only 1 patient is probably due to the law numbers of TG-PTG-specific T cells clone in the majority of T1D patients. Indeed, in this patient the percent of β_7^{hi+} T cells, at baseline, was higher than in the other T1D patients.

a)


Medium
TG-PTG
15%
32%
β7
CD3


Medium
TG-PTG
13%
32%
β7
CD4


CD4 gate
58%
42%
β7
CD45RO

b)

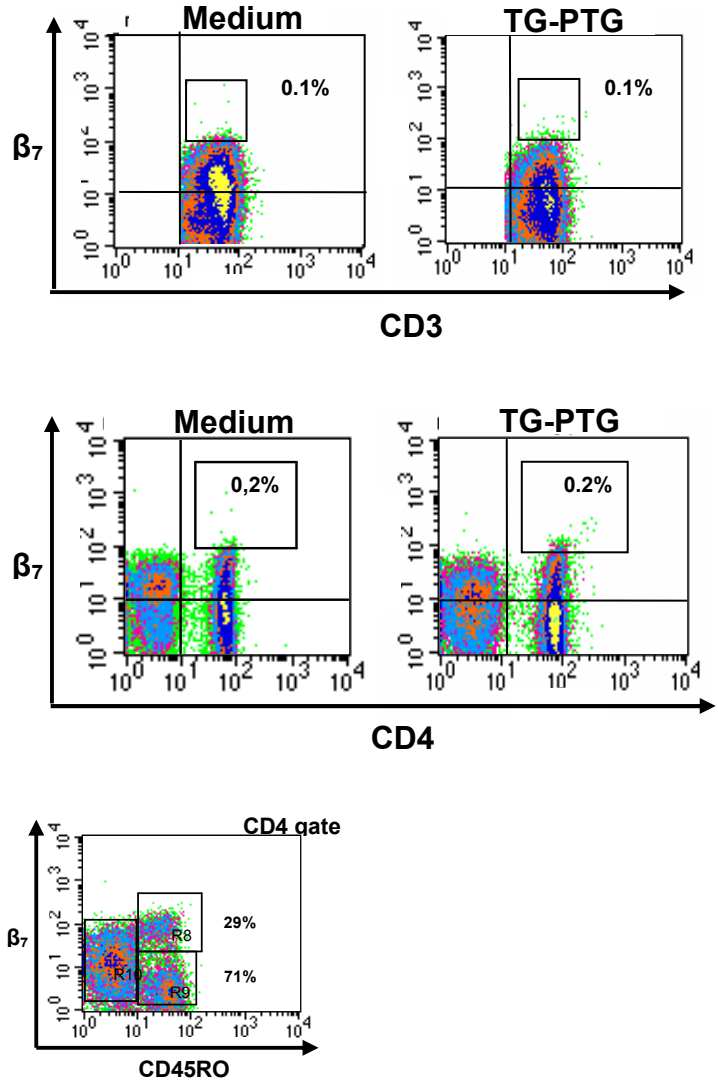


FIGURE 14. Presence of a population of $CD3^{+}\beta_7^{hi+}$ and $CD4^{+}\beta_7^{hi+}$ cells in 1 T1D patient (**a**) and in one healthy control (**b**). Freshly isolated PBMC were cultured with medium alone and with TG-PTG for 5 days. At the end of culture, the cells were harvested and analyzed by flow cytometry after staining with anti-CD3, anti-CD4, anti-CD45RO and anti-β_7 mAbs.

It was of interest to examine the pattern of expression of the β_7

chain in the PBMC. We found that freshly isolated circulating $CD4^+$ T cells consist of 3 distinct population with respect to β_7 expression: negative, low/intermediate, and high. It is interesting that these 3 populations are also distinct with respect to their co-expression of CD45RO. More precisely, the β_7 low/intermediate T cells were primarily $CD45RA^+$ (naïve) cells, whereas the $CD45RO^+$ (primed) T cells exclusively contained a subset of β_7^{hi} cells and a subset of β_7 negative cells.

Clinical Follow-up

During 4 years of follow-up, none of the 25 T1D patients enrolled in the study showed neither gastrointestinal symptoms nor positivity for autoantibody for celiac disease (anti-endomysial and anti-human tissue transglutaminase antibodies). None of them developed other autoimmune diasease.

Discussion and Concluding remarks

The results of this first part of this study indicate that T cells expressing high levels of β_7 integrin are detectable in peripheral blood of CD and T1D patients.

In particular, in CD patients the cells expressing β_7 are both $CD4^+$ and $CD8^+$ lymphocytes, according with the important role of both T cell subsets in the pathogenesis of the disease (**Sollid LM, 2002; Green PH, 2007**). Moreover, we found that β_7 integrin was expressed in both activated and memory T cells. The presence of memory T cells primed in the gut was not completely unexpected since in our study all children came to our observation after a variable period of symptomatic complaint.

This finding is in agreement with a recent report of Ben-Horin et al. (**Ben Horin S et al., 2006**), who demonstrated that a large percentage of CD patients on gluten-free diet has a discrete population of transglutaminated-gliadin specific $CD4^+$ memory T cells, characterized by the expression of high levels of β_7 integrin.

However, differently from this study, we observed a high expression of β_7 also on activated T cells. Since none of our patients was on gluten-free diet at the moment of the enrollment, given that the disease was in an active phase, it is likely that T cells activated in the mesenteric lymph nodes were detectable in peripheral blood while re-circulating to the gut.

Interestingly, our data show an increased expression of the integrin β_7 also on T lymphocyte from T1D children. In this group, differently from CD patients, β_7 was selectively expressed on $CD4^+$ memory T cells.

In addition as reported in the second part of the study, in 1 T1D patient we identified a discrete population of β_7^{hi} $CD4^+$ cells which increased upon antigen stimulation with TG-PTG.

Surprisingly, in both CD and T1D patients the stimulation of T cells for 18 hours via TCR significantly reduced the expression of β_7 on CD4+ memory T cells. Experiments using Annexin V staining performed in some CD patients allowed us to exclude that T cells from patients were more prone to apoptosis (data not shown). Therefore this reduction might indicate that a selective down-regulation of β_7 occurs in gut primed T cells in both CD and T1D children.

We hypothesize that a physiological down-regulation of homing receptors can be induced by immunological and/or inflammatory stimuli with a specific kinetic, which could be the object of future studies.

The importance of $\alpha_4\beta_7$ in gut homing is clearly demonstrated by β_7 KO mice, that present hypocellular Peyer's patches and reduced lymphocyte number in lamina propria (**Wagner N et al., 1996**). Moreover monoclonal antibodies that block the interation between $\alpha_4\beta_7$ and MAdCAM-1 *in-vitro,* inhibit lymphocyte subsets into Peyer's patches (PP) *in vivo* and reduce lymphocyte entry into intestinal lamina propria (**Hamann et al., 1994**). Local

microenvironment such as cutaneous or intestinal, differently direct T cell expression of adhesion and chemoattractant receptors, targeting the effector T cells to the inflamed skin or lamina propria. T cells imprinting occurs rapidly, CD4 T cells in skin draining lymph nodes up regulate P-selectin ligands and down regulate $\alpha_4\beta_7$, while those responding to antigen in intestinal lymph nodes selectively express high level of $\alpha_4\beta_7$ (**Campbell DJ et al., 2002**). Similar results were also reported in humans (**Kantele A et al., 1999**). In this context, experimental observations suggest that local dendritic cells may influence the homing preference of effector T cells (**Mora RJ et al., 2003**).

It was also demonstrated that, following intestinal infection with Rotavirus (**Rott LS et al., 1997**) or Salmonella typhi (**Lundin BS et al., 2002**), the T cells activated by these antigens are programmed for gut trafficking.

These works, together with our data on CD patients suggest that $\alpha_4\beta_7$ could be a good marker of T cell mediated infection and inflammatory disease of the gut.

Nevertheless, further studies are needed to correlate its expression on different subset of T cells with different clinical conditions or stages of the disease.

The question whether gut immune system is involved in the pathogenesis of T1D has been addressed in previous studies performed in NOD mice.

In NOD mice, MadCAM-1 was expressed in the islets at early stages of insulitis; furthermore, most lymphocytes infiltrating the

islets expressed both α_4 and β_7 molecules (**Hanninen A et al., 1993, Yang XD et al., 1994**). Accordingly, in the beginning of autoimmune process, lymphocytes with mucosal homing properties predominate in the islets. Only one study in humans indirectly indicated the involvement of β_7^+ T cells in the development of the specific response to GAD. This study suggests that autoreactive T cells in T1D may re-circulate between the gut and the pancreas (**Paronen J et al., 1997**).

In line with this report, our preliminary data indicate that in the peripheral blood of patients with T1D are present $CD4^+$ memory T lymphocytes expressing gut homing markers.

The finding that $\alpha_4\beta_7$-MAdCAM-1 pathway is central for gut mucosal homing has raised enthusiasm in targeting these molecules as a novel form of precision anti-infiammatory therapy. A humanized anti-α_4-integrin antibody (natalizumab) has been tested in several studies for treatment of Chron's disease and multiple sclerosis (**Targan SR et al., 2007; Polman CH et al., 2006**). The results show for the first time that targeting of lymphocyte homing receptors with monoclonal antibodies can yield clinically useful responses. The manipulation of the gut immune system could be proposed as a tool for the modulation of the autoimmunity against pancreatic β-cells in T1D, however this depends on the improvement of our knowledge on the mechanism of immune activation and tolerance induction in the gut in T1D patients.

In study n° 2 we report of an enhanced cell-mediated immunity against gluten in patients with T1D.

The association between CD and T1D is well established by studies performed in both adults and children (**Maki M et al., 1986**). Such an association may recognize a common immunogenetic basis, but a direct responsibility of gluten in the pathogenesis of autoimmune response cannot be excluded. This has been suggested by the increase risk of developing T1D related to the duration of a gluten containing diet (**Ventura A, and the SIGEP study group, 1999**) and by finding of a fall in autoantibody titer in patients with both CD and T1D once they were placed on a gluten free diet. Approximately 1-16% of patients with T1D have a concomitant celiac disease. Almost without exception, T1D develops before the occurence of celiac disease (**Collin et al., 2002**) and the removal of dietary gluten is associated with a lower frequency of T1D. Likewise, a gluten free diet for 320 days reduces the incidence of T1D from 64% to 15% in nonobese diabetic mice (**Funda DP et al., 1999**).

This data suggest that the withdraw of gluten from the diet might protect from the occurrence of T1D.

The coexistence of these diseases could be explained by molecular mimicry by which gliadin or tissue transglutaminase activates T cells that are cross-reactive with various self antigens. Such inflammatory responses may have the capacity to persist in genetically susceptible hosts and lead to chronic organ-specific autoimmune disease via epitope spreading (**Collin P et al., 2002**). However, it is unclear whether any sequence similarities exist between gliadin or tissue transglutaminase and, for example,

glutamate decarboxylase antibodies associated with T1D (GAD) or insulin. It is also possible that, apart from gliadin, tissue transglutaminase can modify other external or self-antigens by cross-linking or deamidation and thus generate different neoantigens (**Schuppan D, 2000**). These antigens and antibody production can further induce various autoimmune phenomena outside the intestine.

Furthermore, a slightly increased T cell response to gluten has been reported in patients with newly diagnosed T1D (**Klemetti P et al., 1998**).

To address the question whether the response to gluten could be relevant to T1D immuno-pathogenesis, we measured the levels of IFN-γ in the supernatants of the TG-PTG stimulated compared with un-stimulated T cells cultures. We found a increase of IFN-γ levels but not of IL-4 in T1D patients, the some effect was not observed in healthy controls with a positive proliferative response to TG-PTG. These data indicate that the human memory lineage for gliadin-derived antigen is committed to the proinflammatory Th1 phenotype upon re-encounter of gliadin in T1D patient and suggest that T1D is associated with failure in oral tolerance mechanisms, particularly versus gliadin.

The generation of immunological memory is a complex process. Antigen presentation to naïve T cells is postulated to occur in secondary lymphoid organs and is accomplished mainly by professional antigen-presenting cells (APC) which have migrated there from tissue sites where antigen encounter had taken place.

Stimulated, naïve T cells expand and differentiate to effector cells, which recirculate through the blood and home into sites of inflammation in the non lymphoid tissues. Some of these cells subsequently assume a resting state with specific antigen memory, defined as the capacity to readily mount an immune reaction upon subsequent re-encounter with antigen. However, the study of true memory T cells, particularly in humans, has been difficult, chiefly as a result of the inability to distinguish unambiguously among recently activated effector cells, memory precursor, and true memory T cells.

We used PTG or TG-deamidated gliadin as the prime antigen and not a particular peptide sequence; being aware that some author had described various immunodominant, "canonical" epitopes (**Anderson RP et al., 2000**). However, others have shown that, in children, T cell responses are directed toward multiple gliadin epitopes (**Vader W et al., 2002**). Moreover, it is well-established that the different APC population present in fresh PBMC are highly efficient in processing and presenting whole protein antigen (**Inaba K et al., 1986**).

Tissue transglutaminase (TGase) modifies gliadin peptides through deamidation of glutamine residues to negatively charged glutamic acid thus facilitating the binding of gliadin peptides to the peptic groove of HLA DQ2 and DQ8 molecules. Molberg et al. tested the effect of TGase on PT gliadin specific T-cell clones derived from the small intestine or from peripheral blood of celiac disease patients. The author showed that the addition of TGase to

proliferation assays enhanced reactivity and sensitivity to PT-gliadin for all gut derived T cells. Instead, with the exception of one single DQ2 restricted T-cell clone, no increase in PT-gliadin-specific proliferation of PBMC-derived T cells from six CD patients was detected after addition of TGase. These data indicate that gliadin specific T cells that reside in peripheral blood and gut mucosa may recognize distinct epitopes (**Molberg O et al., 1998**).

In this study we found, in T1D patients, a higher proliferative response upon a stimulation with PTG than TG-PTG.

Of note, HLA-DQ2 and DQ8 are postulated to be necessary for the generation of the T cell-dependent, antigliadin responses. Approximately 90% of celiac disease patients share the HLA DR3-DQ2 (encoded by alleles DQA1*0501 and DQB1*0201) and most of the remaining express the HLA DR4-DQ8 haplotype encoded by DQA1*0301 and DQB1*0302 alleles (**Collin P et al., 2002**).

We observed that in the T1D group the mean SI was higher in individuals with HLA $DQ8^+$ or $DQ8^+/DQ2^+$ than in individuals with HLA $DQ2^+$ or with none of the risk HLA molecules.

Recently Tollefsen et al. demonstrated that HLA DQ2 and DQ8 have a preference for binding peptides with negatively charged anchor residues, but that the two HLA molecules employ different criteria for selection of deamidated gluten T cell epitopes. This can result in the selection of distinct epitopes localized in different regions of gliadin protein, but it can also result in the selection of epitopes that combine the DQ2 and DQ8 signatures and are recognized in exactly the same binding register when bound to

DQ2 or DQ8 (**Tollefsen S et al., 2006**).

In this study, we used a control population of healthy, non HLA-matched individuals. Thus, it can be argued that some of the differences between the patient population (usually positive for one or both DQ genes) and the control population (25-35% DQ2 positive) is a result of the different frequencies for these gene products between the two groups. However, we reason that a specific proliferative response in some of the healthy HLA-DQ2$^+$ donors would also indicate the presence of such memory T cells. Moreover, such this observation would not necessarily reduce the significance of a positive response in T1D patient. Future studies may answer whether TG-PTG memory T cells are also found in some healthy HLA-DQ2$^+$ donors and address their particular characteristics. There is some evidence that when such T cells are found in healthy individuals, they do not secrete IFN-γ upon antigen challenge (**O'Keeffe J et al., 1999**).

Moreover, some study observed that biopsies (**Auricchio R et al., 2004**) or PBMC (**Klemetti P et al., 1998**) from HLA DQ2$^+$ or DQ8$^+$ controls subjects did not react with gliadin peptides.

Nevertheless, from a practical viewpoint, there are no indications for withdrawing gluten from diet of T1D patients. A gluten free diet protects mice that are genetically susceptible to diabetes (**Funda DP et al., 1999**). Differently, there are conflicting finding about the clearance of diabetes-related auto-antibodies subsequent to a gluten free diet in humans (**Ventura A et al., 2000; Hummel M et al., 2002**). In view of potential prevention strategies for T1D,

it remains to be established to what extent intestinal inflammation is gluten dependent and if it precedes the occurrence of diabetes.

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