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Impact of Cathepsins on the Activation of Proinsulin-reactive T Cells in Type 1 Diabetes

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Abbreviations

A

ABC	Avidin and biotinylated horse-radish peroxidase macromolecular complex
ABP	Activity-based probe
AEP	Asparagine endoprotease
ANOVA	Analysis of variance
APC	Antigen presenting cells
APL	Altered peptide ligands
ATT	α1-antitrypsin

B

BLC	B lymphoblastoid cells
BSA	Bovine serum albumin

C

Cat	Cathepsin
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CLIP	Class-II associated invariant chain peptide
CPE	Carboxypeptidase E
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte antigen-4

D

DAP	Diphenyl-1-aminoalkylphosponate diaryl ester
DC	Dendritic cells
DCG-04	Generated by **D**oron **C**. **G**reenbaum, University of California
DCins	Proinsulin-derived peptides digested with dendritic cells
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide

DTT	Dithiothreitol
E	
E64d	(2S,3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester
EAE	Experimental autoimmune encephalitis
EBV	Epstein-Barr virus
EBNA1	Epstein-Barr virus nuclear antigen 1
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
F	
FACS	Fluorescence-activated cell sorting
FcR	Antibody Fc (Fragment, crystallized) region receptor
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Fmoc	Fluorenylmethyloxycarbonyl chloride
FoxP3	Forkhead box P3
G	
GAD	Glutamic acid decarboxylase
G-CSF	Granulocyte colony-stimulating factor
H	
HA	Hemagglutinin
HCMV	Human cytomegalovirus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HOBT	NHydroxybenzotrizole
HPLC	High performance liquid chromatography
HPSEC	High performance size exclusion chromatography
HRP	Horseradish peroxidase

HSP	Heat shock protein
I	
IA-2	Insulinoma-associated antigen 2
IAPP	Islet amyloid polypeptide
ICA	Islet cell antibody
IFN	Interferon
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit-related protein
IL	Interleukin
Ii	Invariant chain
iTreg cell	Induced regulatory T cell
K	
kDa	Kilodalton
L	
LAK	Lymphokine-activated killer cells
LAP	Latency-associated peptide
LCMV	Lymphocytic choriomeningitis virus
LHVS	Leucine-homophenylalanine-vinyl sulfone
LPS	Lipopolysaccharide
M	
MACS	Magnetic-activated cell sorting
Mars116	Bt-LC-Suc-Val-Pro-Phe^{P}$(OPh)_2$
MBP	Myelin basic protein
mDC	Myeloid dendritic cells
MHC	Major histocompatibility complex
moDC	Monocyte-derived dendritic cells
MS	Multiple sclerosis
N	
NK cell	Natural killer cell
NMM	N-methylmorpholine

NOD mouse	Non-obese diabetic mouse
NP-40	Nonylphenoxylpolyethoxylethanol
P	
PAIs	Plasminogen activator inhibitors
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Prohormone convertase
PD-1	Programmed death 1
PDVF	Polyvinylidene fluoride
PE	Phycoerythrin
PepA	Pepstatin A
PepA-P	Pepstatin A-penetratin
Pdx1	Pancreatic duodenal homeobox 1
pDC	Plasmacytoid dendritic cells
PPI	Preproinsulin
POD	Peroxidase
PS	Phospholipid phosphatidylserine
PVDF	Polyvinylidene fluoride
R	
RA	Rheumatoid arthritis
Reg II	Regenerating gene II
RPMI	Roswell Park Memorial Institute medium
S	
SCID mouse	Severe combined immunodeficiency mouse
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SRP/SRP-R	Signal recognition particle/signal recognition particle receptor
Suc-VPF	Suc-Val-Pro-Phe^{P}-$(OPh)_2$
T	
TAP	Transporter associated with antigen processing

TBTU	O-Benzotriazole-1-yl-N,N,N',N'-tetramethyluronium tetrafluoroborate
TCR	T cell receptor
T1D	Type 1 diabetes mellitus
Tfh cell	Follicular helper T cell
TGF-β	Transforming growth factor-β
Th	T helper cells
THP-1	Human acute monocytic leukemia cell line
TLR	Toll-like receptor
TMB	3, 3', 5, 5'-Tetramethylbenzidine
TNF-α	Tumor necrosis factor-α
tPA	Tissue plasminogen activators
Treg cell	Regulatory T cell
TTCF	Tetanus toxin C-fragment
U	
U937	Human leukemic monocyte lymphoma cell line
uPA	Urokinase plasminogen activators
V	
VDR	Vitamin D receptor
Z	
ZnT8	Zinc transporter 8

1. Introduction

1.1. A short overview of the immune system

The immune system is composed of a network of cells, tissues, and organs that collectively protect against diseases by identifying and killing pathogens (bacteria, parasites, fungi, and viruses) and tumor cells. To this end, two types of immune responses involved are referred to as innate and adaptive immunity (Parkin and Cohen 2001). The innate response uses phagocytic cells (such as neutrophils, monocytes, macrophages, and dendritic cells), inflammatory mediator-producing cells (basophils, mast cells, and eosinophils), and natural killer cells to neutralize pathogens and prevent infection (Storey and Jordan 2008). However, the adaptive response is characterized by humoral immunity mediated by activated B cells, as well as by CD8+ cytotoxic T lymphocytes (CTL) and CD4+ T helper cells (Th)-mediated cellular immunity. The innate response is rapid, may however damage normal tissues and lacks specificity, whereas the adaptive response is precise, but takes several days or weeks to develop a response. Furthermore, the adaptive immune system develops memory cells, so that subsequent exposure to pathogens results in a more vigorous and rapid response (Storey and Jordan 2008).

1.2. Antigen processing in MHC class I and II pathways

1.2.1. Antigen presenting cells

Antigen presenting cells (APC) are divided into two categories, professional and non-professional APC. B cells, macrophages, and dendritic cells (DC) are known as professional APC, in contrast to non-professional APC, which include fibroblasts (skin cells), thyroid epithelial cells, glial cells, endocrine cells such as pancreatic β cells, and vascular endothelial cells. Professional APC are highly plastic. First, they have the ability to adopt various functional phenotypes depending on the external stimuli and the environmental conditions they undergo. Second, they play an important role in the initiation of adaptive immunity and pathogen clearance. Moreover, they control several processes such as tissue repair, clearance of

apoptotic cells, and maintenance of tolerance to nonharmful self antigens, respectively (Mosser 2003; Steinman et al. 2003).

In particular, professional DC are the most central player in immune responses, they bridge innate and adaptive immunity. DC are exceptionally potent APC under inflammatory conditions, and are also critical in the induction and maintenance of self-tolerance (Hawiger et al. 2001; Bonifaz et al. 2002). It is commonly believed that two classes of DC are recognized based on their function, myeloid DC (mDC) and plasmacytoid DC (pDC), which both play critical roles in linking the innate and adaptive immunity *via* their cytokine production and unique expression patterns of toll like receptors (TLRs) (Robinson et al. 1999; Liu et al. 2001; Kadowaki 2009). Additionally, mDC can be subdivided into at least two subsets, mDC1 (CD1c+CD141-) and mDC2 (CD1c-CD141+) (Dzionek et al. 2000; MacDonald et al. 2002; Piccioli et al. 2007).

1.2.2. MHC class I and II molecules

Based on chemical structure, cell distribution, and function, the major histocompatibility complex (MHC) molecules are divided into two distinct groups (MHC class I and II). Both MHC class I and class II molecules are cell-surface glycoproteins, which act as peptide-binding receptors. MHC class I molecules are expressed in every nucleate cell and recognized by CD8+ T cells. In contrast, MHC class II molecules are expressed by immune cells like B cells, DC, and monocytes/macrophages (Watts 2004). Human MHC molecules are called human leukocyte antigens (HLA) which include three classical class II genes (HLA-DP, -DQ, -DR) and two “non-classical” class II genes (HLA-DM and -DO) (Horton et al. 2004). Most of autoimmune diseases are associated with variations in the MHC gene locus at chromosome 6p21. The MHC class II region is extraordinarily polymorphic, with for example more than 400 different HLA-DRB1 alleles (von Salome et al. 2007). HLA-DRB1*0301, HLA-DRB1*0401, HLA-DQB1*0201, and HLA-DQB1*0302 have been postulated to have predisposing effects on type 1 diabetes mellitus (T1D) (Thomson et al. 1988; Noble et al. 1996).

Expression of MHC class II genes is tightly regulated. Levels of MHC class II expression vary according to the developmental stage of APC. For instance, differentiation of B cells into plasma cells and maturation of DC are characterised by the repression of MHC class II gene expression. Moreover, levels of MHC class II gene expression vary in APC depending on particular stimuli. MHC class II molecules become markedly downregulated in particular physiological conditions, during viral infections, or in B cell lymphomas (Rimsza et al. 2004).

1.2.3. Antigen processing and presentation in the MHC class II pathway

Generally, two distinct pathways of antigen processing exist, the MHC class I and II processing pathways. While the MHC class I pathway generates antigenic peptides that can be presented to CD8+ T cells, the MHC class II pathway presents antigenic peptides to CD4+ T cells (Figure 1). Of note, crosstalk between the MHC class I and class II antigen processing pathways does exist, CD8+ DC are able to deliver exogenous antigens to the MHC class I pathway (cross-presentation) (Villadangos and Schnorrer 2007).

The alpha and beta chains of MHC class II, along with the invariant chain (Ii), are synthesized, assembled in the endoplasmic reticulum (ER), and transported through the Golgi and trans-Golgi apparatus to reach the endosome, where the Ii is degraded to the class II-associated invariant chain peptide (CLIP). On the other hand, antigenic peptides from exogenous sources are loaded onto MHC class II molecules with the assiatance of HLA-DM. The MHC/peptide complex is finally transported to the cell surface where it can be inspected by TCR of CD4+ T cells and initiate the induction of a specific response (Cresswell and Lanzavecchia 2001; van Endert and Villadangos 2007; Colbert et al. 2009). Antigen presentation is not only pivotal for the regulation of protective immune responses against invading pathogens, but is also essential for the maintenance of self-tolerance (Kyewski and Klein 2006).

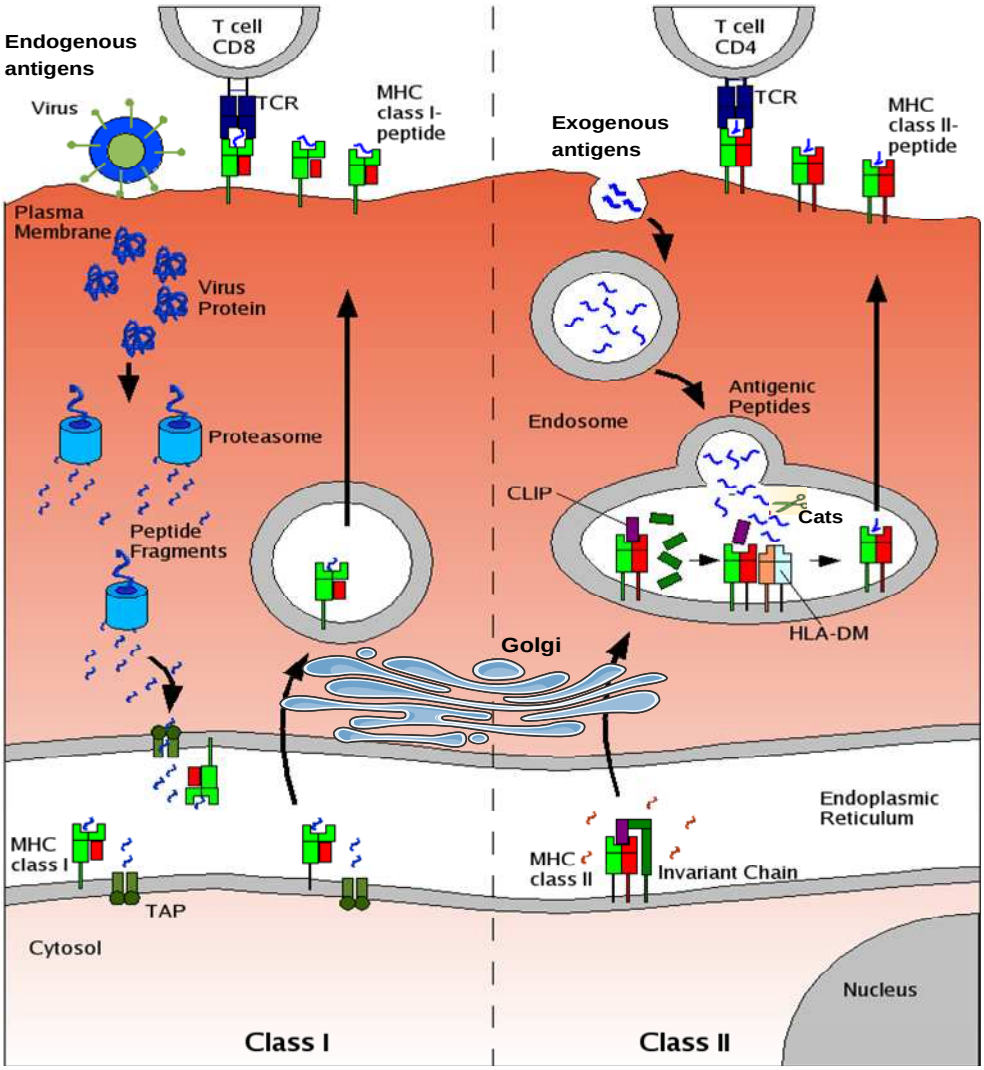


Figure 1: MHC class I and MHC class II antigen processing pathways. Endogenous antigens (such as virus proteins) are generated in the cytosol by the proteasome and then translocated to the endoplasmic reticulum (ER) and loaded to MHC class I molecules. Exogenous antigens are processed in endosome by cathepsins (Cats) and loaded to MHC class II molecules with the assistance of HLA-DM. CLIP: class II-associated invariant chain peptide; HLA: human leukocyte antigens; MHC: major histocompatibility complex; TAP: transporter associated with antigen processing; TCR: T cell receptor. Modified from http://www.lesc.ic.ac.uk/projects/appp.html.

1.3. Proteases involved in antigen/Ii processing of the MHC class II pathway

Cathepsins are proteases which can be found in many types of cells and tissues. In the context of antigen processing, cathepsins are required for the degradation of antigens in order to make them suitable for loading onto MHC class II molecules. The use of specific protease inhibitors and studies using gene knockout mice have been applied to define proteases involved in antigen processing and presentation.

According to the amino acid within their active site, cathepsins are divided into three categories: cysteine, aspartic, and serine protease families.

To date, the proteases responsible for processing of antigens and Ii are still poorly understood. Therefore, further investigation of antigen/Ii processing is indicated. The current knowledge of cathepsins involved in antigen/Ii processing is based on: first, the use of specific protease inhibitors, such as CatG inhibitors (Reich et al. 2009), pepstatin A-penetratin (PepA-P) to inhibit aspartic proteases (Zaidi et al. 2007), or E64d for cysteine protease inhibition (Tamai et al. 1986); second, localization of proteases to particular cells and subcellular compartments; and third, processing of antigens and Ii with defined proteases by using cathepsin knockout mice. However, the *in vivo* situation may not be simply mimicked by *in vitro* experiments or *in vivo* by using knockout mice, since the cathepsin distribution in mice *vs*. humans is known to be different. For example, mouse and human CatG differ significantly in their tryptic activity (Raymond et al. 2010). Furthermore, the Ii degradation is controlled by CatV in the human thymus and CatL in the mouse (Tolosa et al. 2003).

1.3.1. Cysteine proteases

The cysteine proteases of the CA clan include CatB, C, F, H, S, L, V, and X, together with the CD clan-associated asparagine endoprotease (AEP). A list of currently known cysteine cathepsins is given in Table 1 (Brix et al. 2008; Conus and Simon 2010). Over the last decades, numerous studies demonstrated that the cysteine cathepsins are involved in antigen and Ii processing (Bromme et al. 1999; Hsing and Rudensky 2005; Watts et al. 2005; Chapman 2006; Colbert et al. 2009). Experiments, for example, using CatS knockout mice and human B cell lines treated with the CatS specific inhibitor leucine-homophenylalanine-vinyl sulfone (LHVS) have implicated CatS in the last step of Ii degradation to generate CLIP (Riese et al. 1996; Driessen et al. 1999). However, the species- or cell type-specific roles for other cysteine cathepsins in the late stage of Ii processing have been suggested. For instance, CatL was found to be involved in Ii degradation in the mouse thymus,

in analogy to CatV in humans (Nakagawa et al. 1998; Tolosa et al. 2003). CatF was able to process Ii in the lung macrophages of CatS/L double knockout mice (Shi et al. 2000). Moreover, it was reported that both CatS and AEP control the proteolytic processing of myelin basic protein (MBP) *in vitro*, an autoantigen implicated in the inflammatory demyelinating disease multiple sclerosis (MS) (Beck et al. 2001). Further studies showed that AEP can initiate processing of the tetanus toxin C-fragment (TTCF) in B cell lines (Manoury et al. 1998; Antoniou et al. 2000; Watts 2001). In the case of Ii processing, AEP was found to play an important role in initiating Ii cleavage in living cells (Manoury et al. 2003). However, Maehr et al. demonstrated that AEP has no effect in Ii degradation at any stages by using AEP-deficient mice (Maehr et al. 2005a). Collectively, it is still not clear which proteases are important in initiating Ii degradation.

Table 1: Known cysteine cathepsins

Name	Cleavage specificity	Tissue expression	pH
CatB	Endopeptidase, peptidyl-dipeptidase	Ubiquitous	pH 4-6 (optimal), stable up to pH 7
CatC	Dipeptidyl-peptidase, endopeptidase	Ubiquitous	pH 6 (optimal), pH 4-7.5 (stable)
CatF	Endopeptidase	Heart, skeletal muscle, brain, testis, ovary	pH 5.2-6.8 (optimal), pH 4.5-7.2 (stable)
CatH	Endopeptidase, aminomonopeptidase	Brain, kidney, liver; inflamed tonsil	pH 6.8 (optimal), pH 5-8 (stable)
CatK	Endopeptidase	Predominantly in bone (osteoclasts), present in most epithelial tissues	pH 6 (optimal), pH 4-8 (stable)
CatL	Endopeptidase	Ubiquitous	pH 6 (optimal), pH 3-6.5 (stable)
CatO	Endopeptidase	Widely expressed	
CatS	Endopeptidase	Alveolar macrophages, spleen, testis, epithelial cells, CD4+ T cells, APC	pH 6 (optimal), pH 5-7.5 (stable)
CatV	Endopeptidase	Present in brain, corneal epithelium, skin, predominantly in thymus, testis	pH 5.7 (optimal), pH ≤4 to ≥7.2 (stable)
CatW	Pending	Spleen, natural killer cells and cytotoxic T cells	
CatX	Carboxymonopeptidase	Widely expressed, ubiquitously in primary tumors and cancer cell lines	
AEP	Endopeptidase	Thymus, pDC, moDC, BLC	pH 5-5.5 (optimal)

Note: AEP: asparagine endoprotease; APC: antigen presenting cells; BLC: B lymphoblastoid cells; moDC: monocyte-derived dendritic cells; pDC: plasmacytoid dendritic cells.

1.3.2. Aspartic proteases

CatD and CatE are members of the aspartic protease family. While CatD is expressed in all cells (except erythrocytes), CatE expression is restricted to APC (Chain et al. 2005; Zaidi et al. 2007; Burster et al. 2008). In APC, CatE is localized in endosomes, whereas CatD is primarily located in lysosomes (Zaidi and Kalbacher 2008). Both CatD and CatE participate in antigen processing, as shown by using pepstatin A (PepA), an inhibitor of aspartic proteases, *in vitro*, and CatD-deficient mice (Bennett et al. 1992; Deussing et al. 1998; Zhang et al. 2000).

1.3.3. Serine proteases

CatA and CatG belong to the class of serine proteases. Human CatG has both chymotrypsin- and trypsin-like activity and is active across a broad pH spectrum ranging from 5-8 (optimal between pH 7–8). CatG is found in primary human monocytes, B cells, mDC1, mDC2, and pDC, but also in cell lines such as U937 (human leukemic monocyte lymphoma cell line) and THP-1 (human acute monocytic leukemia cell line). Monocyte-derived DC express CatG, but it was found that CatG is not active in these cells (Burster et al. 2005). In the mouse, CatG is expressed in microglia and splenic DC. However, CatG is not expressed in BLC or fibroblast cell lines. Strikingly, exgenous CatG can be internalized into endocytic compartments in a non-CatG-expressing B cell line (Burster et al. 2010a), widening the range of cells where this protease may play a role in antigen and Ii processing. Functional assays have implicated CatG as a critical protease in processing of several antigens and autoantigens (Burster et al. 2010b). Regarding the processing of MBP, it was found that CatG, and not AEP, controls the processing of MBP in primary human B cells (Burster et al. 2004), and another study showed that presentation of hemagglutinin (HA) and TTC was reduced when incubated with a specific CatG inhibitor (Reich et al. 2009). However, whether CatG is involved in Ii prcocessing has not been investigated yet, which will be explored in the present study.

CatA, is a carboxyprotease that releases one amino acid from the C terminus of proteins or peptides, respectively, and has a broad specificity (pH optimum around 4.5 to 6.0) (Pschezhetsky 1998). CatA is mainly expressed in platelets, lymphokine-activated killer cells (LAK), spleen cells, fibroblasts, kidney, liver, melanoma cells, peripheral blood mononuclear cells (PBMC), human alveolar macrophages, and recently found in primary human B cells and mDC1 (Reich et al. 2010). Until now, the precise involvement of CatA in antigen and Ii processing is not known.

1.3.4. Cathepsins and type 1 diabetes

Studies with cathepsin knockout mice have addressed the question of whether cathepsins are directly involved in the development of autoimmunity. For example, experiments by using CatS, B, and L deficient mice indicated that these proteases are important in the onset of autoimmune diabetes (Maehr et al. 2005b; Hsing et al. 2010). In the present study, the impact of cathepsins (particularly CatG) on the activation of proinsulin-reactive T cells from T1D will be elucidated.

1.4. Autoimmunity and type 1 diabetes

1.4.1. Homeostasis and self-tolerance in the immune system

The immune system responds to microbes and eliminates them in a regulated fashion, but it does not respond to self-antigens. Particularly, the immune system is able to maintain balance even when it responds to diverse microbes or it is exposed constantly to self-antigens (van Parijs and Abbas 1998). After a productive response to the foreign antigens, the immune system returns to a status of rest, so that the lymphocytes can be reset to a preimmunized level in numbers and function. This process is called homeostasis. Furthermore, memory allows the immune system to respond effectively to a new antigenic challenge. Normally, individuals maintain a state of unresponsiveness to their own antigens, called self-tolerance (van Parijs and Abbas 1998). In some circumstances, the immune system can turn on itself and target its own cells, tissues and organs for breakdown. This

phenomenon is called autoimmunity or autoreactivity. As a consequence, autoimmune diseases may arise when tolerance to self-tissue breaks down. Autoimmune diseases, like T1D, MS, rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) are characterized by an excessive, inflammatory response against self-antigens, resulting in cell, tissue, or organ destruction. Therefore, to keep self-reactive cells under control, it is crucial to maintain immune homeostasis.

1.4.2. The role of immune cells in the pathogenesis of type 1 diabetes

1.4.2.1. Lymphocytes and type 1 diabetes

Ample evidence documents that contribution of both CD4+ helper and CD8+ cytotoxic T lymphocytes in the pathogenesis of T1D is beyond doubt. It is thought that CD8+ T cells may be more important during the early phases of pathogenesis (DiLorenzo et al. 1998; Groen et al. 2003), whereas CD4+ T cells are required throughout. Furthermore, individual islet-responsive CD4+ and CD8+ T cell clones have been isolated from diabetic animals that can either mediate or accelerate β cell destruction (Daniel et al. 1995; Grewal et al. 1996; Pakala et al. 1997).

There are several ways in which T cell-mediated β cell death might occur. For example, CD8+ T cells could kill pancreatic β cell through MHC class I-mediated cytotoxicity. CD4+ and CD8+ T cells can produce cytokines, such as interferon-γ (IFN-γ), which can induce β cell apoptosis. In addition, IFN-γ can activate macrophages and induce increased pro-inflammatory cytokine production, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Eizirik and Mandrup-Poulsen 2001). This crosstalk between T cells and macrophages undoubtedly exacerbates the immune-mediated stress on β cells and contributes to their destruction (Lehuen et al. 2010). Collectively, these findings have reinforced the notion that T cells play an integral role in the development of T1D.

Although T cells have a pathological role in T1D onset, some T cells have the ability to prevent or mitigate β cell destruction. These cells show a regulatory phenotype [CD4+CD25+Foxp3+ T regulatory (Treg) cells] (Fehervari and Sakaguchi 2004). Furthermore, multiple studies pointed to an essential role for CD4+CD25+Foxp3+

Treg cells in the maintenance of self-tolerance in non-obese diabetic (NOD) mice (Piccirillo et al. 2005; Tang et al. 2006). The question remains, how do Treg cells prevent T1D? It was reported that Treg cells have the potency to control the activation of effector T cells at various stages, inhibit IL-2 production, and traffic to the pancreas to suppress effector T cell functions (Tang et al. 2006; Tang and Bluestone 2006). Moreover, Treg cells can impinge the proper migration of autoreactive T cells (Bluestone 2005). Thus it can be seen that the ideal treatment of autoimmune disease is not depletion of all self-reactive T cells but rather reduction of self-reactive T cells and induction and/or activation of Treg cells.

T cells are clearly pivotal for T1D development, notwithstanding, they are not the only components thought to be important in an autoimmune response (Stadinski et al. 2010a). There are data indicating that other components such as macrophages, B cells, DC subsets, natural killer (NK) cells, and NKT cells are also involved in the destruction of β cells (Willcox et al. 2009).

1.4.2.2. The pathogenic role of macrophages

Early experiments described the presence of macrophages in islet infiltrates of NOD mice, and showed that inhibition of the macrophage influx into the pancreas by blocking an adhesion-promoting receptor on these cells, inhibited the development of T1D (Hutchings et al. 1990). Meanwhile, macrophages have been shown to produce IL-12 which can direct the development of Th1 cells and promote efficient differentiation of diabetogenic CD8+ CTL to destroy pancreatic β cells and lead to T1D onset (Jun et al. 1999).

1.4.2.3. The role of B cells

B cells play a pathogenic role in T1D as an essential APC type (Serreze et al. 1996; Pescovitz et al. 2009; Marino et al. 2011). It was demonstrated that B cells can capture autoantigens (*via* surface immunoglobin), present key antigen-MHC complexes and then trigger diabetogenic T cells (Silveira et al. 2002). B cell depletion, either through gene targeting or antibody treatment, can prevent or delay the development of T1D in NOD mice (Serreze et al. 1996; Hu et al. 2007).

Furthermore, improved β cell function of newly diagnosed T1D patients was obtained by the treatment with the B cell-depleting monoclonal antibody rituximab (Pescovitz et al. 2009). Together, these studies support a pathogenic role for B cells in the development of T1D.

1.4.2.4. The dual role of dendritic cells

mDC1, also called conventional DC (cDC), can take up and process β cell-derived proteins and subsequently initiate a diabetogenic response (Ludewig et al. 1998; Turley et al. 2003). Additionally, pDC have been reported to have a pathogenic role in NOD mice (Stewart et al. 1993; Li et al. 2008; Li and McDevitt 2011). On the other hand, there are some studies supporting a protective role of DC in T1D. Treatment with granulocyte colony-stimulating factor (G-CSF) to recruit DC prevented the development of T1D and increased the numbers of both cDC and pDC in NOD mice. These cells mediated tolerance *via* the effects on CD4+CD25+ Treg cells that suppress pathogenic T cells through the production of transforming growth factor-β (TGF-β) (Kared et al. 2005).

1.4.3. CD4+ T helper cell subsets and their functions in type 1 diabetes

CD4+ T cells can differentiate into a variety of effector subsets based upon the cytokines they produce (Figure 2), including classical Th1 cells and Th2 cells, the recently defined Th17 cells, follicular helper T (Tfh) cells, and induced regulatory T (iTreg) cells. Furthermore, Th22 cells which secret IL-22, were described more recently (Veldhoen et al. 2008; Duhen et al. 2009). A subset of IL-9-producing effector T cells (Th9) distinct from Th1, Th2, or Th17 cells was identified in mice, while it is not well characterized in humans and remains to be determined (Dardalhon et al. 2008; Veldhoen et al. 2008). Th1 cells are characterized by their production of IFN-γ and are involved in cellular immunity against intracellular microorganisms. Th2 cells produce IL-4, IL-5, and IL-13 and are required for humoral immunity to control helminths and other extracellular pathogens. Th17 cells produce IL-17A (here-after referred to as IL-17), IL-17F, and IL-22 and play an important role in clearance of extracellular bacteria and fungi, especially at mucosal

surfaces. iTreg cells characterized by the expression of the forkhead transcription factor (Foxp3) may participate in maintenance of peripheral tolerance and prevention of autoimmunity. Tfh cells are considered to regulate the maturation of B cell responses. Of note, studies did show that CD4+ T helper cells are flexible in their cytokine secretion, which indicates that these cells can change their cytokine profile (Murphy and Stockinger 2010; O'Shea and Paul 2010).

The role of distinct CD4+ T cell subsets on autoimmune diseases such as MS, T1D, and RA, has received considerable attention over the past decade (Lafaille 1998). In the case of T1D, Th1 is generally considered to be pathogenic subset in the development of T1D (Figure 2). There are conflicting data with regard to the role of Th17 in the pathogenesis of T1D (Jain et al. 2008; Honkanen et al. 2010; Ankathatti et al. 2011). Using *in vitro* differentiated Th1 and Th2 cells specific for a pancreatic islet antigen, Katz et al. showed that only Th1 cells were diabetogenic (Katz et al. 1995), and IFN-γ was found to be essential for destruction of β cells and development of T1D (von Herrath and Oldstone 1997). However, it was indicated that IFN-γ, contrary to its inflammatory function, has the potency to modulate inflammation and restore normoglycemia in NOD mice (Jain et al. 2008; Honkanen et al. 2010; Ankathatti et al. 2011). More recently, Beriou et al. reported that patients with autoimmune diabetes present a higher frequency of IL-9+IL-17+ CD4 T cells after IL-1β stimulation, indicating these cells may play a role in human autoimmune diseases (Beriou et al. 2010). On the other hand, Th2 and Treg cells are regulatory subsets of CD4+ T cells that are implicated in protection from T1D. CD4+Foxp3+ Treg cells are critical in the control of β cell autoimmunity in mouse models and humans (Sgouroudis and Piccirillo 2009). Therefore, a successful switch from a Th1 dominated to a Th2 dominated response, and use of CD4+CD25+Foxp3+ Treg cellular therapy to promote immunological tolerance in the absence of long-term immunosuppression might have clinical benefits for individuals suffering from pre-T1D or recent onset T1D.

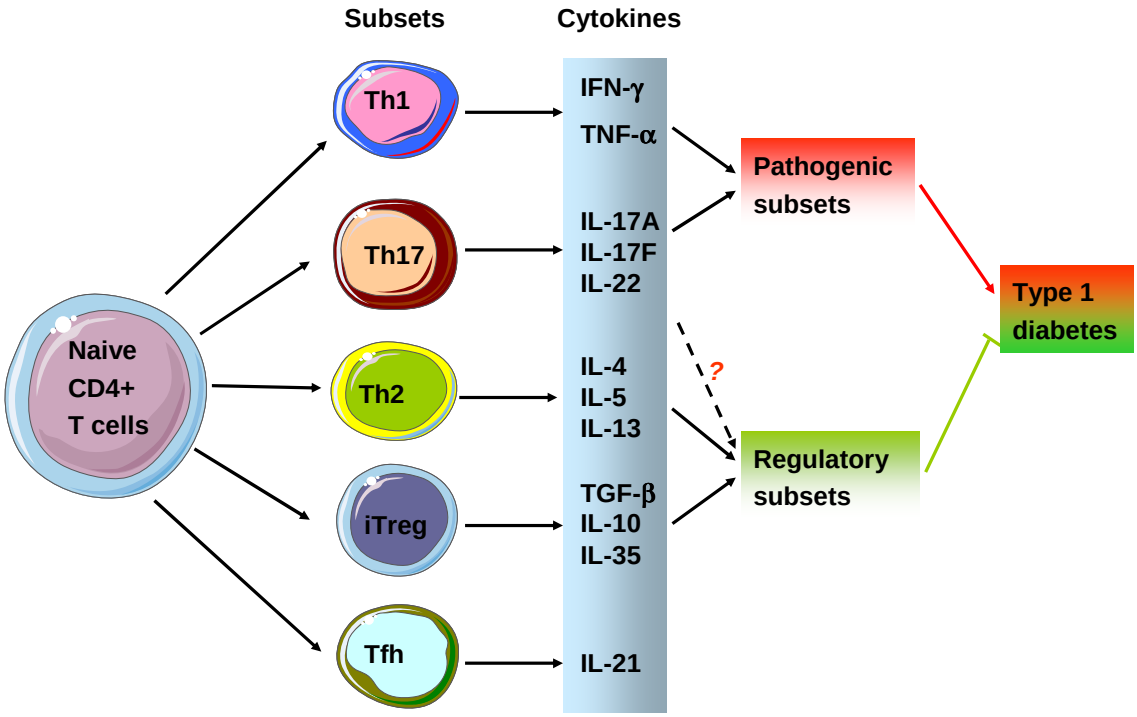


Figure 2: The classical primary polarization of diverse CD4+ T cell subsets and their roles in the development of T1D. Naive CD4+ T cells differentiate into a variety of effector subsets based upon the cytokines they produce, Th1, Th2, Th17, iTreg, and Tfh. Generally, Th1 and Th17 cells are considered to be pathogenic in the development of T1D, while Th2 and Treg cells are implicated in protection from T1D. However, Th17 cells might play a protective role in the development of T1D. IFN-γ: interferon-γ; IL: interleukin; iTreg cells: induced regulatory T cells; T1D: type 1 diabetes; Tfh cells: follicular helper T cells; Th: T helper cells; TGF-β: transforming growth factor-β; TNF-α: tumor necrosis factor-α.

Chemokines might also play a role in insulitis and β cell destruction. Altered expression of chemokines and their receptors in T1D has been indicated recently (Eizirik et al. 2009). Chemokines exert their biological functions by interacting with chemokine receptors and induce leukocyte migration and activation (Charo and Ransohoff 2006). Chemokine receptors, for instance chemokine (C-C motif) receptor 2 (CCR2), a receptor for C-C motif ligand 2 (CCL2), can present distinct activation states depending on the chemokine ligands it binds (Ogilvie et al. 2004). Therefore, it is conceivable that CCL2 may play a dual role in T1D and its complications (Guan et al. 2011). However, many studies did show that Th1 cell-derived chemokines, CCL3, CCL4, and CCL10, were elevated in the serum of patients with new-onset T1D (Shimada et al. 2001; Nicoletti et al. 2002; Hanifi-Moghaddam et al. 2006). On the other hand, CCL22 was recently found to be

a novel therapeutic target of T1D due to its potential role in recruitment of Tregs to islets and attenuation of β cell destruction (Montane et al. 2011). Meanwhile, therapeutic strategies, antagonists of chemokine receptors, are in development for the treatment of autoimmune diseases. Of note, CCR2 antagonists with high selectivity have been identified by high-content analysis assay (Kredel et al. 2011).

1.4.4. Self-antigens in type 1 diabetes

A number of islet proteins have been identified as potential autoantigens in T1D (Stadinski et al. 2010a). Specific autoantibodies have been used to follow the progression of T1D in mice and humans. Most of autoantigens are shared between patients and NOD mice. The expanding list of autoantigens is presented in Table 2 (Lieberman and DiLorenzo 2003). Of note, insulin and its prohormone precursor proinsulin are the ones to be truly β cell specific among the potential autoantigens in T1D (Chentoufi and Polychronakos 2002; Zhang et al. 2008). Furthermore, accumulating evidences suggest that proinsulin rather than its processed products (insulin and C peptide) might be a candidate for an early autoantigen in T1D (Chen et al. 2001; Narendran et al. 2003).

Antigen-specific immunotherapies, using insulin, GAD65, and altered peptide ligand derived from the insulin peptide B9-23, have been performed and some have been applied for their utility in the prevention and treatment of T1D (Assan et al. 1985; Lampeter et al. 1998; Raz et al. 2001; Herold et al. 2002; Herold et al. 2005; Alleva et al. 2006; Ludvigsson et al. 2008). Data from our group showed that proinsulin- and GAD-derived peptides may have immunomodulatory effects on PBMC from T1D (Boehm et al. 2009; van Aalst et al. 2010). However, a clinical trial by using GAD vaccine found no protective effect on β cell function of new-onset T1D (Wherrett et al. 2011). So far, only a handful of trials using antigen-based immunotherapy have been shown to preserve C-peptide in individuals with new-onset T1D (Orban et al. 2011; Sherry et al. 2011). Strikingly, Tasi and colleagues found that the therapy by using disease-relevant peptide-MHC complex coated with nanoparticles was able to both blunt T1D progression in prediabetic

mice and even reverse disease in overtly diabetic mice (Tsai et al. 2010). Nevertheless, there is still a long and winding road to conquer T1D and to optimize the path from bench to bedside.

Table 2: The list of self antigens in T1D patients and NOD mice

T1D patients	NOD mice
Proinsulin and insulin	Preproinsulin, proinsulin, and insulin
GAD	GAD65 and GAD67 (Elliott et al. 1994)
IA-2 and IA-2β	IA-2 and IA-2β
ICA69	ICA69
HSP60	HSP60
CPE	S100β
ZnT8 (Chimienti et al. 2004)	IGRP (Lieberman et al. 2003)
Pdx1 (Li et al. 2010)	Pdx1 (Li et al. 2010)
	IAPP
	RegII (Gurr et al. 2007)
	Chromogranin A (Stadinski et al. 2010b)

Note: CPE: Carboxypeptidase E; GAD: glutamic acid decarboxylase; HSP60: heat shock protein 60; IA: insulinoma antigen; IAPP: islet amyloid polypeptide; ICA: islet cell autoantigen; IGRP: islet-specific glucose-6-phosphatase catalytic subunit-related protein; Pdx1: pancreatic duodenal homeobox 1; Reg II: regenerating gene II; ZnT8: zinc transporter 8.

1.4.5. Autoantigen epitopes for diabetogenic T cells

CD4+ and CD8+ T cell recognition of islet autoantigenic epitopes is a key step in the autoimmune cascade of T1D. Furthermore, epitope recognition is also an important target for antigen-specific immunotherapy. The discovery of T cell epitopes is therefore a vital component of T1D research, in both humans and mice, and also an important aim in the present study.

Since the initial discovery of autoantibody is insulin, multiple studies have been conducted to characterize T cell response to insulin, proinsulin, and preproinsulin peptides (Congia et al. 1998; de Marquesini et al. 2008; Skowera et al. 2008; Durinovic-Bello et al. 2010). The biosynthetic pathway and structural model of proinsulin is shown in Figure 3 (Weiss 2009). Many peptide epitopes derived from β cell proteins have been described (de Marquesini et al. 2008; Wong et al. 2009; Durinovic-Bello et al. 2010). At least two immunodominant T cell epitopes within the

proinsulin molecule have been published, including C18-A1 (P17) and A5-A21 (P21) (Durinovic-Bello et al. 2002; Durinovic-Bello et al. 2006). In addition, prominent CD4+ and CD8+ T cell epitopes have been identified, including insulin peptide B:9-23, a preproinsulin signal peptide and insulin B10-18, B18-27, and A12-20 (Pinkse et al. 2006; Velthuis et al. 2010).

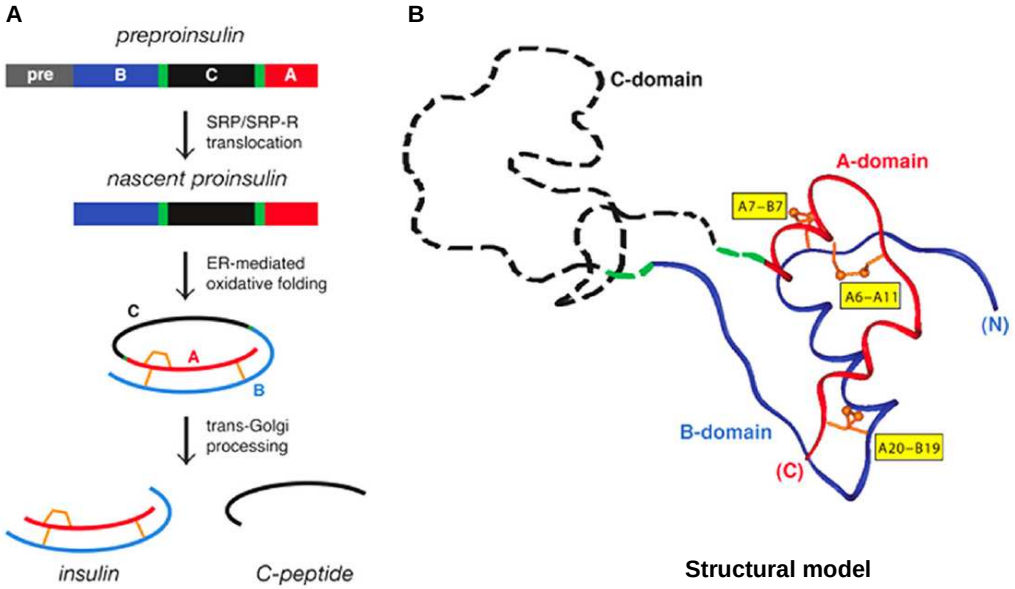


Figure 3: Biosynthetic pathway and structural model of proinsulin. A) Biosynthetic pathway of proinsulin starts with preproinsulin (upper), which is composed of a signal peptide (gray), B-domain (blue), dibasic BC junction (green), C-domain (black), dibasic CA junction (green), and A-domain (red). The unfolded preproinsulin undergoes specific disulfide pairing to generate nascent proinsulin (center) in the ER. BC and CA junctions are cleaved by prohormone convertases (PC1 and PC2) and CPE, resulting in the formation of mature insulin and the C-peptide (lower). SRP/SRP-R: signal recognition particle/signal recognition particle receptor. B) Structural model of proinsulin. The A- and B-domains are shown in red and blue, respectively, the disordered connecting domain is shown by the dashed black line, three disulfide bridges (A6-A11, A7-B7, and A20-B19) are labeled in yellow boxes. Taken from Weiss MA, J Biol Chem. 2009, 284 (29): 19159-63.

1.5. Vitamin D and type 1 diabetes

Vitamin D, the essential vitamin in calcium and bone metabolism, has beneficial effects also on β cell function and normal immunity. Vitamin D receptors (VDRs) are present in pancreatic β cells and vitamin D is essential for insulin secretion (Norman et al. 1980). Multiple evidences suggest a role for vitamin D in the pathogenesis and prevention of T1D. Its activated form, $1\alpha,25(OH)_2D_3$, has immunomodulatory

capacity (Figure 4) and can prevent T1D in animal models (Zella et al. 2003). Moreover, vitamin D can modify T cell differentiation, modulate dendritic cell action, and induce cytokine secretion by shifting the balance to a Treg cell phenotype (van Halteren et al. 2002; Jeffery et al. 2009; Baeke et al. 2010; von Essen et al. 2010). However, it is unknown whether vitamin D can regulate cathepsins. Therefore, this question will be addressed in this study.

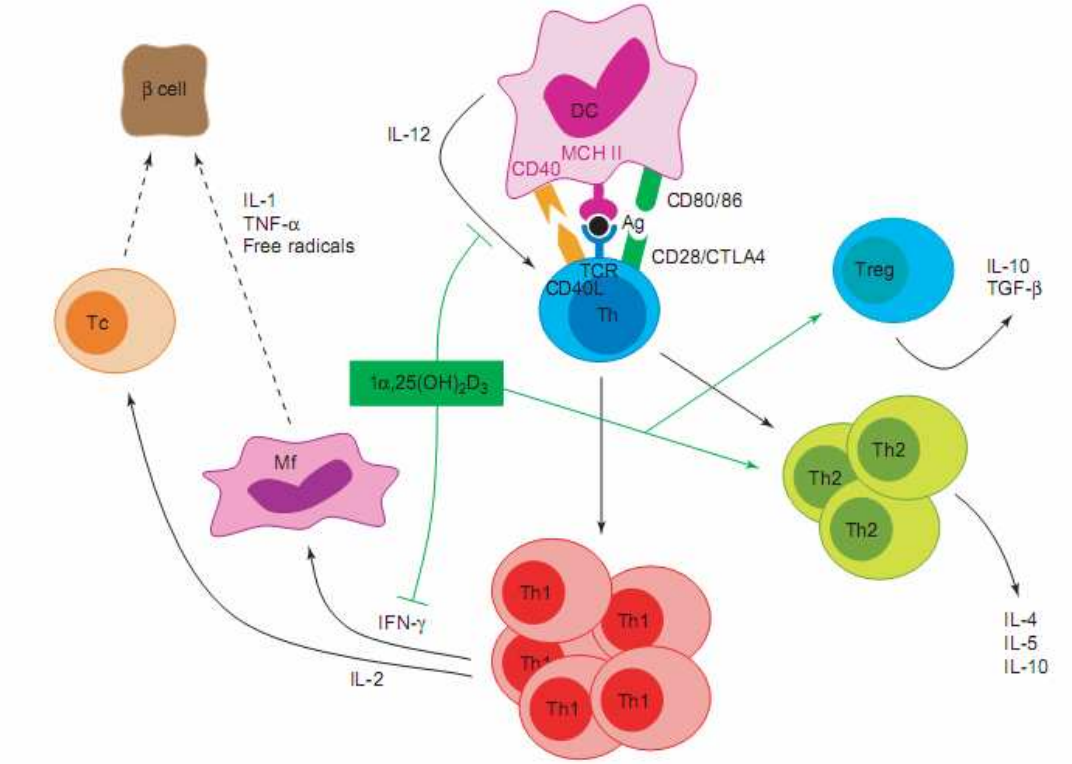


Figure 4: The immunomodulatory effector of 1α,25(OH)$_2$D$_3$. 1α,25(OH)$_2$D$_3$ inhibits the production of the cytokine IL-12, thereby indirectly shifting the polarization of T cells from a Th1 towards a Th2 phenotype. In addition, 1α,25(OH)$_2$D$_3$ has immunomodulatory effects directly on T cell, by inhibiting the production of the Th1 cytokines (IL-2 and IFN-γ) and stimulating the production of Th2 cytokines. Strikingly, 1α,25(OH)$_2$D$_3$ favours the induction of Treg cells. These immunomodulatory effects of 1α,25(OH)$_2$D$_3$ can lead to the protection of target tissues, such as islet β cells in autoimmune diseases. DC: dendritic cells; IL: interleukin; IFN-γ: interferon-γ; TCR: T cell receptor; TGF-β: transforming growth factor-β; Th: T helper cells; TNF-α: tumor necrosis factor-α. Taken from Methieu and Badenhoop, Trends in Endocrinology and Metabolism, 2005, 16 (6): 261-6.

In humans, epidemiology suggested that vitamin D intake is associated with T1D prevention. For instance, the use of vitamin D-containing supplements in early childhood was associated with a reduced risk of T1D in one case control study and

one cohort study (Dahlquist et al. 1999; Hypponen et al. 2001). In the Norwegian study, the use of cod liver oil but not vitamin D supplementation during pregnancy or in the first year of child life was associated with lower risk of T1D in children (Stene and Joner 2003). These findings are further supported by results from a meta-analysis, which concluded the protective effects of vitamin D supplementation in early childhood in the development of T1D (Zipitis and Akobeng 2008). Moreover, maternal intake of vitamin D during pregnancy was related to decreased risk of early β cell autoimmunity (Brekke and Ludvigsson 2007).

1.6. Viral infection as a potential trigger of autoimmune diseases such as type 1 diabetes

The relationship between autoimmune diseases and infection by different microbial agents, bacteria, viruses, fungi, and parasites, has been well-investigated and well-known in the medical literature. Many environmental factors can trigger autoimmunity, and among them the role of infectious agents is pivotal. In the case of T1D, environmental factors, especially viruses, are thought to play an important role in the initiation or acceleration of T1D. Data from correlative clinical studies as well as experimental studies using rodent models for T1D strongly suggest that viral infections can be associated with human T1D. These viruses include enterovirus (particularly coxsackie B) (Salminen et al. 2003; Richer and Horwitz 2009), rubella (Menser et al. 1978; Ou et al. 2000), mumps (Helmke et al. 1980; Hyoty et al. 1988), rotavirus (Honeyman et al. 1998; Honeyman et al. 2000; Honeyman et al. 2010), herpesviruses (cytomegalovirus and Epstein–Barr virus) (Pak et al. 1988; Parkkonen et al. 1994; Tirabassi et al. 2010), and varicella zoster virus (der Werf et al. 2007). Mechanisms by which these viruses may trigger T1D remain unclear.

Although some viruses have been shown to promote T1D pathogenesis, viral infections have also been shown to have a protective role in T1D (Lehuen et al. 2010). For example, Infection of Coxsackie virus B3 or lymphocytic choriomeningitis virus (LCMV) were found to delay diabetes onset and reduce disease incidence in prediabetic NOD mice, which is achieved by triggering

CD4+CD25+ Treg cells (Filippi et al. 2009). This dual role of viruses in the pathogenesis of T1D depends on the timing of the infection and the type of pathogen. In addition, the different effects of viruses on β cells or other components involved in T1D might be due for this dual role. Nevertheless, it is obvious that viral infection belongs to environment factors that may contribute to the development of T1D.

1.7. Study purposes

The purposes of the present work are as following:

(1) Proinsulin is a major autoantigen in T1D, and proinsulin-specific T cells play an important role in the autoimmune process, therefore, the first aim is to determine proteases involved in proinsulin processing and presentation by MHC class II molecules.

(2) The identification of T cell epitopes is crucial for understanding the autoimmune process and may lead the way to a successful treatment of T cell driven autoimmune diabetes, thus, the second aim is to identify novel proinsulin-derived T cell epitopes by using PBMC from T1D patients.

(3) T1D is a proinflammatory state characterized by increased levels of circulating biomarkers of inflammation and TLRs play a critical role in the pathogenesis of T1D, therefore, the third aim is to analyze whether the exposure of PBMC with proinflammatory cytokines or TLR1-9 ligands might increase cathepsin activity.

(4) Vitamin D, as an immunomodulator, might be beneficial for T1D, therefore, the fourth aim is to characterize the regulation of endocytic cathepsins by vitamin D in primary APC.

(5) A link between Epstein–Barr virus (EBV) infection and the onset of T1D has been shown, therefore, the next aim is to address the impact of EBV infection on proinsulin processing and cathepsin activity.

(6) CatG plays a role in antigen processing, however, the role of CatG in Ii processing, a critical step in MHC class II loading, is unknown, thus, whether CatG is involved in the initial step of Ii processing in primary APC will be investigated.

2. Materials and methods

2.1. Materials

2.1.1. T cell assay and enzyme-linked immunosorbent assay

Bovine serum albumin (BSA), pH 7.0	SERVA Electrophoresis GmbH, Heidelberg, Germany
Blocking buffer	1% BSA in PBS
Cell culture medium	10% FCS, 100 U/ml penicillin/streptomycine in RPMI 1640 medium
Cell culture plate	48-well multiwell plate
DCins peptides	Synthesized by Hubert Kalbacher, University of Tübingen, Tübingen, Germany
Delipidized bovine serum	R&D Systems, Minneapolis, MN, USA
DuoSet ELISA kits (TNF-α, IFN-γ, IL-6, IL-17, IL-22, TGF-β1)	R&D Systems, Minneapolis, MN, USA
Fetal calf serum (FCS)	Invitrogen, Carlsbad, CA, USA
HEPES	Sigma-Aldrich, Taufkirchen, Germany
PBS (1x), liquid, pH: 7.4	Invitrogen, Carlsbad, CA, USA
PBS (10x), liquid, pH: 7.2	Invitrogen, Carlsbad, CA, USA
Penicillin/streptomycine	Invitrogen, Carlsbad, CA, USA
RPMI 1640 medium (1x), liquid	Invitrogen, Carlsbad, CA, USA
Reagent diluent A	1% BSA in PBS
Reagent diluent B	0.1% BSA, 0.05% Tween 20 in PBS
Reagent diluent C	1% BSA, 0.05% Tween 20, and 1.4% delipidized bovine serum in PBS
Stop solution (1 M H_2SO_4)	Sigma-Aldrich, Taufkirchen, Germany
Substrate solution	1:1 mixture of reagent A (H_2O_2) and reagent B (tetramethylbenzidine) (R&D Systems, Minneapolis, MN, USA)
1x TMB ELISA substrate solution	eBioscience, San Diego, CA, USA

TGF-β1 activation solution	1 N HCl (Riedel de Haën, Seelze, Germany)
TGF-β1 neutralization solution	1.2 N NaOH, 0.5 M HEPES
Tween 20	Sigma-Aldrich, Taufkirchen, Germany
48-well multiwell plate	BD, Franklin Lakes, NJ, USA
96-well half area ELISA microplate	Greiner Bio-One, Frickenhausen, Germany
Polypropylene tubes, conical bottom (50 ml, 15 ml)	Greiner Bio-One, Frickenhausen, Germany
Washing buffer (PBS-T)	0.05% Tween 20 in PBS

2.1.2. Multiparameter FACS

Anti-human CD4 Alexa Fluor 700	BD, Franklin Lakes, NJ, USA
Anti-human CD25 eFluor 450	eBioscience, San Diego, CA, USA
Anti-human CD127 PE	BD, Franklin Lakes, NJ, USA
Anti-human Foxp3 Alexa Flour 488	eBioscience, San Diego, CA, USA
Anti-human HLA-DR PE/Cy7	Biolgend, San Diego, California, USA
Anti-mouse Ig, κ/negative control compensation particles set	BD, Franklin Lakes, NJ, USA
Blocking buffer	1% BSA in PBS
Cell culture dish	Greiner Bio-One, Frickenhausen, Germany
Cell culture plate, 48 wells	Corning, NY, USA
Cytofix/cytoperm fixation and permeabilization solution	BD, Franklin Lakes, NJ, USA
FITC Annexin V apoptosis detection kit	BD, Franklin Lakes, NJ, USA
PE/Cy7 mouse IgG2a, κ isotype control	Biolgend, San Diego, California, USA
Perm/wash buffer	BD, Franklin Lakes, NJ, USA
Round-bottom tube (5 ml)	BD, Franklin Lakes, NJ, USA
96-well polystyrene cell culture microplate, clear	Greiner Bio-One, Frickenhausen, Germany

2.1.3. Cell isolation, cell stimulation, active site label, and western blot

Amersham hybond-P membrane	GE Healthcare, Chalfont St. Giles, UK
Anti-β-actin antibody	Sigma-Aldrich, Taufkirchen, Germany
Anti-CatD antibody	Calbiochem, Schwalbach, Germany
Anti-CLIP antibody	Kind gift from Hubert Kalbacher, University of Tübingen, Tübingen, Germany
Anti-EBNA	Antibodies-online GmbH, Aachen, Germany
Anti-HLA-DR antibody (CHAMP)	Kind gift from Lawrence J Stern, University of Massachusetts, MA, USA
Beads buffer	0.5% BSA, 2 mM EDTA in PBS
Buffy coats, HCMV-seronegative	Red cross blood bank Ulm
Cathepsin G from human sputum	Sigma-Aldrich, Taufkirchen, Germany
Cathepsin G inhibitor I	Calbiochem, Schwalbach, Germany
CD1c (BDCA-1) dendritic cell isolation kit	Miltenyi Biotec, Auburn, CA, USA
CD11b microbeads	Miltenyi Biotec, Auburn, CA, USA
CD14 microbeads	Miltenyi Biotec, Auburn, CA, USA
CD304 (BDCA-4/neuropilin-1) microbead kit	Miltenyi Biotec, Auburn, CA, USA
Cell culture flask	BD, Franklin Lakes, NJ, USA
Cholecalciferol	Sigma-Aldrich, Taufkirchen, Germany
$1\alpha,25(OH)_2D_3$	Sigma-Aldrich, Taufkirchen, Germany
Citric acid	Sigma-Aldrich, Taufkirchen, Germany
Coomassie plus reagent	Pierce Biotechnology, Rockford, IL, USA
DAP22c	MP Biomedicals, Solon, OH, USA
DCG-04	Kind gift from Matthew Bogyo, Stanford University, Palo Alto, CA, USA
Dulbecco's modified eagle medium (DMEM), 1x, liquid	Invitrogen, Carlsbad, CA, USA
DTT	SERVA, Heidelberg, Germany

ECL advance blocking reagent	GE Healthcare, Chalfont St. Giles, UK
ECL plus western blotting	GE Healthcare, Chalfont St. Giles, UK
ECL HRP chemoluminescent substrate reagent kit	Invitrogen, Carlsbad, CA, USA
EDTA	Sigma-Aldrich, Taufkirchen, Germany
E64d	Enzo Life Science, Loerrach, Germany
Ficoll-paque plus	GE Healthcare, Chalfont St Giles, UK
Hemocytometer	Carl Roth GmbH, Karlsruhe, Gemany
Hydrogen chloride	Sigma-Aldrich, Taufkirchen, Germany
Hyperfilm ECL	GE Healthcare, Chalfont St. Giles, UK
IL-1β	R&D Systems, Minneapolis, MN, USA
IL-4	PeproTech, Hamburg, Germany
IL-17	PeproTech, Hamburg, Germany
IFN-γ	PeproTech, Hamburg, Germany
LPS	Sigma-Aldrich, Taufkirchen, Germany
LS column	Miltenyi Biotec, Auburn, CA, USA
Luminol TM-6 solution A/B	GE Healthcare, Chalfont St. Giles, UK
Lysis buffer	20 mM Tris pH 7.0, 150 mM NaCl, 1% NP-40
MACS separator	Miltenyi Biotec, Auburn, CA, USA
Mars116	Synthesized by Marcin Sieńczyk, Warsaw University of Technology, Wroclaw, Poland
β-mercaptoethanol	Sigma-Aldrich, Taufkirchen, Germany
Methanol	Sigma-Aldrich, Taufkirchen, Germany
Me-Val-Pro-Phe^{p} $(OPh)_2$ (MeSucVPF)	Synthesized by Jozef Oleksyszyn and Marcin Sieńczyk, Warsaw University of Technology, Wroclaw, Poland
Novex 12% tris-glycine mini gel, 1.0 mm, 12 wells	Invitrogen, Carlsbad, CA, USA
Novex tris-gycine SDS-running buffer (10x)	Invitrogen, Carlsbad, CA, USA

Novex tris-glycine SDS-transfer buffer (25x)	Invitrogen, Carlsbad, CA, USA
NuPAGE novex 10% bis-tris midi gel	Invitrogen, Carlsbad, CA, USA
NuPAGE LDS sample buffer (4x)	Invitrogen, Carlsbad, CA, USA
NuPAGE MOPS SDS running buffer (20x)	Invitrogen, Carlsbad, CA, USA
NuPAGE transfer buffer (20x)	Invitrogen, Carlsbad, CA, USA
NP-40	Sigma-Aldrich, Taufkirchen, Germany
Pan T cell isolation kit	Miltenyi Biotec, Auburn, CA, USA
Pepstatin A (PepA)	Calbiochem, Darmstadt,Germany
Pepstatin A-penetratin (PepA-P)	Kind gift from Hubert Kalbacher, University of Tübingen, Tübingen, Germany
Precision plus protein standards kaleidoscope	Bio-Rad, Hercules, CA, USA
Reaction tube 1.5 ml	Eppendorf, Hamburg, Germany
Roti block	Carl Roth GmbH, Karlsruhe, Gemany
Trypan blue	Sigma-Aldrich, Taufkirchen, Germany
Sodium chloride	Sigma-Aldrich, Taufkirchen, Germany
Stericup and steritop vacuum filter cups	Millipore Corporation, Billerica, MA, USA
Suc-Val-Pro-Phe^{p} $(OPh)_2$ (SucVPF)	Synthesized by Jozef Oleksyszyn, Warsaw University of Technology, Wroclaw, Poland
Suc-Val-Pro-Phe-pNA	Synthesized by Marcin Sieńczyk, Warsaw University of Technology, Wroclaw, Poland
TGF-β1	PeproTech, Hamburg, Germany
TNF-α	PeproTech, Hamburg, Germany
TNF-α	eBioscience, Frankfurt, Germany
Tris	Sigma-Aldrich, Taufkirchen, Germany
VECTASTAIN Elite ABC system	Vector labs, Inc., Burlingame, CA, USA

West pico stable luminol solution	Thermo Fisher Scientific, Rockwood, IL, USA
West pico stable peroxide solution	Thermo Fisher Scientific, Rockwood, IL, USA
X-ray cassette 24x30	Rego X-ray, Augsburg, Germany

2.1.4. Laboratory equipments

BD LSRII flow cytometer	BD Biosciences, Franklin Lakes, NJ, USA
Centrifuge (minispin)	Eppendorf, Hamburg, Germany
Centrifuge (multifuge 3S-R)	Heraeus, Hanau, Germany
Costar stripette serological pipette	Corning Inc., Corning, NY, USA
Eppendorf reference pipettes	Eppendorf, Hamburg, Germany
Freezer	Liebherr, Bulle, Schweiz
Heating block (Thermostat 5320)	Eppendorf, Hamburg, Germany
Incubator (BBD 6220)	Heraeus, Hanau, Germany
Laminar flow hood (HA 2448)	Heraeus, Hanau, Germany
Magnetic stirrer (Ikama Rec-G)	Ika Labortechnik, Staufen, Germany
Microscope (Olympus IMT-2)	Olympus, Tokyo, Japan
Microtiterplate reader (EL808)	BioTek, Winooski, Vermont, USA
Nitrogen tank (Chronos 400)	Messer, Sulzbach ,Germany
Pipet-boy	INTEGRA Biosciences, Fernwald, Germany
Power supply (EBS 500/400)	Bosch, Gerlingen, Gemany
Refridgerator	Liebherr, Bulle, Schweiz
Semi-dry electroblotting system	Thermo Fisher Scientific, Rockwood, IL, USA
Shaker (Rotamax 120)	Heidolph, Schwabach, Germany
Water (3043)	Köttermann, Hänigsen, Germany
XCell surelock mini-cell	Invitrogen, Carlsbad, CA, USA

2.2. Methods

2.2.1. Peptide synthesis

Lang et al. described that porcine insulin-derived intermediates did not activate T cells when using a fixed B cell line as a source of APC. In contrast, a non-fixed B cell

line was able to activate T cells, demonstrating that intermediates required further processing within the endocytic compartments in order to become T cell epitopes (Lang et al. 1996). Therefore, the destination of non-reduced proinsulin within the antigen-processing compartment was mimicked in an *in vitro* digestion experiment by incubating proinsulin with purified cathepsins, CatD, G, S, and V, for 2 h at 37℃. In addition, primary human B cell- or mDC1-derived lysosomal cathepsins were also used to digest proinsulin. The resulted peptides were synthesized by the solid phase Fmoc strategy on a multiple peptide synthesizer Syro II (MultiSynTech, Witten, Germany). Activation and coupling were performed with O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU), NHydroxybenzotrizole (HOBT), and N-methylmorpholine (NMM) (Merck, Darmstadt, Germany). Peptides were purified by reversed-phase high performance liquid chromatography (HPLC) using a C18 column 125×8 (Grom, Herrenberg, Germany) and analyzed by mass spectrometry (Reflex IV, Bruker Daltonics, Bremen, Germany).

2.2.2. T cell assay

T cell assay is a major method to assess the functional response of CD4+ T cells to various stimuli. T cells can be stimulated to proliferate by using antigens *in vitro,* and cytokine secretion is measured to evaluate their response. In the present study, TNF-α and IFN-γ represent Th1-type cytokines, IL-17 and IL-22 represent Th17-type cytokines, IL-6 or TGF-β1 represent Th2- or Treg-type cytokines, respectively. The procedure of T cell assay used here is described in Figure 5. New onset adult T1D donors were defined as a clinical diagnosis (<1 year). PBMC from both T1D donors and healthy control donors were Ficoll-isolated from heparinized blood as described in 2.2.6 and cryo-preserved in liquid nitrogen. Cryo-preserved PBMC were thawed at 37℃ and washed in 10 ml comple te roswell park memorial institute medium (RPMI) 1640 (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin/streptomycin). This wash procedure was repeated once and PBMC (1×10^6 cells/well) were plated to 48-well cell culture plate in a total volume of 400 µl per well

with or without indicated stimuli. After an incubation period of five days, supernatants were collected to detect cytokine secretion by enzyme-linked immunosorbent assay (ELISA). The use of PBMC for *in vitro* studies was approved by local ethics committee.

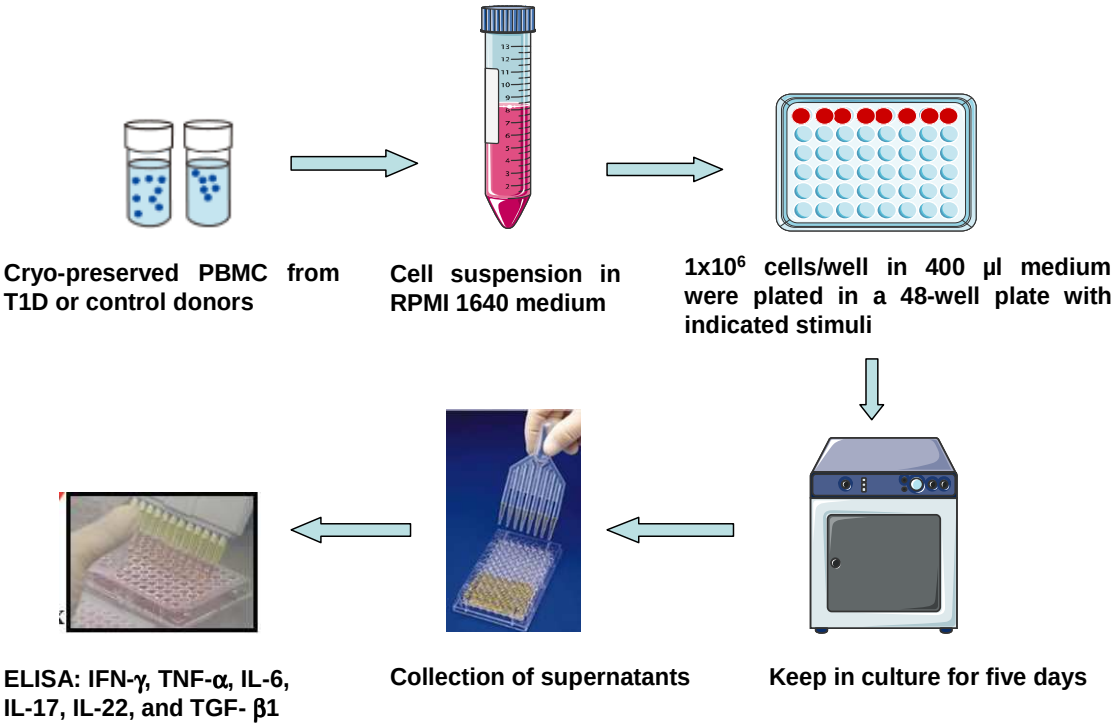


Figure 5: The procedure of T cell assay. Cryo-preserved PBMC from T1D or control were suspended in RPMI 1640 medium, then plated in 48-well plate with 400 µl medium in the presence or absence of indicated stimuli. After incubation of five days, supernatants were collected to detect cytokine production by using ELISA. PBMC: peripheral blood mononuclear cells, T1D: type 1 diabetes, RPMI: roswell park memorial institute medium, ELISA: enzyme-linked immunosorbent assay

2.2.2.1. T cell assay using cathepsin inhibitors

In an initial experiment, the class of proteases involved in proinsulin processing and presentation was determined. To this end, previously published cell permeable cathepsin inhibitors, such as cathepsin G inhibitor I (CatGinh), pepstatin A-penetratin (PepA-P), and E64d in a final concentration of 10 µM, respectively, were co-incubated with proinsulin (10 µg/ml) and PBMC from T1D or control donors (HLA-DRB1*0401/0301) for five days (37℃, 6% CO_2, 94% relative humidity). A final

concentration of 0.1% DMSO served as a vehicle control. Supernatants were collected and stored at -20℃ for detection of cyto kine secretion (IFN-γ, TNF-α, IL-17, IL-22, IL-6, and TGF-β1) by ELISA. Proinsulin was used in this functional T cell assay, since in prior publication T cells did not respond to insulin protein (Mannering et al. 2009).

2.2.2.2. T cell assay with proinsulin-derived peptides (DCins peptides)

DCins peptides (DCins1-16) or P17 (10 µg/ml, 6 µM) were co-cultured with PBMC from T1D (HLA-DRB1*0401/0701, HLA-DRB1*0301/0301) or HLA matched control donors for five days. P17 known as a T cell epitope severed as a positive control. Supernatants were collected to determine the level of secreted cytokines by ELISA as described in 2.2.3.

2.2.2.3. T cell assay with vitamin D

To test the hypothesis that vitamin D might affect the antigen processing machinery in primary APC, PBMC from T1D or control donors (HLA-DRB1*0401/0301) were pulsed with proinsulin (10 ug/ml) in the presence or absence of vitamin D (the prohormone cholecalciferol, vitamin D_3 or the active form of vitamin D, $1\alpha,25(OH)_2D_3$, 100 ng/ml) for five days. DMSO in a final concentration of 0.1%, was used as a vehicle control. Supernatants were harvested to detect cytokine secretion as mentioned above.

2.2.2.4. T cell assay with EBV supernatant

A link between EBV infection and the onset of T1D has been reported in a rare number of cases. There is some evidence that EBV may be potentially capable of triggering T1D by molecular mimicry. On the other hand, it could be speculated that EBV infection has an influence on diabetogenic T cell activation *via* regulation of cathepsin activity. To test this hypothesis, primary human B cells (CD19+), mDC1 (CD1c+), and CD4+ T cells were freshly isolated from buffy coats of healthy blood donors using immunomagentic beads by magnetic-activated cell sorting (MACS) which is described under 2.2.6. B cells or mDC1 (3×10^5 cells/well) were plated in a

48-well plate with untouched CD4+ T cells (2.4×10^6 cells/well). Cells were pulsed with 80 µg/ml proinsulin with or without EBV supernatant (1×10^9 genomes/ml) in 400 µl of complete RPMI 1640 medium. CatGinh, E64d, or PepA were added to a final concentration of 10 µM. DMSO was added as a vehicle control (final concentration 0.1%). After five days of incubation, supernatants were collected and cytokine production (IFN-γ, TNF-α, IL-17, IL-22, IL-6, and TGF-β1) was analyzed by ELISA.

2.2.3. Enzyme-linked immunosorbent assay

Cytokine levels (IFN-γ, TNF-α, IL-17, IL-22, IL-6, and TGF-β1) in the supernatants of T cell assays were measured by DuoSet ELISA development kit following the protocols as described in the company's manual (R&D Systems, Minneapolis, MN, USA). First, a capture antibody specific to the cytokine of interest was bound to a 96-well microtiter plate to create the solid phase by using 100 µl of appropriately diluted antibody (Table 3) in phosphate buffered saline (PBS) and incubated overnight at 4℃ or 1 h at room temperature. Unbound antibody was removed by aspirating and washing the plate with washing buffer (PBS-T, 0.1% Tween 20 in PBS). This process was repeated twice for a total of three washes. Subsequently, blocking buffer (1% BSA in PBS) was added to prevent non-specific binding of proteins for 1 h incubation at room temperature. Aspiration/wash procedure was repeated three times and afterwards T cell assay supernatants were added in quadruplets. Dissociation of the latency-associated peptide (LAP) from the homodimer TGF-β1 was achieved by adding 1 M HCl (final concentration 0.2 M) to the T cell assay supernatants and 10 min of incubation at room temperature. Subsequently, neutralization was performed by adding 1.2 N NaOH (final concentration 0.25 M) and 0.5 M HEPES (final concentration 0.1 M). A seven point standard curve was created by using two-fold serial dilutions which started at 1000 pg/ml. Followed by an incubation of 2 h, the plate was washed and 100 µl of reagent diluents with the secondary biotin-conjugated detection antibody was added to incubate for 2 h (Table 3). Unbound detection antibody was removed by washing for three times, 100 µl of detection reagent (streptavidin-horseradish peroxidase (HRP))

was added in 1:200 dilution in the respective reagent diluent (Table 3) which was washed again after 20 min reaction in the dark. Then 1:1 mixture of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) and hydrogen peroxide was added. The color developed in proportion to the amount of bound analyte. Color development was stopped before saturation by adding 50 µl of 2 N sulfuric acid H_2SO_4 (final concentration 0.67 N). Finally, optical density of each well was measured by using a microplate reader set to 450 nm. The working condition was performed at room temperature if not indicated otherwise.

Table 3: Concentrations of ELISA working solutions

Cytokine	Capture-antibody concentration [µg/ml]	Detection-anibody concentration [ng/ml]	Standard concentration [pg/ml]	Streptavidin-HRP diluent	Reagent diluent
IFN-γ	4	50	1000 - 30	1:200	B
TNF-α	4	350	1000 - 30	1:200	A
IL-17	4	150	1000 - 30	1:200	A
IL-22	2	250	1000 - 30	1:200	A
IL-6	2	200	1000 - 30	1:200	A
TGF-β1	2	300	1000 - 30	1:200	C

Note: Reagent diluent A: 1% BSA in PBS; B: 0.1% BSA, 0.05% Tween 20 in tris-buffered saline; C: 1% BSA, 0.05% Tween 20, 1.4% delipidized bovine serum in PBS; HRP: horseradish peroxidase; IFN-γ: interferon-γ; IL: interleukin; TGF-β1: transforming growth factor-β1; TNF-α: tumor necrosis factor-α.

2.2.4. Multiparameter FACS

2.2.4.1. Detection of apoptosis by Annexin V

There is a consensus that proinflammatory cytokines like TNF-α are direct mediators of β cell death manifested by both apoptosis and necrosis (Ortis et al. 2008; Ortis et al. 2010). Apoptosis is the predominant form of cytokine-induced β cell death in human islets. Since DCins6 and DCins12 peptides can significantly stimulate the secretion of TNF-α in PBMC from HLA-DRB1*0401/0701 T1D donors, apoptosis induced by TNF-α secretion by DCins6/12 was tested. Apoptosis detection was analyzed by FITC Annexin V apoptosis detection kit I (BD Biosciences). In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner leaflet of the plasma membrane to the outer

leaflet, thereby exposing PS to the external environment. Annexin V is a calcium-dependent phospholipid-binding protein that has a high affinity for PS, and is useful for identifying apoptotic cells with exposed PS. Those stained both Annexin V and PI positive are late apoptotic or dead cells, positive for Annexin V and negative for PI are early apoptotic cells, and both negative are viable cells.

BON cells ($1x10^5$ cells/well), a human carcinoid cell line from pancreas, were used as a surrogate β cell line, 80% confluence were resuspended in 500 µl of complete DMEM medium (DMEM supplemented with 10% FCS, 100 U/ml penicillin /streptomycin) and treated with supernatants from T cell assay with indicated DCins peptides (DCins6, 12, and 10) or P17. DCins10 was used as a “non responder”, while TNF-α 20 ng/ml served as a positive control. After incubation of 24 h, cells were harvested and washed twice with cold PBS, then resuspended in 1x binding buffer at a concentration of $1x10^6$ cells/ml. 5 µl of FITC Annexin V and 5 µl PI for each sample were added, and incubated for 15 min at room temperature in the dark, afterwards, 400 µl of 1x binding buffer was added and subsequently detected by multiparameter fluorescence-activated cell sorting analysis (FACS) (BD Biosciences).

2.2.4.2. Detection of Treg cells by multiparameter FACS

It was reported that diabetogenic T cells can trigger the expansion of Treg cells by TNF-α release, a cytokine traditionally considered to be proinflammatory (Grinberg-Bleyer et al. 2010). Therefore, a possible effect of boosting Treg cells by TNF-α induced by DCins6/12 peptides was determined. DCins6 and DCins12 peptides, which induced highest *in vitro* secretion of TNF-α, were selected for Treg cell stimulation. DCins10 was used as a negative control, P17 as a positive control. PBMC from T1D and controls (HLA-DRB1*0401/0701) treated with DCins 6, 10, 12, and P17 for five days were harvested, followed by incubation with blocking buffer (1% BSA in PBS) for 10 min at room temperature and then incubated for 15 min with CD4-Alexa Flour 700, CD25-eFlour 450, and CD127-PE. After washing procedure, cells were incubated for 20 min with BD fix/perm (BD Biosciences) for intracellular

staining. An additional 10 min incubation with 10% human AB serum diluted in BD perm/wash was performed to block non-specific binding. Cells were stained with FoxP3-Alexa Flour 488 for 20 min and analyzed by using a BD LSR II flow cytometry. CD4+CD127lo/-CD25+ T cells were gated for Treg cells which was described before (Skrindo et al. 2008; Putnam et al. 2009).

2.2.4.3. Detection of MHC class II molecules by multiparameter FACS

It has been proposed that MHC class I and II molecules and the antigen processing pathway might be a target of EBV proteins to escape CD8+ and CD4+ T cell detection. Furthermore, EBV lytic infection is associated with the reduction of MHC class I and II expression (Keating et al. 2002). Therefore, the expression of MHC class II molecules on cell surface was analyzed after EBV exposure by flow cytometry. EBV-exposed B cells, mDC1 or pDC were stained with HLA-DR-PE/Cy7 antibody for 15 min at room temperature, then measured by a BD LSR II flow cytometer.

2.2.5. PBMC isolation

PBMC isolation was performed by ficoll density gradient centrifugation (Figure 6). Blood from HCMV-negative buffy coats was diluted 1:2 in PBS and 25 ml thereof overlaid carefully on 15 ml Ficoll-Paque Plus solution (GE Healthcare, Uppsala, Sweden). The centrifugation at 2000 rpm for 30 min resulted in the formation of different layers containing dinstict cell types due to differential migration. The interface layer containing the PBMC fraction was transferred, and washed three times with PBS by centrifugation at 1700 rpm for 5 min, followed by aspiration of the supernatant and resuspension in PBS. All of the following centrifugation steps were performed at 1700 rpm at 5 min if not indicated otherwise.

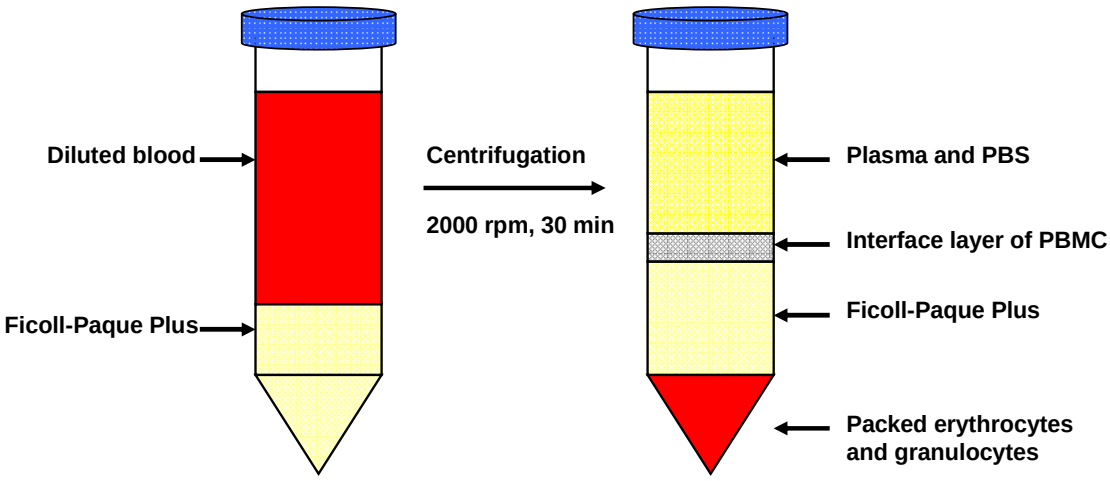


Figure 6: Isolation of PBMC by ficoll density gradient centrifugation. PBMC from either T1D patients or controls were isolated by using Ficoll-Paque Plus, the interface layer of PBMC was collected for further experiments. PBMC: peripheral blood mononuclear cells; T1D: type 1 diabetes.

2.2.6. MACS cell separation

MACS technology has been described to be the standard method for cell separation. It is based on MACS microbeads, specific monoclonal antibodies conjugated to superparamagnetic particles, which are only 50 nanometers in size. Freshly isolated PBMC were incubated with indicated microbeads for 15 min at 4℃, then cells labeled with MACS microbeads were retained in the magnetic field in a MACS column (LC column) placed in a MACS separator (Figure 7). The positively labeled fraction as well as the non-labeled (negative) fraction can be directly be used for downstream applications, such as cell analysis, stimulation, or functional assays.

2.2.6.1 Separation of CD19+ B cells, CD1c+ mDC1, and CD304+ pDC

The isolation of B cells (CD19+) and myeloid dendritic cells (mDC1, CD1c+) was performed using MACS separation kit for CD1c (BDCA-1+) (Miltenyi Biotec, Auburn, CA, USA). The surface marker CD1c+ is expressed on the major subset of mDC1 in the blood. A small subset of resting CD19+ B cells also expresses CD1c like mDC1, therefore mDC1 isolation is accomplished in two magnetic separation steps depleting B cells prior to isolation of mDC1.

Freshly isolated PBMC were resuspended in 4 ml of beads buffer (0.5% BSA, 2 mM EDTA in PBS). Subsequently, 500 µl of FcR blocking reagent, anti-CD19 microbeads and anti-CD1c-biotin each were added and incubated for 15 min at 4℃. Afterwards, cells were washed with beads buffer and resuspended in 5 ml of beads buffer for MACS separation.

For MACS separation, a LS column was placed in the magnetic field of a magnetic MACS separator. The column was equilibrated by rinsing with 3 ml of beads buffer and the cell suspension was applied into the column. The total effluent containing unlabeled cells was collected. The column was washed twice with 3 ml of beads buffer. After removing the column from the magnetic separator, the CD19+ B cells were eluted by adding 5 ml beads buffer to the column and applying a plunger. Cell elusion was repeated once. The collected effluent was centrifuged and the cell pellet resuspended in 4 ml of beads buffer. Labeling of CD1c+ mDC1 was achieved by using 500 µl of anti-biotin microbeads and incubating for 15 min at 4℃. Afterwards mDC1 were isolated according to CD19+ B cell separation as described above. The effluent after mDC1 isolation was collected to isolate pDC. After centrifugation, cell pellet was resuspened in 4 ml beads buffer and 500 µl of CD304 (BDCA-4/neuropilin-1) microbeads was added for incubation of 15 min at 4℃, then the isolation steps were the same as CD19+ B cell separation.

2.2.6.2 Separation of CD14+, CD11b+, and CD4+ cells

PBMC were isolated from buffy coats of healthy blood donors, suspended in 4 ml of beads buffer, then 500 µl of anti-CD14 microbeads was added and incubated for 15 min at 4℃. Afterwards, cells were washed with bead s buffer and resuspended in 5 ml of beads buffer for MACS separation as described above. The effluent was collected for isolation of CD11b+ macrophages and CD4+ T cell subsequently by using anti-CD11b microbeads and pan T cell microbeads, respectively.

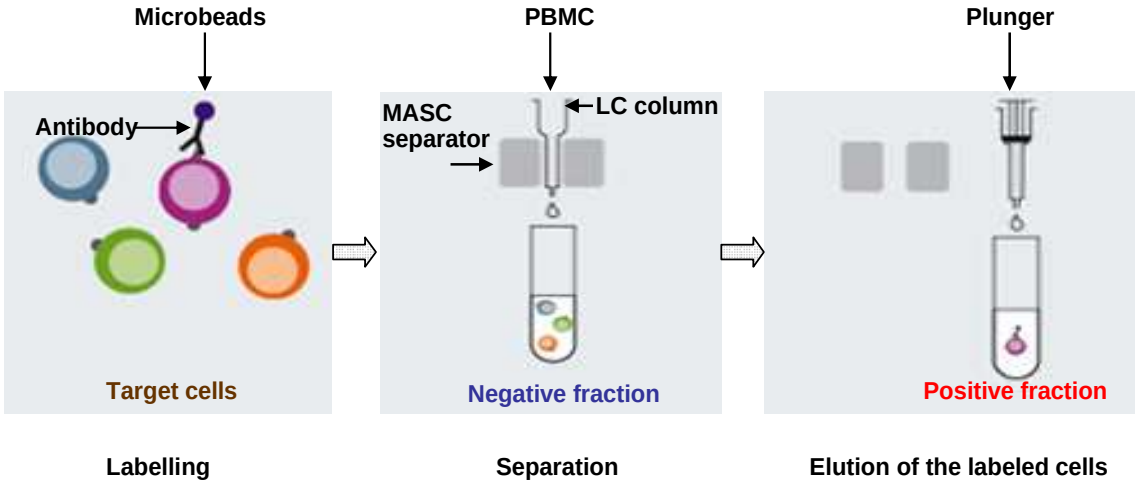


Figure 7: MACS cell separation. PBMC were labeled with indicated MACS microbeads for 15 min at 4℃. Cells were separated on a LC column pla ced in the magnetic field of a MACS separator and and the flow-through was collected as negative fraction. Then the MACS column was removed from the magnetic field and the magnetically retained cells as positively selected cells were eluted with beads buffe by using a plunger. PBMC: peripheral blood mononuclear cells; MACS: magnetic-activated cell sorting. Modified from http://www.miltenyibiotec.com/.

2.2.7. Exposure of primary cells with different stimuli

2.2.7.1. Cytokines or TLR ligands exposure to primary cells

Since higher CatG activity in PBMC from T1D patients was found, the question remains, which factor might be responsible for the high levels of CatG activity in PBMC? As putative regulators of CatG activity, cytokines and TLR ligands were tested. MACS-separated cells were counted using a hemocytometer and cell viability was assessed by adding trypan-blue in a 1:10 ratio. Equal numbers of cells (5-10 millions each) were splitted according to the experimental design. Freshly isolated PBMC, B cells, or mDC1 were stimulated with various cytokines, IL-1β (20 ng/ml, R&D System, Minneapolis, MN, USA), IL-4, IL-17, TNF-α, IFN-γ, TGF-β1 (20 ng/ml, PeproTech, Hamburg, Germany) in complete RPMI 1640 medium for 24 h at 37℃, in addition, TNF- α purchased from eBioscience (20 ng/ml, 50 ng/ml, 100 ng/ml, eBioscience, Frankfurt, Germany) and TLR ligands (Imgenex, San Diego, CA, USA) were tested as well. In other experiments, freshly isolated PBMC, CD14+, CD11b+, or CD4+ cells were treated with lipopolysaccharides (LPS) (0.1 ug/ml, 0.5

ug/ml, 1 ug/ml, 5 ug/ml, 10 ug/ml, Sigma-Aldrich, Taufkirchen, Germany) in complete RPMI 1640 medium for 24 h at 37℃. Cell pe llets were collected to perform active site label of active cathepsins and immunoblot of anti-HLA-DR antibody.

2.2.7.2. Exposure of B cells or mDC1 with vitamin D

For a better understanding of the activity of proteases after vitamin D exposure, freshly isolated B cells and mDC1 from controls were treated with vitamin D (30 ng/ml or 100 ng/ml, cholecalciferol or $1\alpha,25(OH)_2D_3$, Sigma-Aldrich, Taufkirchen, Germany), in complete RPMI 1640 medium for 24 h at 37℃. In the case of T1D-derived B cells and mDC1, cells were only treated with active vitamin D ($1\alpha,25(OH)_2D_3$,100 ng/ml) due to limited cell numbers. Since vitamin D was dissolved in DMSO, DMSO served as a vehicle control in the final concentrations of 0.03% and 0.1%, respectively. After incubation of 24 h, cell pellets were harvested for active site label and western blot by using anti-HLA-DR antibody.

2.2.7.3. Exposure of primary cells with Epstein-Barr virus

Human PBMC were freshly isolated from buffy coats of healthy blood donors, washed with PBS and adjusted to cell density of 16×10^6 cells/ml in complete RPMI 1640 medium. In addition, 4×10^6 PBMC were prepared to generate a B cell line. For EBV infection, PBMC were cultured in the presence of medium containing EBV supernatant (1×10^9 genomes/ml, EBV supernatant was previously obtained from the productive EBV B cell line B95-8) for different time points of 1, 5, 12, 14, 21 days at 37℃. Simultaneously, cells were cultured w ith medium without EBV as a control experiment. At each time point, PBMC were harvested for active site label of active cathepsins and immunoblot (anti-CatD antibody, anti-HLA-DR antibody, and anti-EBNA antibody).

The cathepsin activity differs significantly between immortalized EBV-transformed BLC and primary B cells (Burster et al. 2004), hence, the putative involvement of EBV in cathepsin regulation in primary APC was elucidated. In a separate experiment, B cells (CD19+), mDC1 (CD1c+), and pDC (CD304+) were positively

selected from buffy coats of healthy blood donors using the appropriate magnetic cell separation kit following the manufacturer's protocol as described in 2.2.6. Equal numbers of cells were splitted according to the experimental design. B cells, mDC, or pDC were incubated with or without EBV supernatant ($1x10^9$ genomes/ml) in complete RPMI 1640 medium for 24 h at 37℃. Cell pe llets were harvested to perform active site label of active cathepsins.

2.2.8. Cell lysis and protein quantification by Bradford assay

Cells were washed twice with cold PBS, then 30 µl lysis buffer (20 mM Tris pH 7.0, 150 mM NaCl, and 1% NP-40) was added and lysed on ice for 30 min. Cell debris was removed by centrifugation at 14,000 rpm for 10 min at 4℃ and the protein-rich supernatants were collected. Total protein quantification was achieved by colorimetric measurement using the coomassie plus kit (Pierce Biotechnology, Rockford, IL, USA). This method exploits the immediate shift in absorption maximum which occurs from 465 nm to 595 nm when coomassie dye binds to protein. Protein concentrations were estimated by comparison to absorption obtained from a series of two-fold standard protein dilutions of BSA ranging from 20 µg to 0.625 µg.

2.2.9. Active site label of endocytic proteases

In order to detect active proteases, active site-based labelling was performed by using activity-based probes (ABPs). ABPs can bind to the active center of proteases and then active proteases can be detected via avidin/POD in a western-blot-based assay. The schematic diagram and typical result of active site label was depicted in Figure 8. For detection of serine proteases, DAP (DAP22c) or Mars116 (Bt-LC-Suc-Val-Pro-Phe^P$(OPh)_2$) were used as ABPs. 20 µg of the cell lysate were incubated with DAP (2 µM) in PBS (pH 7.4) for 1 h at room temperature (Oleksyszyn and Powers 1991). In case of limited protein levels, 5 µg of the cell lysate were incubated with Mars116 (4 µM) in PBS (pH 7.4) for 1 h at room temperature. Alternatively, in order to visualize activity of cysteine proteases, DCG-04 (kindly donated by M. Bogyo, Stanford University, Palo Alto, CA, USA) was used as ABP. 5

µg of cell lysate were incubated with DCG-04 (10 µM) in reaction buffer (1 mM EDTA, 50 mM citrate [pH 5.0], and 50 mM DTT) for 1 h at room temperature (Greenbaum et al. 2000). Afterwards, samples were diluted in 4x sample loading buffer (0.01% bromphenol blue, 150 mM Tris, 2% SDS, 192 mM glycine, 10% β-mercaptoethanol) and boiled at 95℃ for 5 min to denature and linear ize proteins. Subsequently, samples were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 150 V for 2 h. A hybond polyvinylidene fluoride (PVDF) membrane was pre-wetted in 100% methanol for 1 min and equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Proteins were transferred to the PVDF membrane using semi-dry blotting procedure for 1 h at 170 mA.

After blotting, non-specific binding sites were blocked by immersing the membrane in blocking buffer (ECL advance blocking reagent in PBS-T) for 1 h at room temperature or overnight at 4℃, then the membrane was washed for 15 min thereby exchanging the wash buffer (PBS-T) three times. Visualization of active proteases bound to the PVDF membrane was accomplished by using the biotin/avidin system of VECTASTAIN Elite ABC kit (Vector Laboritories, Inc., Burlingame, CA, USA). This procedure is based on the extremely high affinity of biotin for the glycoprotein avidin, which results in a virtually irreversible binding of avidin to biotin. Avidin is conjugated to the biotinylated enzyme HRP. One drop of each VECTASTAIN ABC system reagent A and B, respectively, was added to 20 ml of PBS-T, and incubated for 30 min at room temperature. This allowed the avidin and biotinylated HRP macromolecular complex to preform. The membrane was incubated with the VECTASTAIN solution for 1 h with gentle agitation at room temperature. The ECL plus detection reagents (GE Healthcare, Buckinghamshire, UK) provided the chemiluminescent detection of the cathepsins. This method is based on the oxidation of the cyclic diacylhydrazide, luminol. HRP catalyses the oxidation of luminol generating acridinium ester intermediates. These intermediates react with hydrogen peroxide triggering a chemiluminescent emission at about 430 nm. The resulting light was detected on audioradiograpy films.

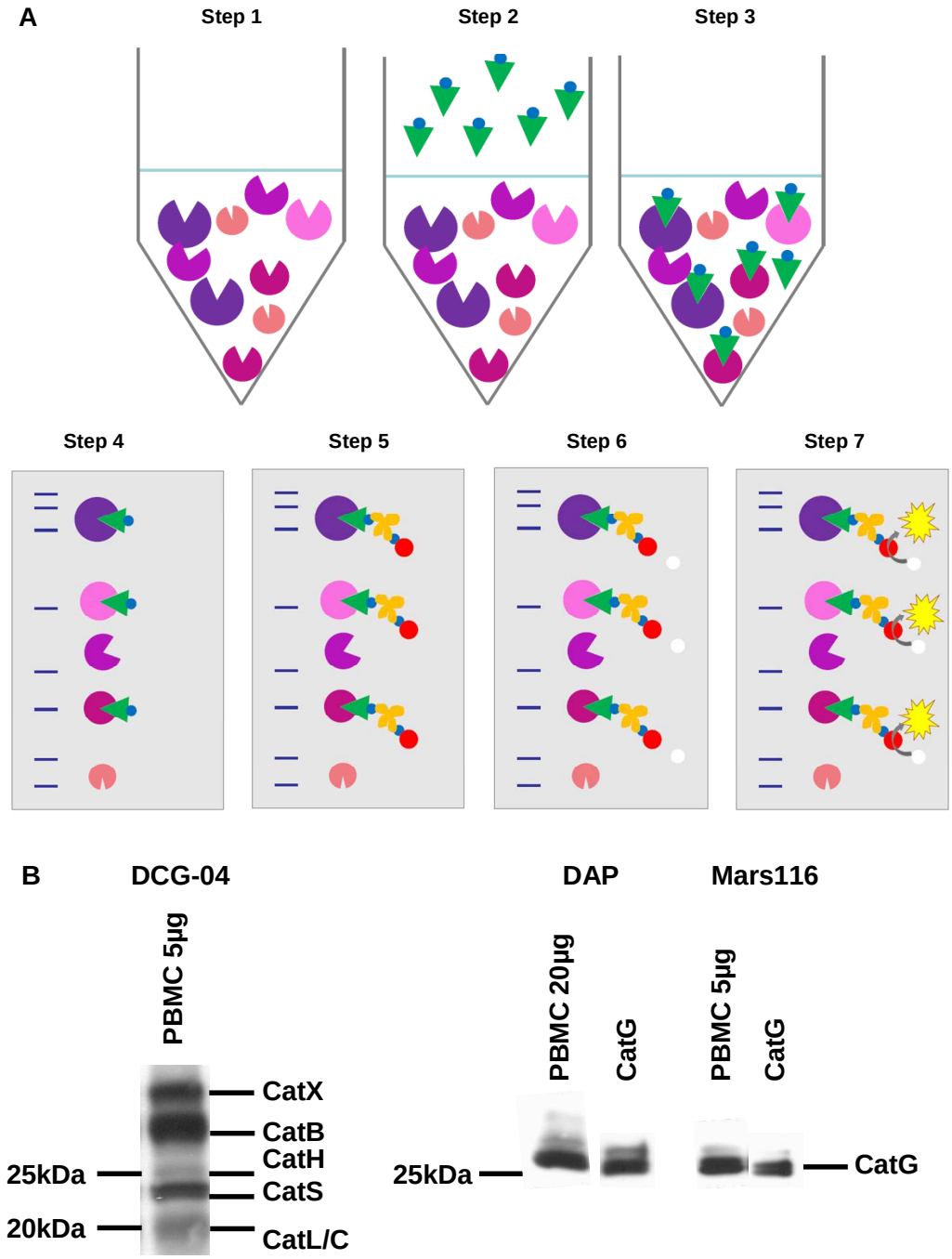


Figure 8: The schematic diagram and typical result of active site-based labeling. A) The schematic diagram of active site label. Step 1: Sample preparation, step 2: Addition of activity-based probes, step 3: Covalent binding of the activity-based probes to the samples, step 4: Separation of proteins via SDS-PAGE, transfer to PDVF membrane, step 5: Incubation with complex of avidin and biotinylated peroxidase, step 6: Addition of luminol, step 7: Light reaction and visualization of active proteases (Taken from A. Weigel). B) Active site label of PBMC. 5 µg cell lysate of PBMC were measured for their cysteine cathepsin activity by using DCG-04 (left panel), CatG activity was detected by using DAP (20 µg cell lysate) or Mars116 (5 µg cell lysate) (right panel). PBMC: peripheral blood mononuclear cells; PDVF: polyvinylidene fluoride; SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis.

2.2.10. Western blot

20 µg of protein were loaded on SDS-PAGE, blotted, and blocked as described in 2.2.9. Proteins of interest were detected by incubating the membranes with specific antibodies, like anti-CatD antibody (1:1000 dilution), anti-HLA-DR antibody (CHAMP) (1:5000 dilution), anti-CLIP antibody (1:1000 dilution), anti-EBNA antibody (1:1000), or anti-β-actin (1:25000 dilution) in PBS-T for 1 h at room temperature. The membranes were then incubated with HRP-conjugated specific secondary antibodies (1:5000 dilution) for 1 h in PBS-T at room temperature followed by three times of washing with PBS-T. Bound HRP-labeled antibodies were visualized using ECL plus detection kit as described above.

2.2.11. Determination of CatG activity

Kinetic measurement of CatG activity was accomplished by adding 15 µg of the indicated samples to the colorimetric substrate Suc-Val-Pro-Phe-pNA (200 µM, M. Sieńczyk, University of Wroclaw, Wroclaw, Poland) in buffer (0.5 M $MgCl_2$ in PBS, pH 7.4). The enzyme assays were performed in duplicates at 37℃, and the absorption was determined at 405 nm.

2.2.12. Preloading of BLC with CatG and inhibition of CatG in intact cells

It is well characterized that CatG plays a critical role in antigen processing (Burster et al. 2010b). Cysteine cathepsins, on the other hand, are critical in the degradation steps of invariant chain (Ii). Whether the serine protease CatG is involved in Ii degradation has not been investigated yet. In order to determine whether CatG plays a potential role in Ii degradation, human B cells expressing HLA-DRB1*0401/0401 were immortalized with EBV to generate a BLC. BLC cell lysate were incubated with different amounts of purified CatG (0.6 ng, 1.2 ng, 2.5 ng, 5.0 ng, 10 ng, 20 ng, 40 ng, 80 ng) (Sigma-Aldrich, Taufkirchen, Germany) under pH 7.4 or 5.0 conditions at 37℃ for 2 h, anti-CLIP im munoblot assay was used to visualize Ii-derived intermediates.

Previous investigations showed that exogenously added CatG can be internalized

into endocytic compartments of BLC (Burster et al. 2004). Therefore, purified CatG was added to viable BLC in order to observe Ii processing within intact cells. In a separate experiment, BLC were pretreated with different amounts of purified CatG (18, 9, 4.5, and 2.25 µg/ml) for 4.5 h at 37℃. Afterwards BLC were prepar ed for western blot by lysis in 10 mM Tris (pH 7.5), 150 mM NaCl, 0.5% NP-40, and CatG inhibitor (10 µM), to avoid post lysis degradation of Ii and followed by adjustment for equal total protein content (quantified by the Bradford assay as described in 2.2.8). Human primary B cells and mDC1 differ from BLC in their cathepsin distribution. Therefore, freshly isolated B cells, mDC1, or BLC were treated with several cell permeable CatG inhibitors (CatGinh, SucVPF, MeSucVPF), or E64d in a final concentration of 10 µM for 72 h and cell lysate were subjected to SDS-PAGE. Ii intermediates were analyzed by using anti-CLIP antibody.

2.3. Statistical analysis

Data depict means ± S.E.M. Statistical analysis was performed using one-way ANOVA (post test, Dunnett) or unpaired two-tailed Student's *t*-test (GraphPad Prism 5 software). A value of $p<0.05$ was considered to be significant.

3. Results

3.1. Downregulation of proteases within APC led to reduced proinsulin-reactive T cell activation

Proinsulin induced an adequate T cell activation in PBMC from T1D patients (HLA-DRB1*0401/0301) as analyzed by TNF-α secretion (Figure 9). In contrast, proinsulin did not activate T cells from control donors (HLA-DRB1*0401/0301), indicated by the high TNF-α secretion in the control sample (no antigen; no Ag). Co-treatment of PBMC with CatG inhibitor (CatGinh) resulted in reduced frequencies of T cell stimulation in T1D donors indicated by a 70% reduction of TNF-α release. E64d, PepA-P, and DMSO did not significantly decrease the secretion of TNF-α. Additionally, further proinflammatory cytokines, IFN-γ, IL-17, and IL-22, were analyzed, and found that inhibition of CatG activity downregulated these cytokine secretion significantly by 65-90% in PBMC from T1D patients (Figure 9 and Figure 10). E64d only affected IL-17 secretion with a 60% reduction in PBMC from T1D, PepA-P and DMSO had no effect in all the donors tested. In the case of IL-6 and TGF-β1, no differences were found in neither T1D nor control donors (Figure 11).

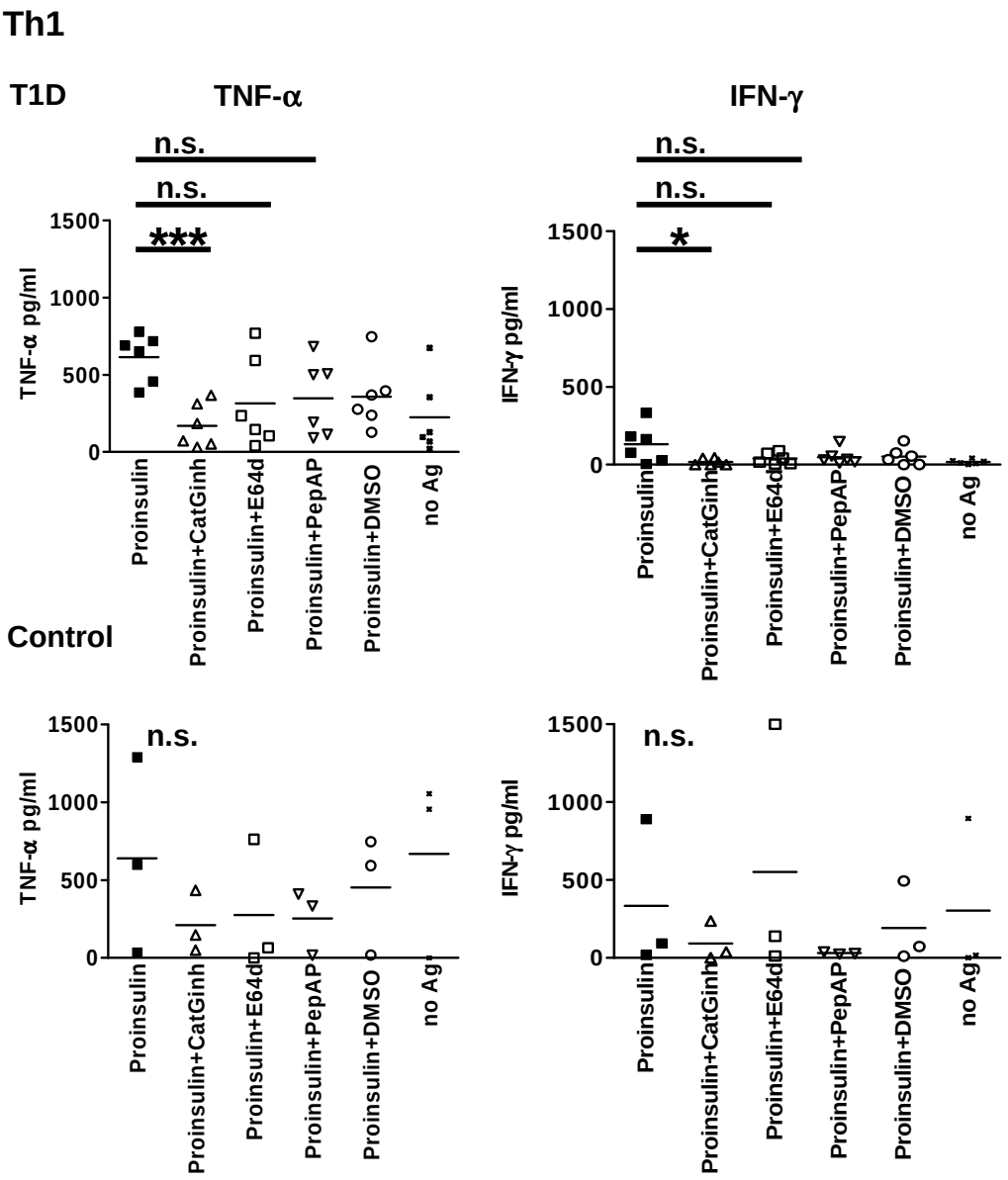


Figure 9: Th1-type cytokine secretion in the regulation of proinsulin presentation after treatment with selected inhibitors. PBMC from T1D and control donors (HLA-DRB1*0401/0301) were incubated with proinsulin (10 μg/ml) in the presence of CatG inhibitor (CatGinh), E64d, or pepstain A-penetratin (PepA-P) at 10 μM, respectively, for five days. DMSO served as a vehicle control in a final concentration of 0.1%. Th1-type cytokine production (TNF-α and IFN-γ) was analyzed by ELISA. ELISA was done in quadruplicates. T1D (n=6) *vs.* controls (n=3). Statistatical analysis was performed by using the unpaired, two-tailed Student's *t*-test. n.s., not significiant, * significant at $p<0.05$, *** significant at $p<0.001$. ELISA: enzyme-linked immunosorbent assay; HLA: human leukocyte antigens; IFN-γ: interferon-γ; PBMC: peripheral blood mononuclear cells; T1D: type 1 diabetes; Th: T helper cells; TNF-α: tumor necrosis factor-α.

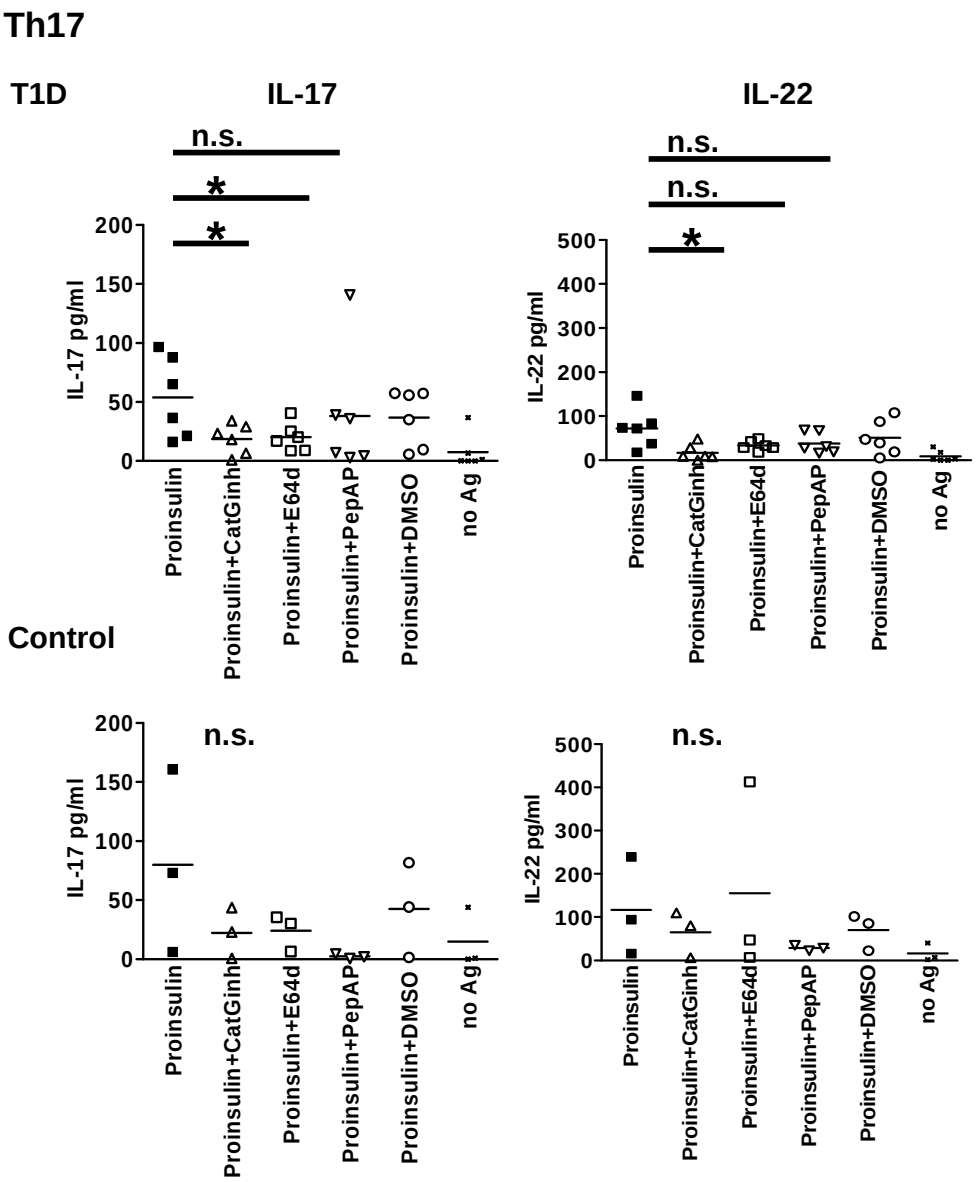


Figure 10: Th17-type cytokine secretion in the regulation of proinsulin presentation after treatment with selected inhibitors. PBMC from T1D and control donors (HLA-DRB1*0401/0301) were incubated with proinsulin (10 μg/ml) in the presence of CatG inhibitor (CatGinh), E64d, or pepstain A-penetratin (PepA-P) at 10 μM, respectively, for five days. DMSO served as a vehicle control in a final concentration of 0.1%. IL-17 and IL-22 were analyzed by ELISA. ELISA was done in quadruplicates. T1D (n=6) *vs.* controls (n=3). Statistatical analysis was performed by using the unpaired, two-tailed Student's *t*-test. n.s., not significiant, * significant at $p<0.05$. ELISA: enzyme-linked immunosorbent assay; HLA: human leukocyte antigens; IL: interleukin; PBMC: peripheral blood mononuclear cells; T1D: type 1 diabetes; Th: T helper cells.

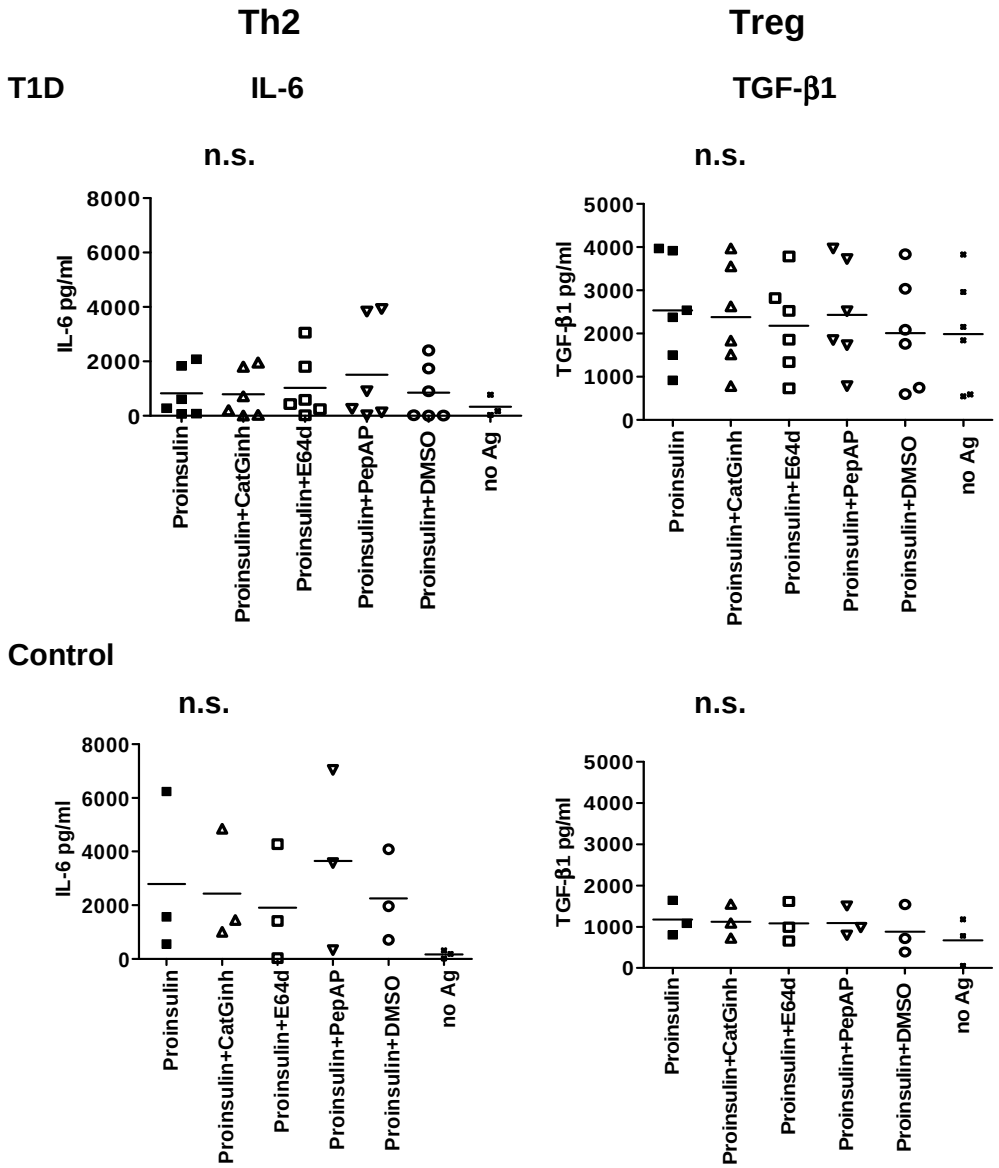


Figure 11: Th2- and Treg-type cytokine secretion in the regulation of proinsulin presentation after treatment with selected inhibitors. PBMC from T1D and control donors (HLA-DRB1*0401/0301) were incubated with proinsulin (10 µg/ml) in the presence of CatG inhibitor (CatGinh), E64d, or pepstain A-penetratin (PepA-P) at 10 µM, respectively, for five days. DMSO served as a vehicle control in a final concentration of 0.1%. IL-6 and TGF-β1 were analyzed by ELISA. ELISA was done in quadruplicates. T1D (n=6) *vs.* controls (n=3). Statistatical analysis was performed by using the unpaired, two-tailed Student's *t*-test. n.s., not significiant. ELISA: enzyme-linked immunosorbent assay; HLA: human leukocyte antigens; IL-6: interleukin-6; PBMC: peripheral blood mononuclear cells; T1D: type 1 diabetes; TGF-β1: transforming growth factor-β1; Th: T helper cells; Treg: regulatory T cell.

3.2. Processing of proinsulin *in vitro*

The proteolytic degradation pattern of proinsulin by B cell- or mDC1-derived lysosomal cathepsins, and purified CatG was identified. Intact proinsulin was found to be degraded into distinct intermediates. The tryptic, chymotryptic, and Leu-ase activity is restricted to human CatG compared to the chymotryptic activity of murine CatG (Raymond et al. 2010), therefore human CatG cleaved proinsulin after positively charged and aromatic amino acids as well as after leucine. Processing of proinsulin was increased at neutral pH compared to acidic conditions, as expected, since CatG displays maximal proteolytic activity at neutral pH measured by 2- to 5-fold increase in absorption compared to pH 5.0 (Figure 12). Having determined cleavage sites using purified CatG, the digestion pattern of proinsulin by using primary human B cell- or mDC1-derived lysosomal was analyzed. The majority of cleavage sites within proinsulin resembled those obtained with purified CatG (one key cleavage between LA at position C24/C25) (Figure 13).

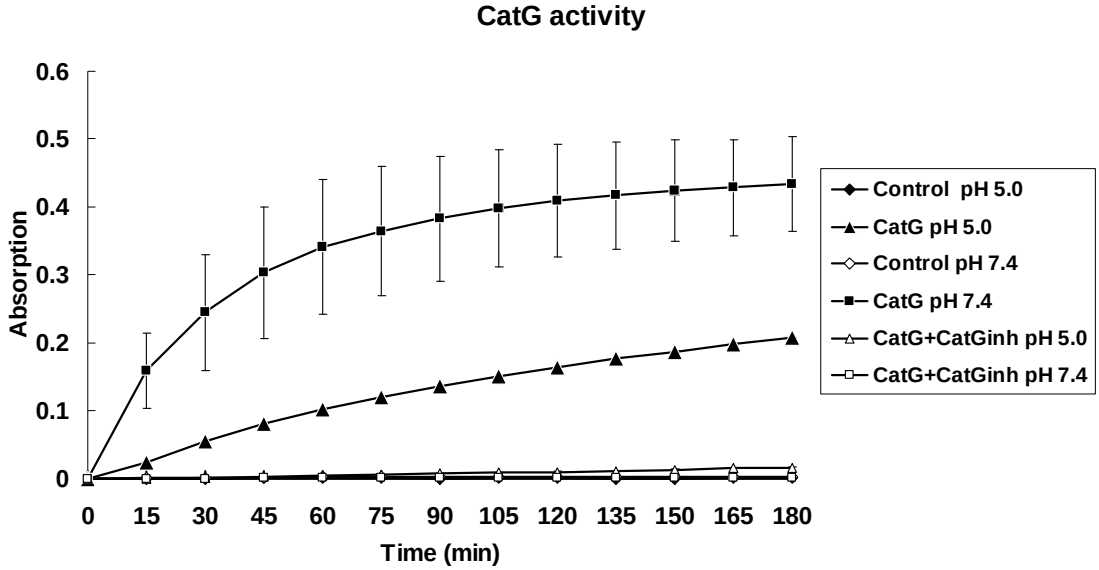


Figure 12: CatG activity under pH 5.0 or pH 7.4 conditions. CatG activity was analyzed by using the colorimetric substrate Suc-VPF-pNA (200 µM). CatG inhibitor was used in a final concentration of 1 µM. One of two independent experiments is shown. Experiments were done in duplicate, standard deviation was very low for CatG pH 5.0 and therefore not visible.

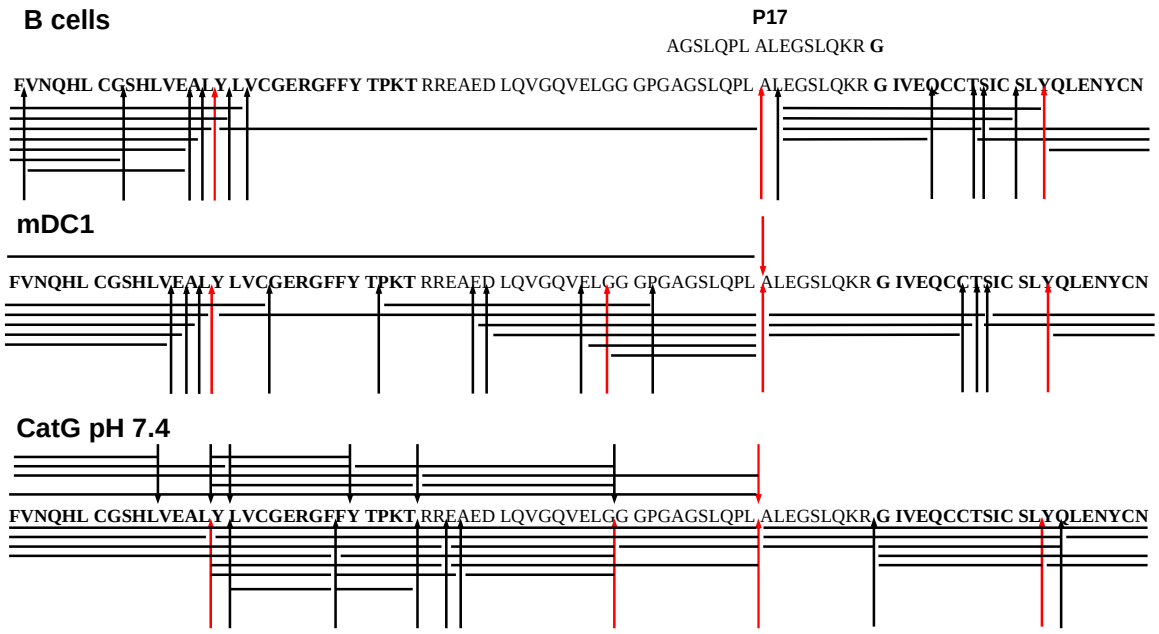


Figure 13: Processing of proinsulin by B cell- and mDC1-derived lysosomal cathepsins, or purified CatG. Proinsulin was incubated with B cell- and mDC1-derived lysosomal cathepsins, or purified CatG in vitro, respectively. The resulting fragments were analyzed by HPLC and mass spectrometry (LC-MS-MS and additionally MALDI-TOF-MS). P17 represents a known T cell epitope. Bars indicate identified fragments and arrowheads designate cathepsin cleavage sites, those arrowheads in red represent the same cleavage sites between B cell- or mDC1-derived lysosomal cathepsins and purified CatG. HPLC: high performance liquid chromatography; LC-MS-MS: liquid chromatography coupled with tandem mass spectrometry; MALDI-TOF-MS: matrix assisted laser desorption ionization/time of flight mass spectrometry.

3.3. T cells in PBMC from type 1 diabetes donors were activated by distinct antigenic peptides from *in vitro* processed proinsulin

3.3.1. CatG-generated proinsulin peptides induced distinct TNF-α secretion in PBMC from type 1 diabetes patients

Since DC are the APC par excellence to activate T cells, therefore the fragments obtained after proteolytic degradation of proinsulin using mDC1-derived lysosomal cathepsins were synthesized and named DCins1-16 (Figure 14). Strikingly, the generated fragment DCins10 was equal to a published peptide, which was protective in T1D and bond to DQB1*0602 (Ettinger and Kwok 1998). Thus, DCins10 was used as a “non-responder” and was compared to other DCins peptides in a T cell assay.

DCins1 (A14-A21) YQLENYCN	**DCins7** (C13-C24) GGGPGAGSLQPL	**DCins13** (C4-C24) DLQVGQVELGGGPGAGSLQPL
DCins2 (C25-A6) ALEGSLQKRGIVEQC	**DCins8** (B1-B12) FVNQHLCGSHLV	**DCins14** (A8-A21) TSICSLYQLENYCN
DCins3 (B1-B13) FVNQHLCGSHLVE	**DCins9** (C11-C24) ELGGGPGAGSLQPL	**DCins15** (A9-A21) SICSLYQLENYCN
DCins4 (B1-B14) FVNQHLCGSHLVEA	**DCins10** (B1-B15) FVNQHLCGSHLVEAL	**DCins16** (B1-B19) FVNQHLCGSHLVEALYLVC
DCins5 (C25-A8) ALEGSLQKRGIVEQCCT	**DCins11** (B28-C13) PKTRREAEDLQVGQVELGGG	**P17** (C18-A1) AGSLQPLALEGSLQKRG
DCins6 (C25-A7) ALEGSLQKRGIVEQCC	**DCins12** (C3-C24) EDLQVGQVELGGGPGAGSLQPL	

Figure 14: Sequences of the resulting intermediates (DCins peptides) after proinsulin digest. Sequences in red are CatG-generated fragments, which are DCins1, DCins2, DCins5, DCins6, DCins7, DCins9, DCins12, and DCins13.

It was found that levels of TNF-α secretion were 3- to 4-fold higher in response to DCins6 and DCins12 compared to DCins10 in HLA-DRB1*0401/0701 expressing T1D donors, and none of these peptides tested elevated the secretion of TNF-α in control donors (Figure 15A). In addition, there were no substantial differences in the levels of TNF-α incubated with the indicated DCins peptides in HLA-DRB1*0301/0301 T1D donors (Figure 15B). Several DCins peptides induced significant secretion of TNF-α with a 1-fold increase on average in controls, however, only one control donor was tested because of limited HLA-matched donors. Other cytokines such as IFN-γ, IL-17, and IL-22 were detected, but no differences between the DCins peptides were found in neither T1D nor control donors. Of note, in several donors the level of cytokines was below the threshold of the assay. In the case of IL-6 and TGF-β1, no significant differences were observed. Strikingly, DCins6 as well as the C-terminal end of DCins12 and DCins13 were generated by CatG after proteolytic digest of proinsulin (Figure 14), but only DCins6 and 12 induced distinct T cell activation from HLA-DRB1*0401/0701 T1D donors.

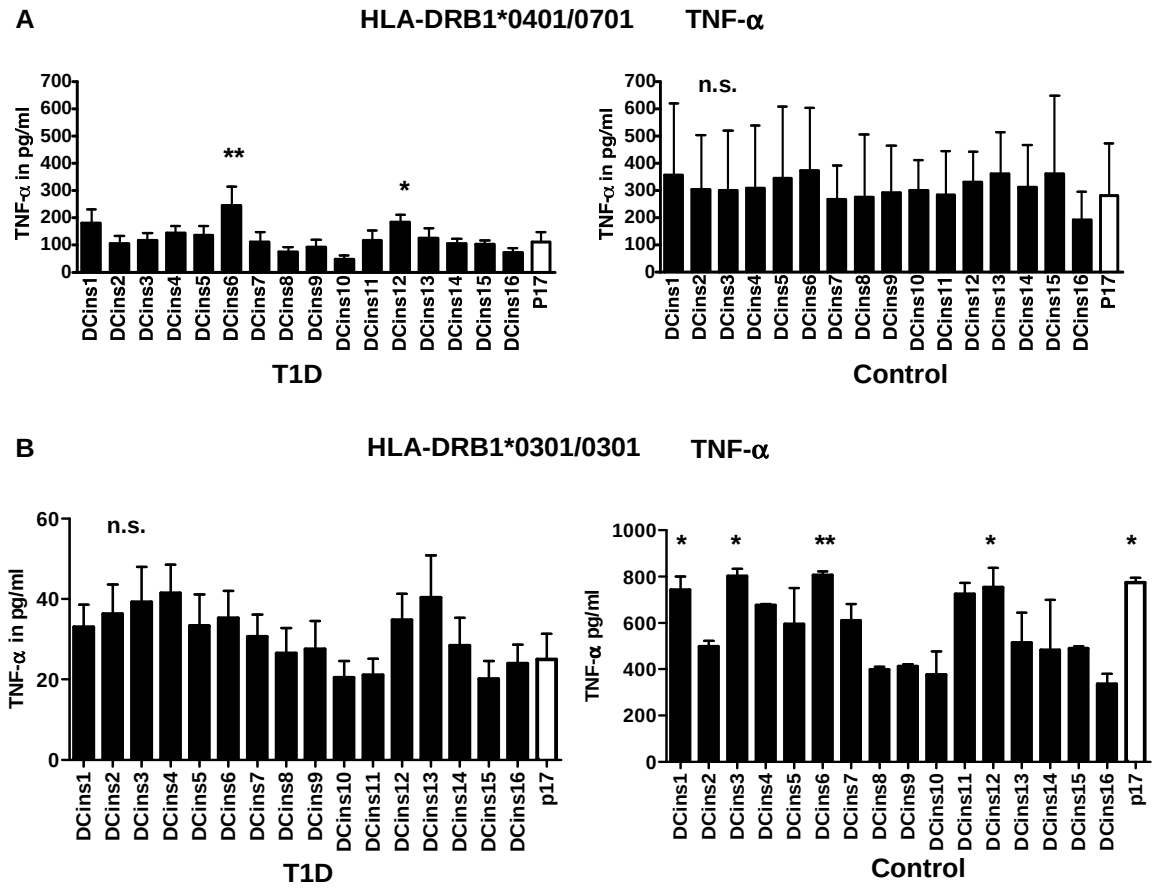


Figure 15: TNF-α secretion in the functional T cell assay with DCins peptides using human PBMC from T1D patients or controls. PBMC from T1D patients were incubated with DCins peptides or P17 (10 µg/ml) for five days. A) HLA-DRB1*0401/0701, n=6 T1D donors, n=2 controls. B) HLA-DRB1*0301/0301, n=5 T1D donors, n=1 control. P17 served as a positive control. TNF-α scretion was monitored using ELISA. ELISA was done in quadruplicate. Significance was determined by one-way ANOVA, post test Dunnett. Compared to DCins10, n.s., not significant, * significant at $p<0.05$, and ** significant at $p<0.01$. ANOVA: analysis of variance; ELISA: enzyme-linked immunosorbent assay; FITC: fluorescein isothiocyanate; HLA: human leukocyte antigens; PBMC: peripheral blood mononuclear cells; T1D: type 1 diabetes; TNF-α: tumor necrosis factor-α.

3.3.2. Increased TNF-α secretion did not induce apoptosis in BON cells

Proinflammatory cytokine, TNF-α, is one of the direct mediators to induce apoptosis and necrosis of β cells. DCins6 and DCins12 stimulated significant TNF-α secretion in the supernatants, however, It was found here that they did not induce apoptosis or death, compared to DCins10 (Figure 16).

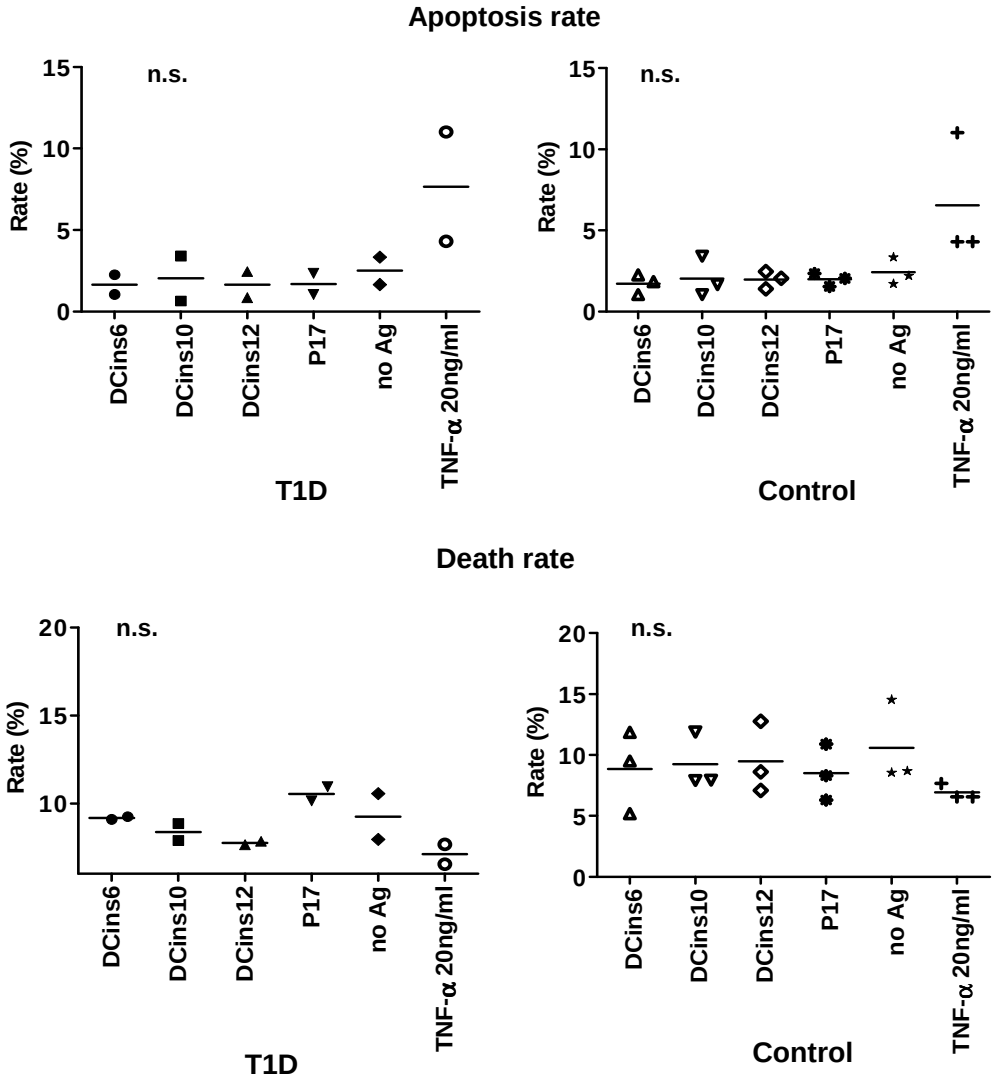


Figure 16： Apoptosis and death rate of BON cells induced by DCins peptides. BON cells were incubated with supernatants from T cell assay with indicated DCins peptides for 24 h. HLA-DRB1*0401/0701, n=2 T1D donors, n=3 controls. Apoptosis was detected by FITC-Annexin V. TNF-α 20 ng/ml served as positive control. Compared to DCins10, n.s, not significant. HLA: human leukocyte antigens; PBMC: peripheral blood mononuclear cells; T1D: type 1 diabetes; TNF-α: tumor necrosis factor-α.

3.3.3. TNF-α induced by DCins peptides did not boost Treg cells

Next, a possible effect of boosting Treg cells by TNF-α, which was induced by DCins6/12 supernatants, was determined. As shown in Figure 17, DCins6 and DCins12 peptides did not boost Treg cells in T1D or controls, compared to DCins10.

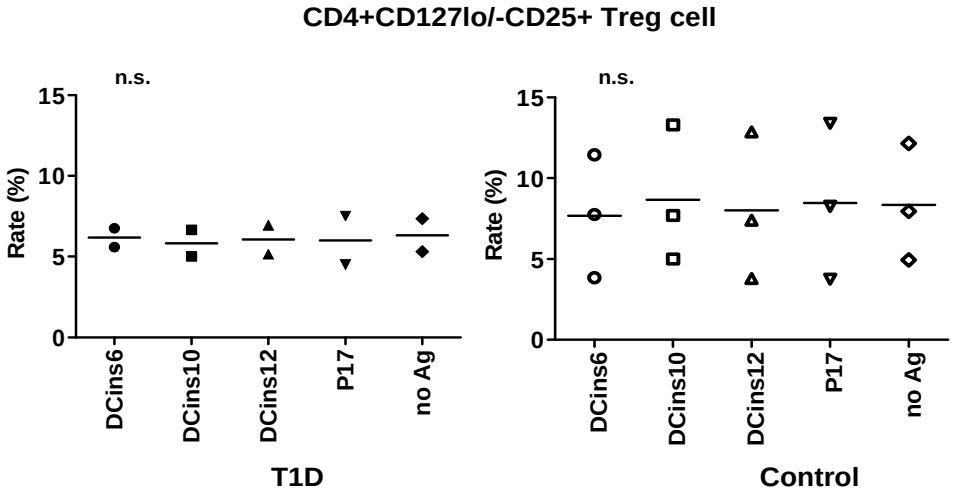


Figure 17: Treg cell stimulation by using supernatants from T cell assay with DCins peptides. DCins6, 10, 12 peptides or P17 were incubated with PBMC from T1D or controls (HLA-DRB1*0401/0701). Cells were harvested and stained for flow cytometry analysis. CD4+CD127lo/-CD25+ cells were gated as Treg cells. T1D donors n=2, controls n=3. Compared to DCins10, n.s, not significant. HLA: human leukocyte antigens; PBMC: peripheral blood mononuclear cells; T1D: type 1 diabetes; Treg cell: regulatory T cell.

3.4. Regulation of cathepsins after exposure to cytokines or TLR ligands

Higher CatG activity in PBMC from T1D patients was found, however, none of the cytokines tested (IL-4, TGF-β1, IL-1β, IL-17, TNF-α, and IFN-γ) altered CatG activity, whereas LPS slightly increased CatG activity in PBMC (Figure 18). Comparable levels of CatG activity were found in PBMC, CD14+, CD11b+, and CD4+ cells from the same donor after LPS treatment (titration from 0.1 µg/ml to 10 µg/ml) (Figure 19A and B). Notably, CatX, B, and S activity were considerably increased in CD14+ cells with concentration-dependent effects by LPS (Figure 19C). While other TLR ligands did not alter CatG activity in PBMC (Figure 20A), it was observed that TLR1/2, TLR3, and TLR5 upregulated the activity of CatX, B, and S (Figure 20B). In addition, the cathepsin activity of B cells or mDC1 after cytokine exposure was detected, CatG and CatD were downregulated in mDC1 by IFN-γ (Figure 21A and B) and CatG was reduced after LPS exposure in mDC1 (Figure 21C), these cytokines did not show any significant effects on B cells. Furthermore, the activity of cysteine proteases was not altered by these cytokines in mDC1 (Figure 21D). In contrast, MHC class II molecules were upregulated after IFN-γ exposure (Figure 21E).

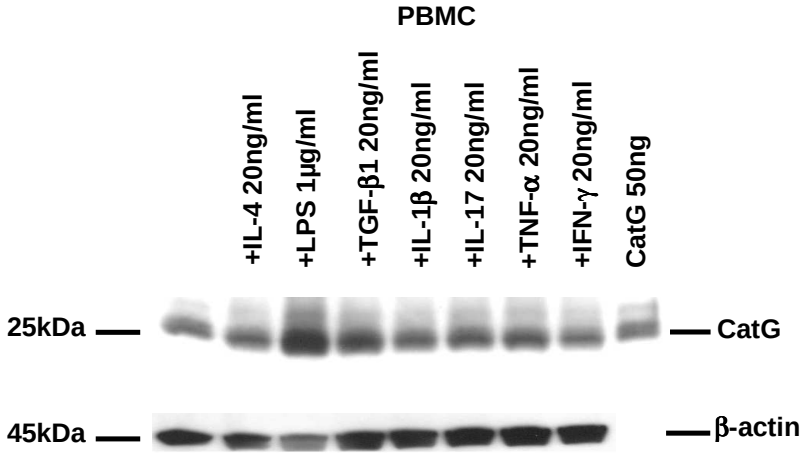


Figure 18: Regulation of cathepsin G in PBMC after cytokine or LPS exposure. Freshly isolated PBMC from buffy coats (n=3) were treated with LPS (1µg/ml) or selected cytokines (IL-4, TGF-β1, IL-1β, IL-17, TNF-α, and IFN-γ, 20 ng/ml) for 24 h. CatG activity of 20 µg cell lysate was detected by using DAP.One of three indepedent experiments is shown. IFN-γ: interferon-γ; IL: interleukin; LPS: lipopolysaccharides; PBMC: peripheral blood mononuclear cells; TGF-β1: transforming growth factor-β1; TNF-α: tumor necrosis factor-α.

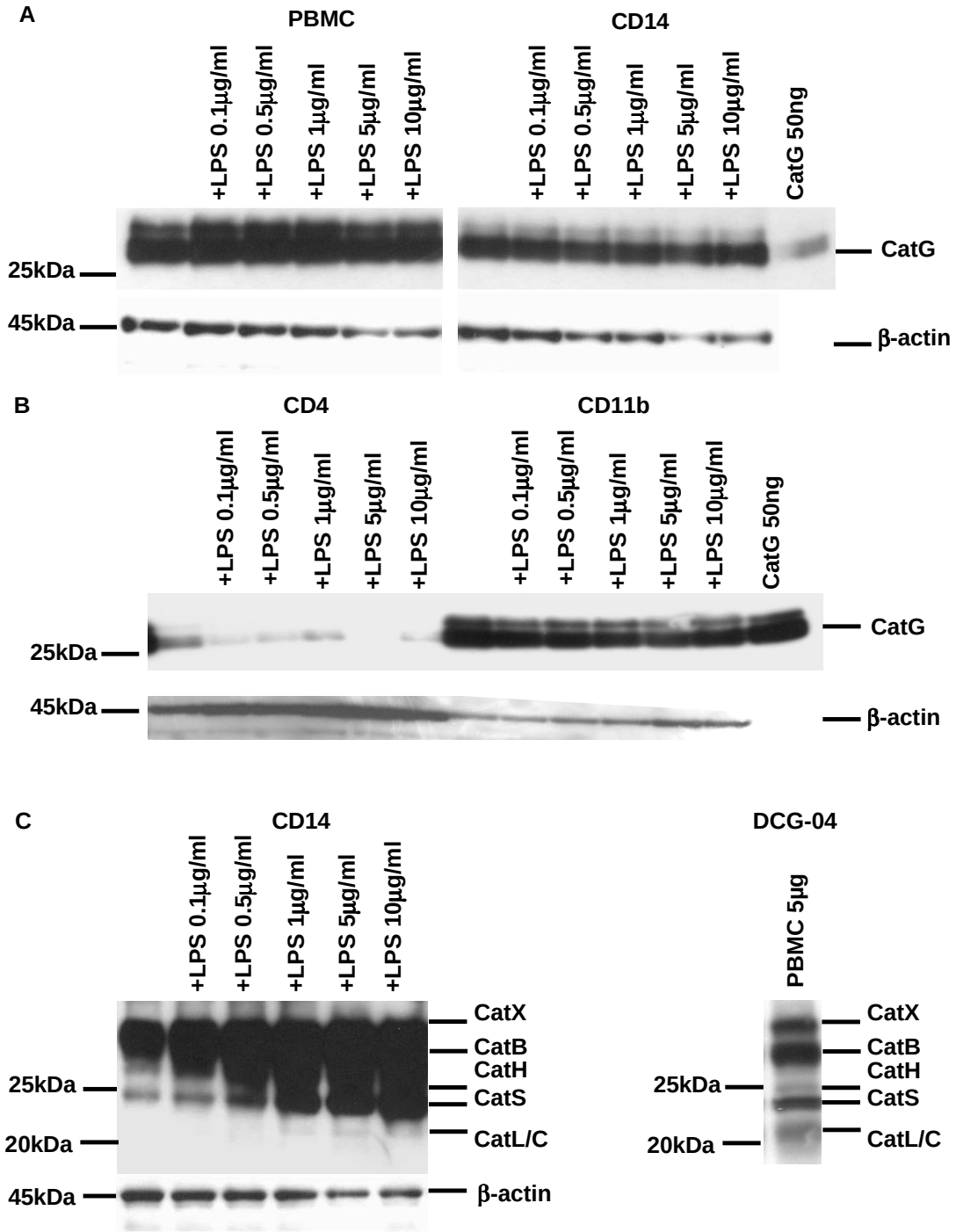


Figure 19: Regulation of cathepsins after LPS exposure. A) and B) Different concentration of LPS was incubated with freshly isolated PBMC, monocytes (CD14+), macrophages (CD11b+), or CD4+ T cells for 24 h, CatG activity of 20 µg cell lysate was detected by using DAP. C) 5 µg cell lysate of CD14+ monocytes were measured for their cysteine cathepsin activity by using DCG-04 after LPS exposure. One of three indepedent experiments is shown. LPS: lipopolysaccharides; PBMC: peripheral blood mononuclear cells.

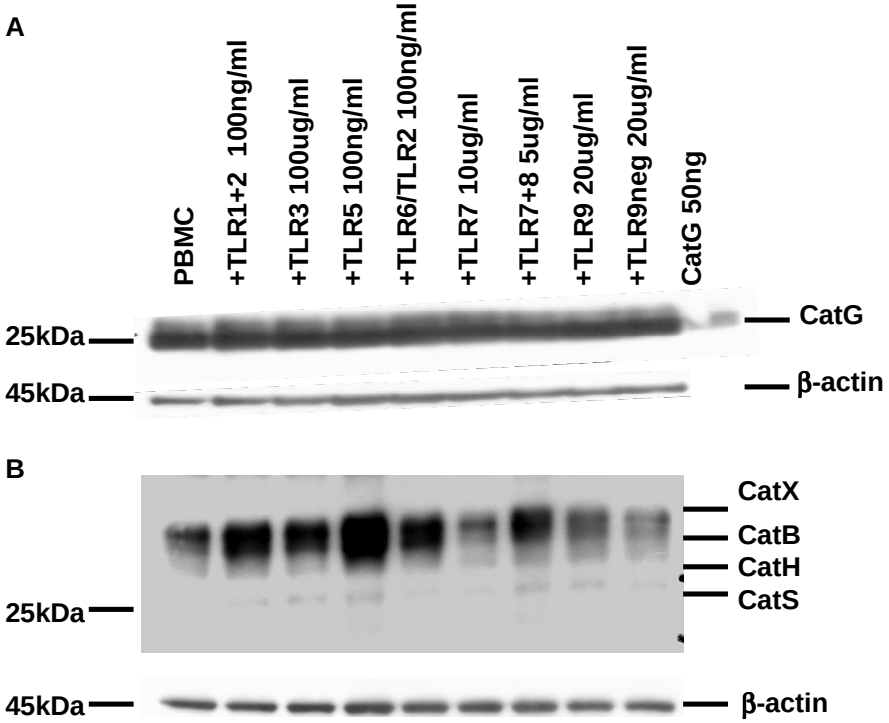


Figure 20: Regulation of cathepsins after stimulation with TLR ligands. A) CatG activity (20 µg cell lysate) and B) cysteine protease activity (5 µg cell lysate) were determined in freshly isolated PBMC after treatment with different TLR ligands (TLR1/2, TLR3, TLR5, TLR6/TLR2, TLR7, TLR8, TLR9, and TLR9 negative control) for 24 h. One of two different experiments n=2 donors is shown. PBMC: peripheral blood mononuclear cells; TLR: toll like receptor.

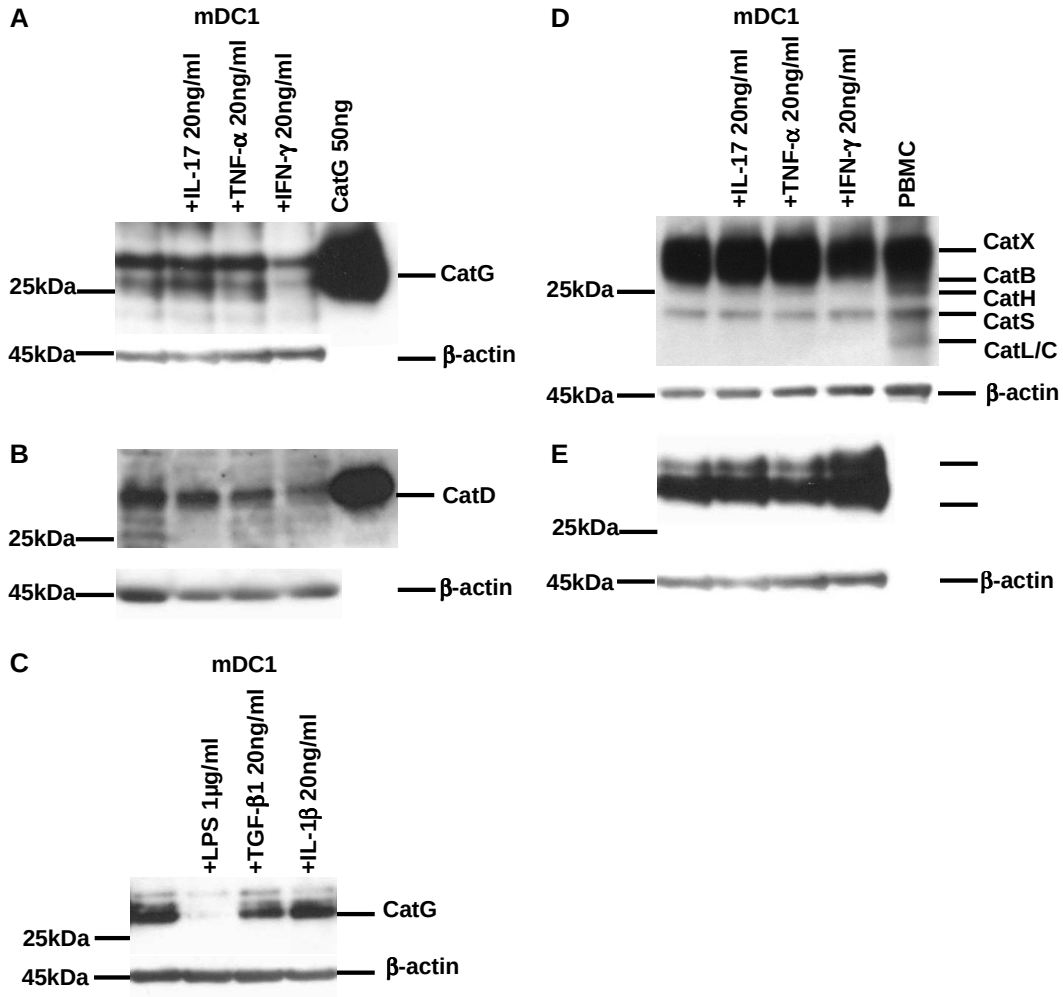


Figure 21: Regulation of cathepsins in mDC1. Primary mDC1 were isolated from PBMC and incubated with either IL-17, TNF-α, or IFN-γ. A) DAP active site label to detect CatG activity by using 20 µg cell lysate, n=3, B) CatD immunoblot (20 µg cell lysate) by using anti-CatD antibody, n=3, C) CatG activity (20 µg cell lysate) was analyzed by DAP labeling after LPS, TGF-β, IL-1β stimulation, n=2, D) DCG-04 active site label (5 µg cell lysate) to detect activity of cysteine proteases, n=3, and E) MHC class II immunoblot (20 µg cell lysate) by using anti-HLA-DR antibody (CHAMP), n=3. One representative blot is shown (for TNF-α, n=6). IFN-γ: interferon-γ; IL: interleukin; LPS: lipopolysaccharides; mDC: myeloid dendritic cells; MHC: Major histocompatibility complex; PBMC: peripheral blood mononuclear cells; TGF-β1: transforming growth factor-β1; TNF-α: tumor necrosis factor-α.

3.5. Vitamin D reduced proinsulin presentation in type 1 diabetes and decreased CatG activity in mDC1 from healthy donors

Proinsulin induced an adequate T cell response in PBMC from T1D patients. In contrast, addition of 1α,25$(OH)_2D_3$ reduced secretion of Th1-type cytokines (TNF-α and IFN-γ) and Th17-type cytokines (IL-17 and IL-22) by 70-85% (Figure 22 and

Figure 23), no differences of IL-6 or TGF-β1 were detected (Figure 24). Prohormone cholecalciferol, vitamin D_3 and vehicle control, DMSO had no significant effect on the secretion of all tested cytokines. Of note, PBMC from control donors showed no response indicated by secretion of cytokines even with no antigen.

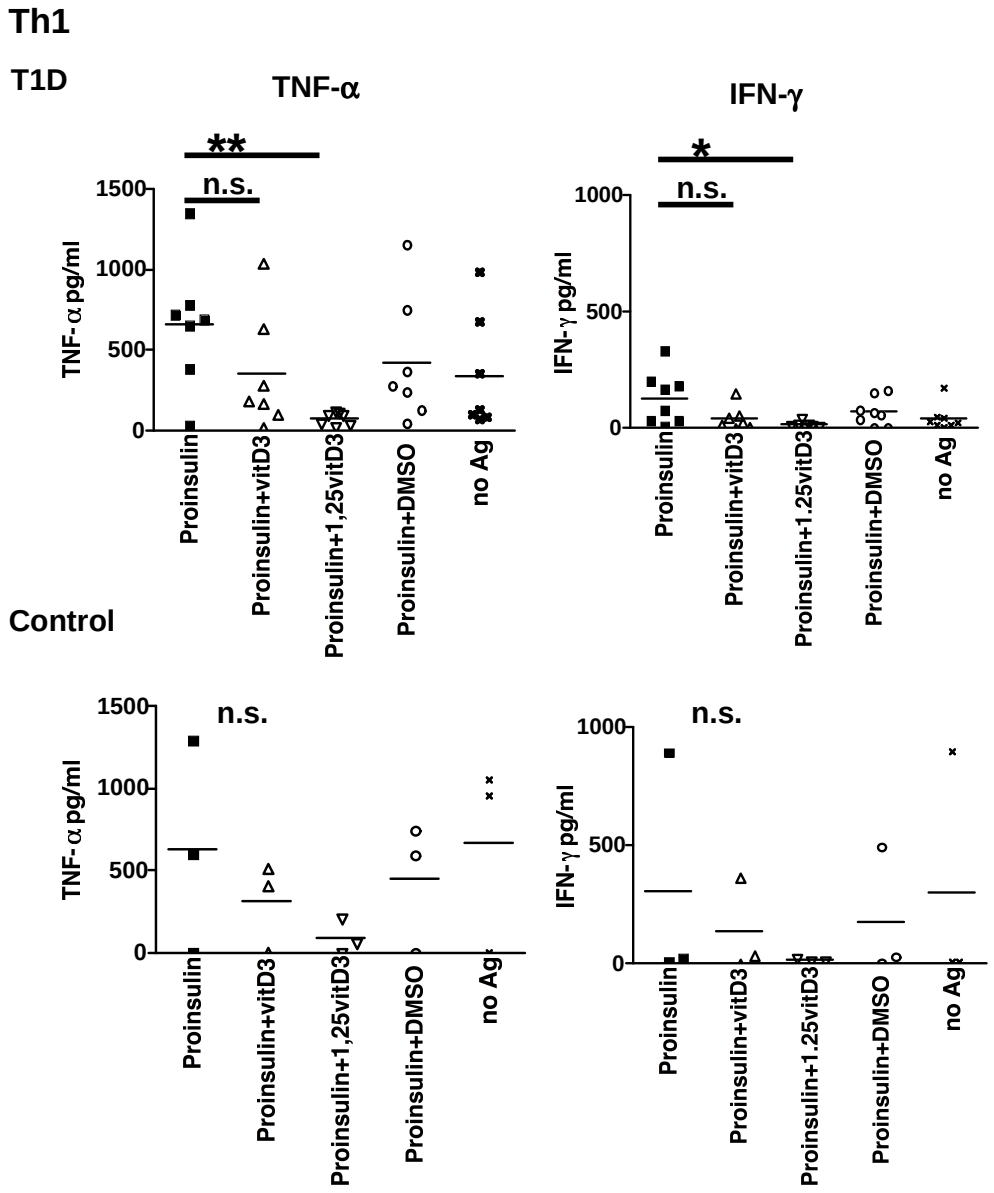


Figure 22: Th1-type cytokine secretion in the regulation of proinsulin presentation by vitamin D. PBMC from T1D (n=6) or control (n=3) donors were incubated with proinsulin (10 µg/ml) with or without vitamin D (cholecalciferol or 1α,25$(OH)_2D_3$, 100 ng/ml), DMSO (final concentration 0.1%) served as a vehicle control. Cytokine production (TNF-α and IFN-γ) was analyzed by ELISA in quadruplicate. Statistical analysis was performed by using the unpaired, two-tailed Student's *t*-test. n.s., not significant, * significant at $p<0.05$, and ** significant at $p<0.01$. ELISA: enzyme-linked immunosorbent assay; IFN-γ: interferon-γ; PBMC: peripheral blood mononuclear cells; T1D: type 1 diabetes; Th: T helper cells; TNF-α: tumor necrosis factor-α.

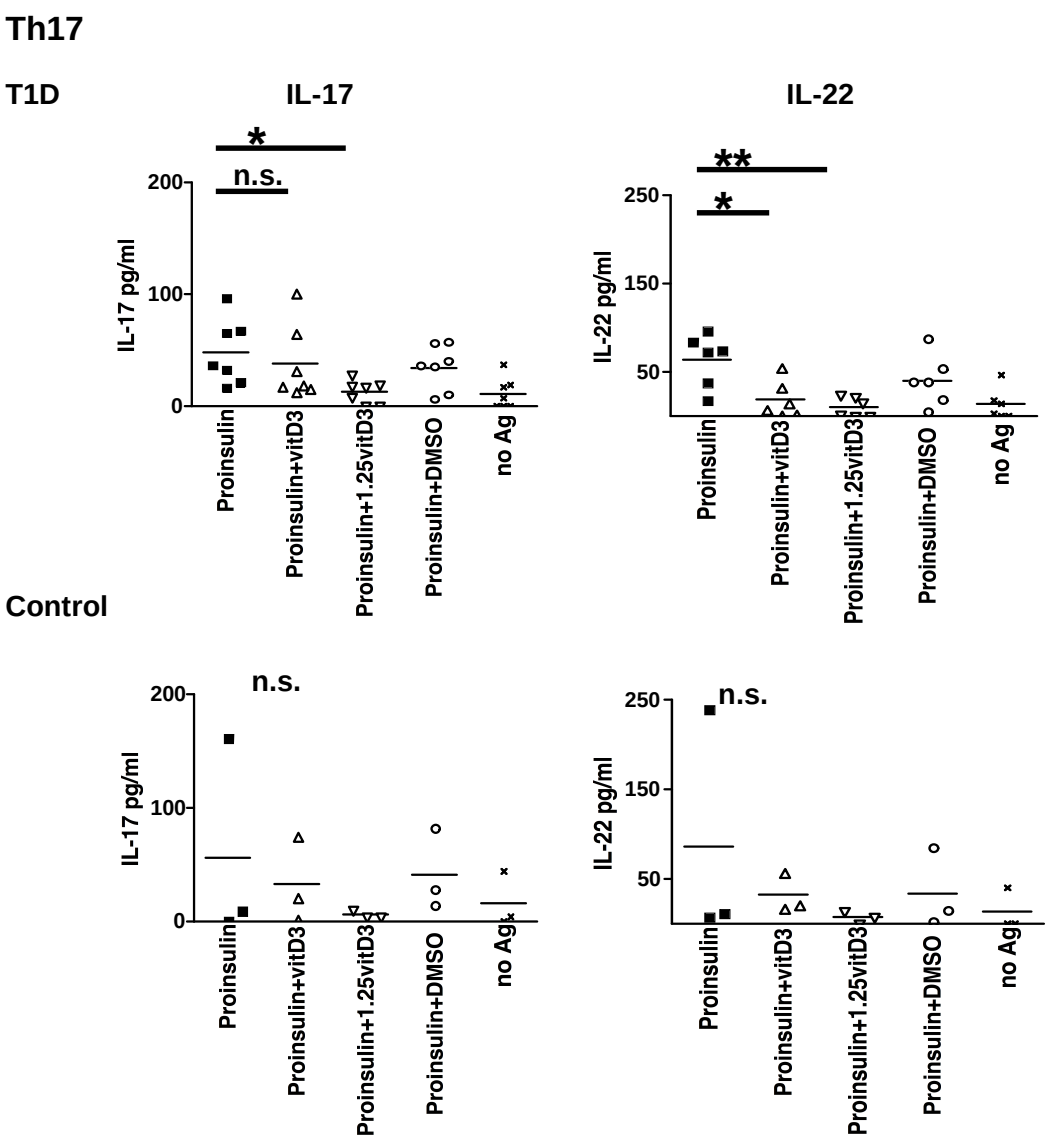


Figure 23: Th17-type cytokine secretion in the regulation of proinsulin presentation by vitamin D. PBMC from T1D (n=6) or control (n=3) donors were incubated with proinsulin (10 µg/ml) with or without vitamin D (cholecalciferol or $1\alpha,25(OH)_2D_3$, 100 ng/ml), DMSO (final concentration 0.1%) served as a vehicle control. IL-17 and IL-22 were analyzed by ELISA in quadruplicate. Statistical analysis was performed by using the unpaired, two-tailed Student's *t*-test. n.s., not significant, * significant at $p<0.05$, and ** significant at $p<0.01$. ELISA: enzyme-linked immunosorbent assay; IL: interleukin; PBMC: peripheral blood mononuclear cells; T1D: type 1 diabetes; Th: T helper cells.

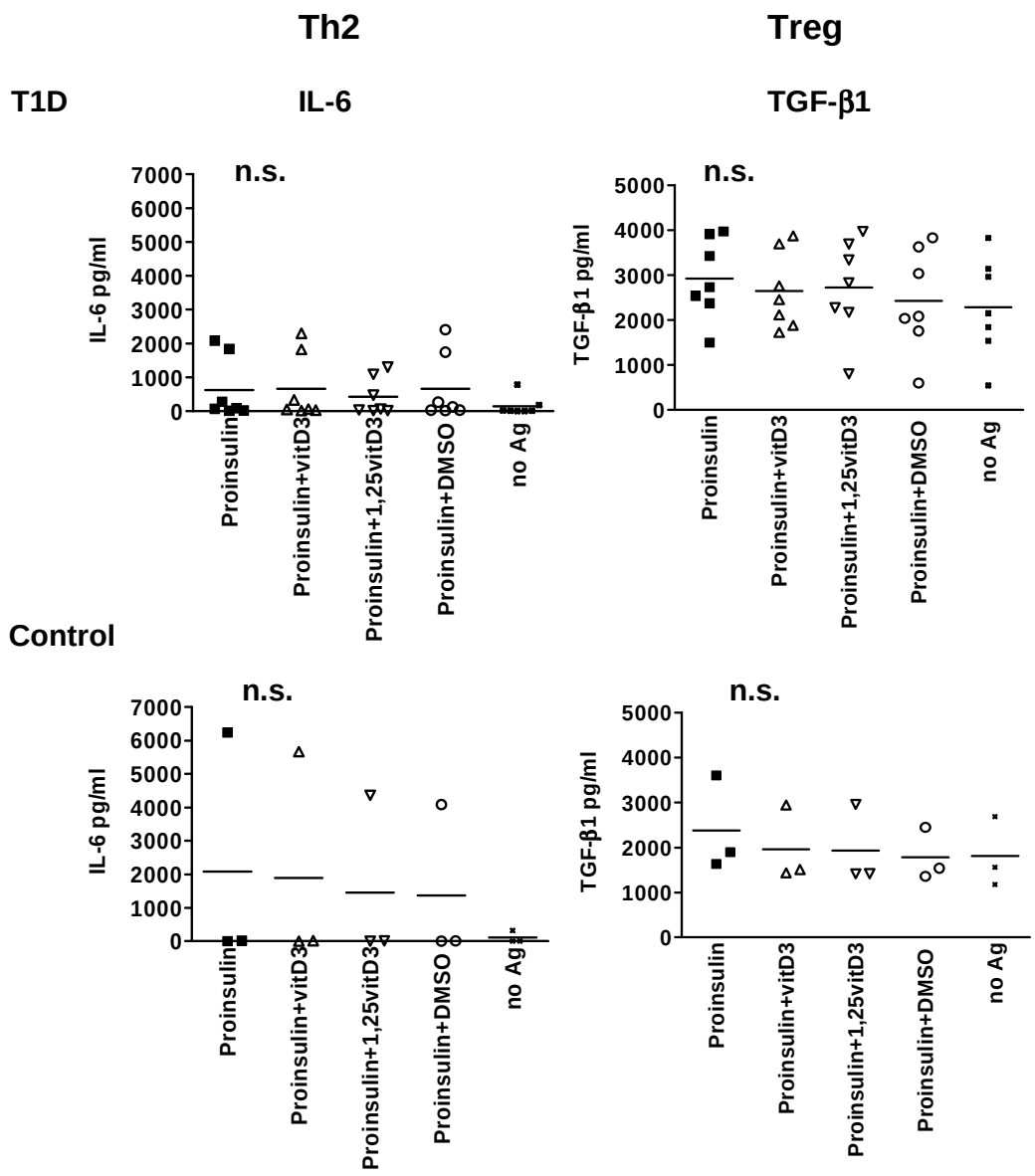


Figure 24: Th2- and Treg-type cytokine secretion in the regulation of proinsulin presentation by vitamin D. PBMC from T1D (n=6) or control (n=3) donors were icubated with proinsulin (10 µg/ml) with or without vitamin D (cholecalciferol or 1α,25(OH)$_2$D$_3$, 100 ng/ml), DMSO (final concentration 0.1%) served as a vehicle control. IL-6 and TGF-β1 were analyzed by ELISA in quadruplicate. Statistical analysis was performed by using the unpaired, two-tailed Student's *t*-test. n.s., not significant. ELISA: enzyme-linked immunosorbent assay; IL: interleukin; PBMC: peripheral blood mononuclear cells; T1D: type 1 diabetes. TGF-β1: transforming growth factor-β1, Th: T helper cells; Treg cell: regulatory T cell.

In a further experiment, for a better understanding of the activity of proteases after vitamin D exposure, B cells and mDC1 from T1D or control donors were treated with physiological serum concentrations of vitamin D$_3$ or 1α,25(OH)$_2$D$_3$. Downregulation of CatG activity in mDC1 was only found in control donors, but not in T1D-derived mDC1, however, vitamin D had no effect on CatG activity in B cells from T1D and

control (Figure 25A). Cysteine protease activity was not altered under the conditions used in either B cells or mDC1 from T1D or controls (Figure 25B), in contrast to the levels of MHC class II molecules, which were reduced after treatment with $1\alpha,25(OH)_2D_3$ only in control donors (Figure 26). No differences of cathepsin activity and MHC class II molecule expression were found between the vehicle control DMSO (Figure 27).

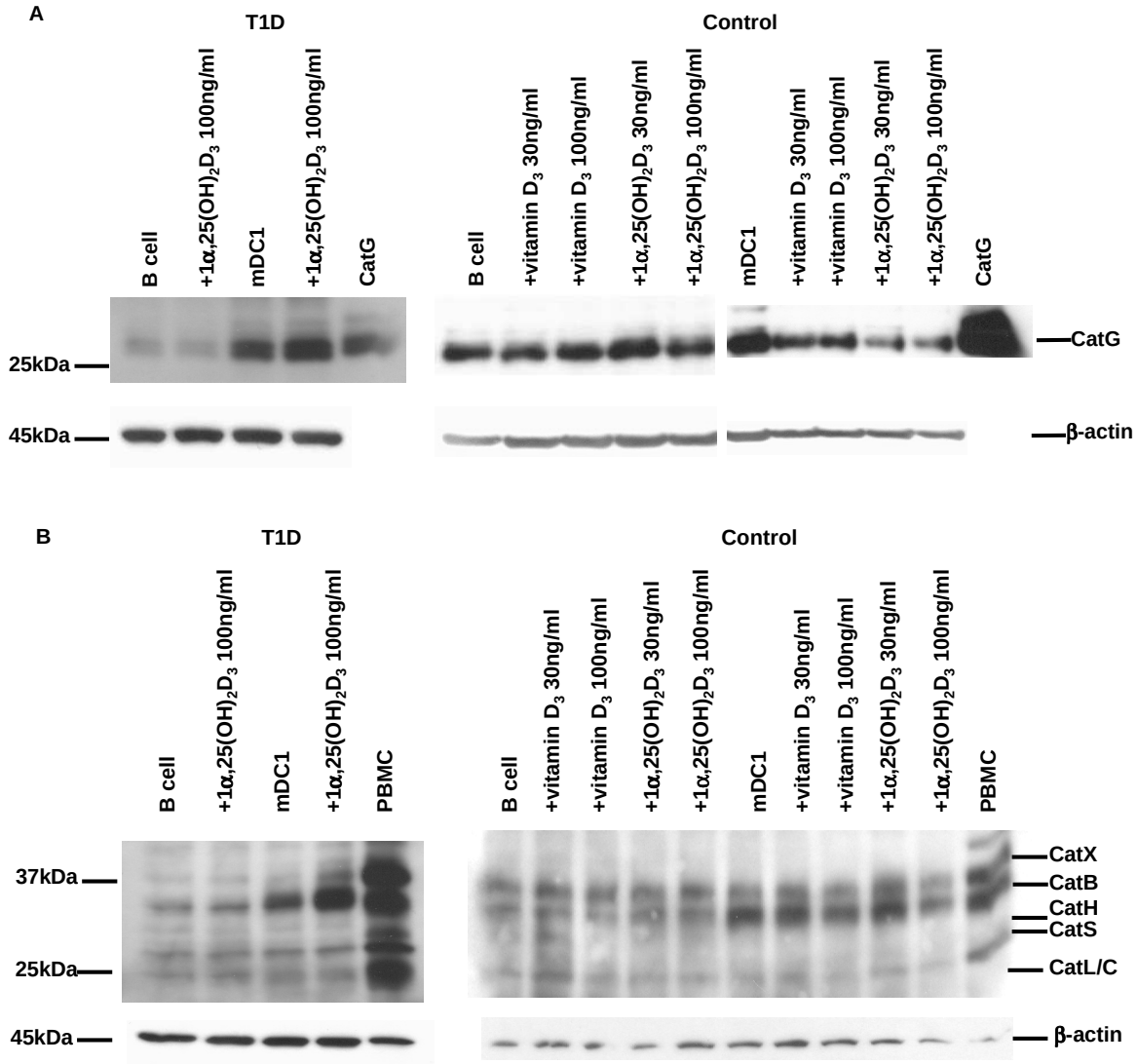


Figure 25: Regulation of cathepsin activity by vitamin D. A) Freshly isolated B cells or mDC1 were co-cultured with different concentrations (30 and 100 ng/ml) of vitamin D_3 and $1\alpha,25(OH)_2D_3$ for 24 h. Equal amounts of cell lysate (20 µg or 5µg) were incubated with the active site probe DAP or Mars116 to visualize active CatG, B) DCG-04 to visualize cysteine proteases by using 5 µg cell lysate, One representative active site label out of n=4 T1D and n=5 controls is shown. mDC: myeloid dendritic cells ; T1D: type 1 diabetes.

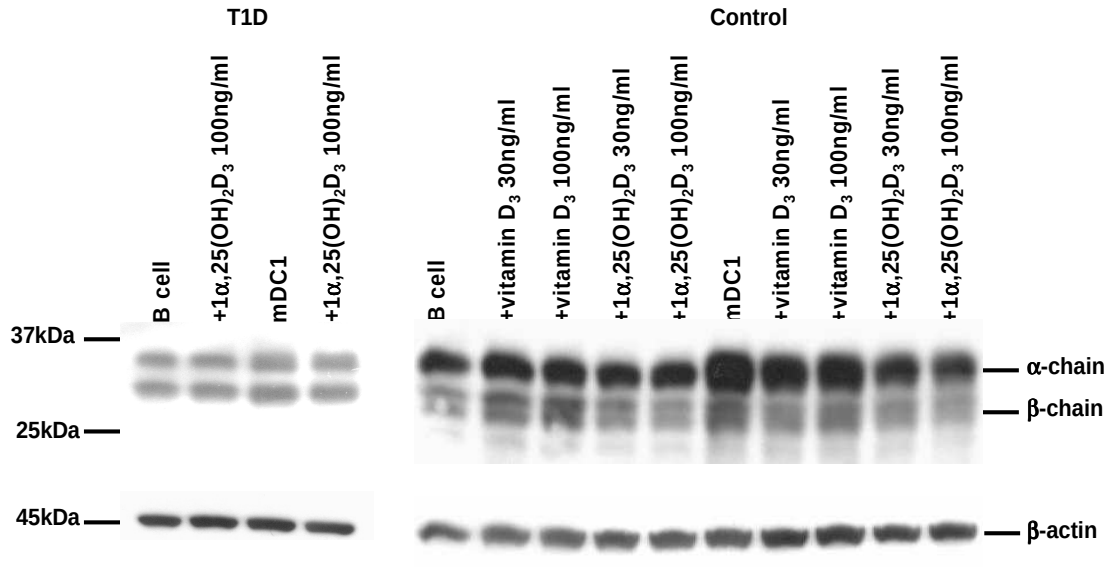


Figure 26: Regulation of MHC class II molecule expression by vitamin D. Freshly isolated B cells or mDC were co-cultured with different concentrations (30 and 100 ng/ml) of vitamin D_3 and $1\alpha,25(OH)_2D_3$ for 24 h. immunoblot of anti-HLA-DR antibody (CHAMP) was used to visualize both α and β chain of MHC class II by using 20 µg cell lysate. One representative immunoblot out of n=4 T1D and n=5 controls is shown. mDC: myeloid dendritic cells; MHC: major histocompatibility complex; T1D: type 1 diabetes.

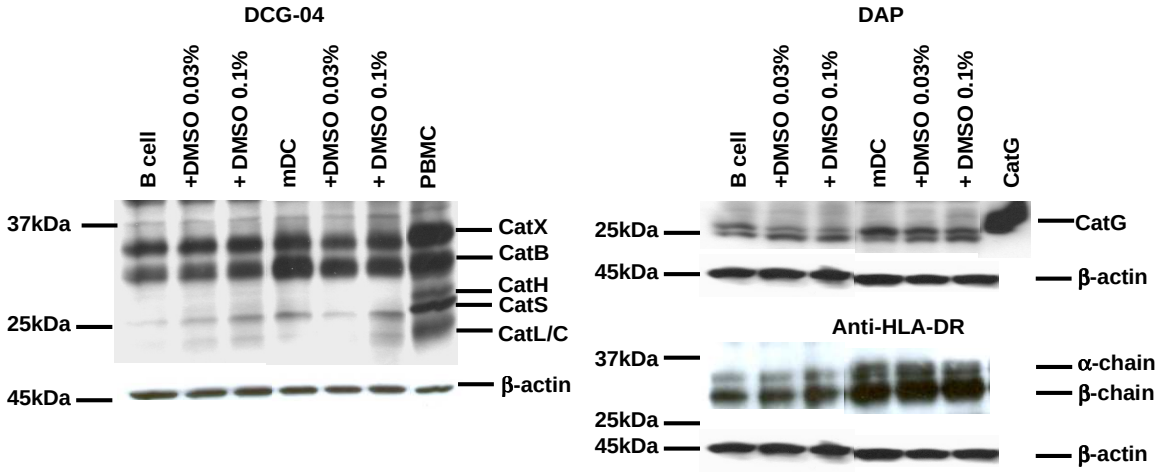


Figure 27: Regulation of cathepsin activity and MHC class II molecule expression by vehicle control DMSO. Freshly isolated B cells or mDC1 were incubated with vehicle control DMSO in the final concentrations of 0.03% and 0.1% respectively. DCG-04 (left panel), DAP (right upper panel), and immunoblot with anti-HLA-DR antibody (CHAMP) (right lower panel) were performed by using 5 µg, 20 µg and 20 µg of the cell lysate, respectively. Two different experiments n= 2 different donors. DMSO: dimethyl sulfoxide; mDC: myeloid dendritic cells; MHC:major histocompatibility complex; T1D: type 1 diabetes.

3.6. Regulation of T cell activation by EBV

Strikingly, EBV invoked activation of proinsulin-reactive T cells indicated by increased TNF-α secretion, compared to no EBV supernatant exposure, and this effect can be downregulated to baseline by using CatG inhibitor, and to a lesser extent E64d (Figure 28). PepA and the vehicle control had no effect. Other cytokines were analyzed, and it was observed that the secretion of IFN-γ, IL-17, and IL-22 was reduced by CatG inhibitor and E64d after EBV exposure, however, these data did not reach statistical significance (Figure 29). No difference in the levels of IL-6 and TGF-β1 was found (Figure 29). The same conditions were used for mDC1 as a source of APC, however, the secretion of cytokines tested showed no significant differences.

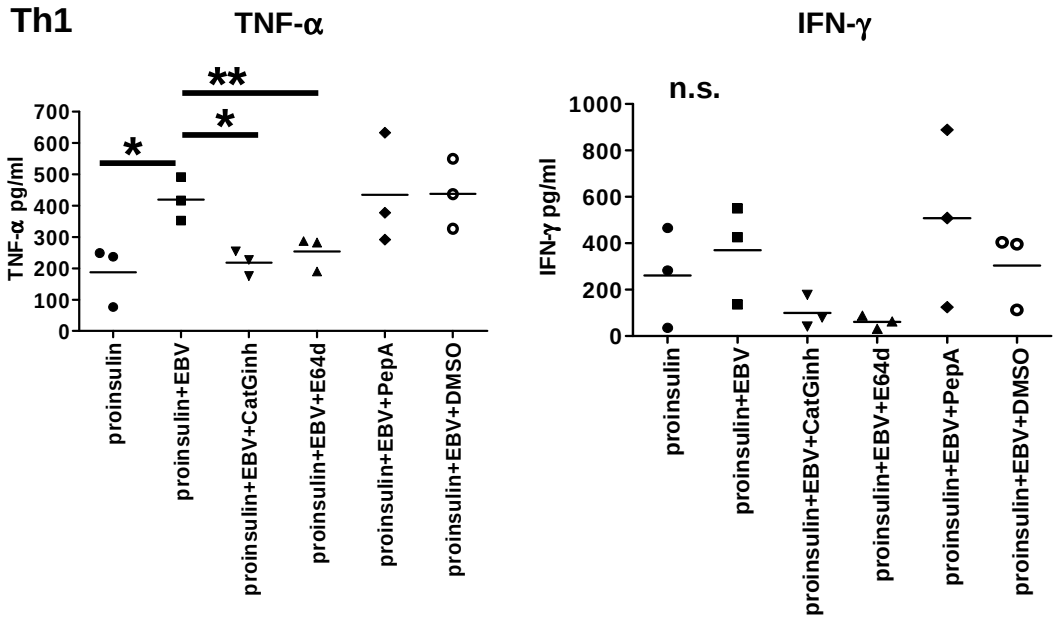


Figure 28: Th1-type cytokine secretion in the regulation of proinsulin-reactive T cells by EBV. Human B cells and CD4+ T cells were freshly isolated from healthy donors (n=3), pulsed with proinsulin (10 µg) and EBV in the presence of CatG inhibitor, E64d, or PepA in a final concentration of 10 µM to do functional T cell assay. DMSO severed as a vehicle control in a final concentration of 0.1%. Th1-type cytokine production (TNF-α and IFN-γ) was analyzed by ELISA in quadruplicate. Statistical analysis was performed by using the unpaired, two-tailed Student's *t*-test. n.s., not significant, * significant at $p<0.05$. EBV: Epstein-Barr virus; ELISA: enzyme-linked immunosorbent assay; IFN-γ: interferon-γ; Th: T helper cells; TNF-α: tumor necrosis factor-α.

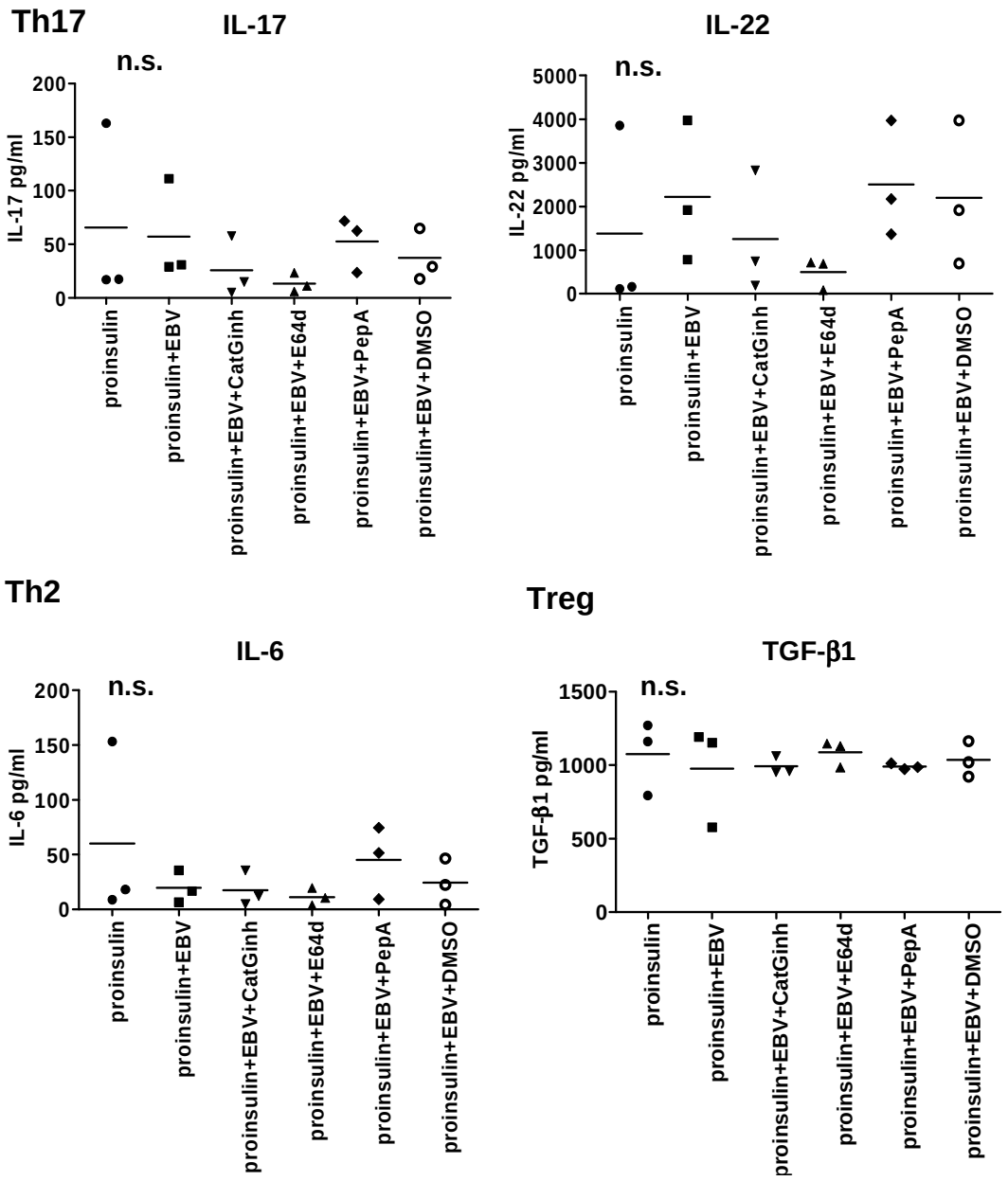


Figure 29: Cytokine secretion in the regulation of proinsulin-reactive T cells by EBV. Human B cells and CD4+ T cells were freshly isolated from healthy donors (n=3), pulsed with proinsulin (10 µg) and EBV in the presence of CatG inhibitor, E64d, or PepA in a final concentration of 10 µM to do functional T cell assay. DMSO severed as a vehicle control in a final concentration of 0.1%. Cytokine production (IL-17, IL-22, IL-6, and TGF-β1) was analyzed by ELISA in quadruplicate. Statistical analysis was performed by using the unpaired, two-tailed Student's *t*-test. n.s., not significant. EBV: Epstein-Barr virus; ELISA: enzyme-linked immunosorbent assay; IL: interleukin; Th: T helper cells; TGF-β1: transforming growth factor-β1.

3.7. Cathepsin regulation in primary APC after EBV stimulation

Next, the mitigation of T cell activation, which might be due to the activity of proteases located in the endocytic compartments of APC, was addressed. No differences were detected regarding CatG activity in B cells, mDC1, or pDC after EBV exposure (Figure 30A). Focusing on cysteine proteases, EBV exposure did not alter cysteine protease activity in these cells (Figure 30B). However, EBV was found to downregulate the expression of MHC class II molecules in B cells, but not in mDC1 as shown in Figure 30C.

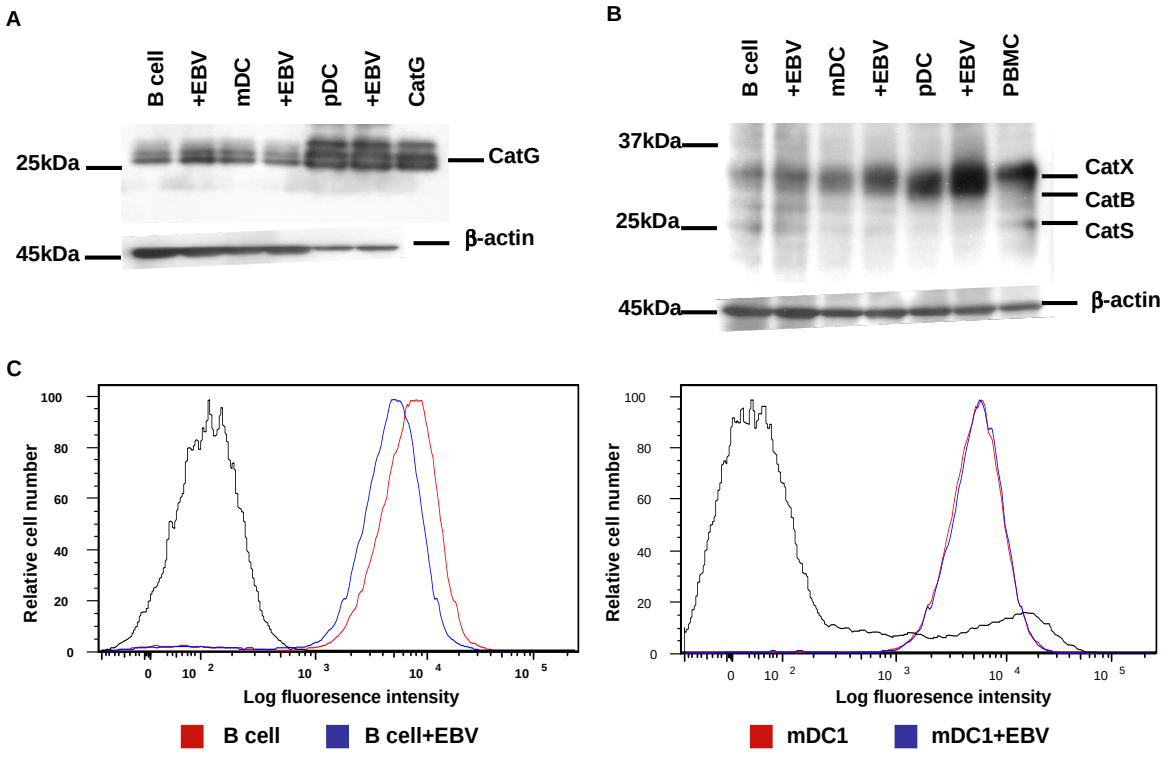


Figure 30: Regulation of cathepsin activity in APC after EBV exposure. Freshly isolated B cells, mDC1, or pDC were co-cultured with EBV supernatant for 24h, Equal amounts of cell lysate 5 µg were incubated with the active site probe to visualize A) active CatG or B) cysteine proteases, n=4. C) Cells were stained with anti-human HLA-DR-PE-Cy7 antibody to detect MHC class II expression by flow cytometry. The gray curve represents cell populations stained with PE-Cy7 isotype control, the red and blue curves represent cell populations stained with HLA-DR-PE-Cy7 antibody. X axis stands for fluorescence intensity logarithmic scale and Y axis stands for relative cell number. MHC class II expression of B cells was decreased by EBV exposure indicated by a left shift of the curve and there was no difference in mDC1, n=3. APC: antigen presenting cells; EBV: Epstein-Barr virus; mDC: myeloid dendritic cells; HLA: human leukocyte antigens; MHC: major histocompatibility complex; pDC: plasmacytoid dendritic cells.

In furthe experiments, the influence of EBV in alteration of the cathepsin activity after B cell transformation was investigated. It was found that the activity of CatX was upregulated starting after 12 days of culture and was downregulated afterwards, in contrast to CatB and CatS, where both were downregulated after EBV exposure (Figure 31A). Focusing on CatG activity, which was reduced starting at day five and was undetectable after day 12 (Figure 31B). Levels of MHC class II molecules were detected and it was demonstrated in Figure 31C that MHC class II molecules were reduced by EBV. In addition, EBNA1 immunoblot showed that regression of EBV transformation took place at day 12 (Figure 32).

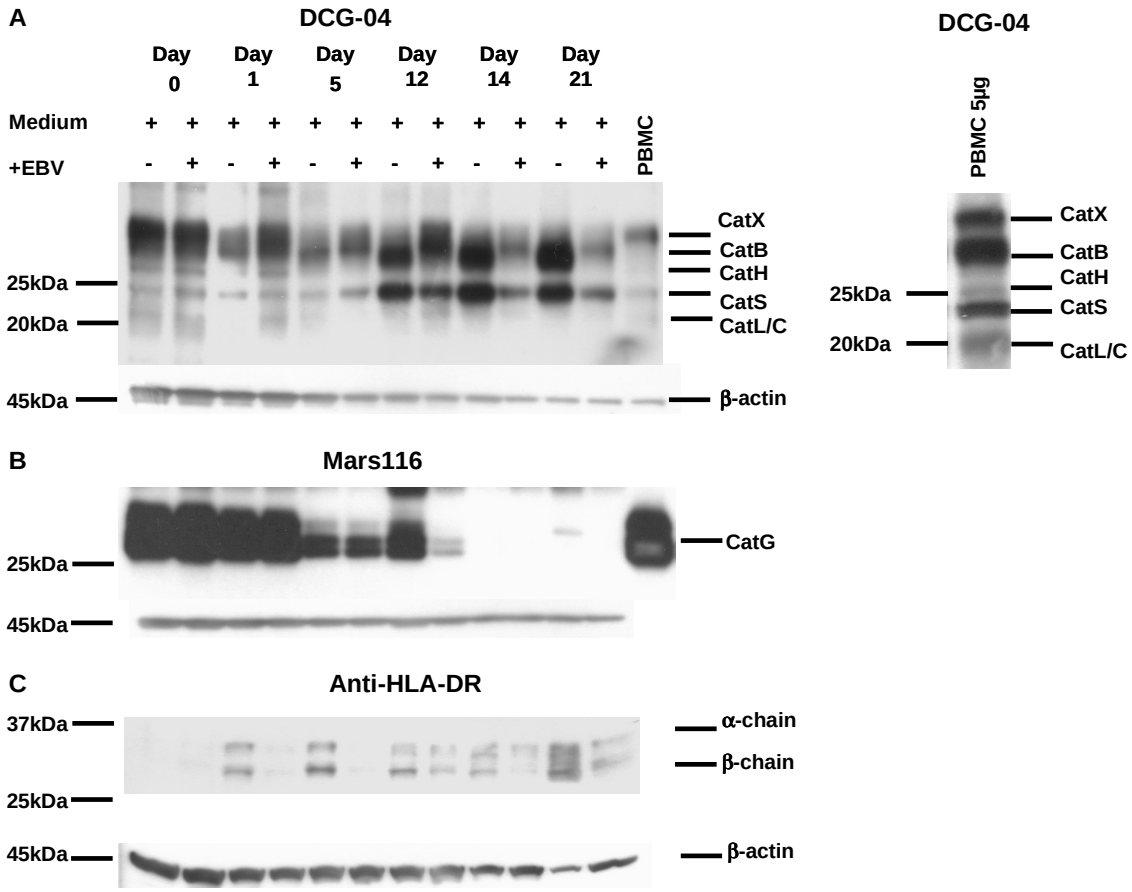


Figure 31: Regulation of cathepsin activity after B cell transformation with EBV. Freshly isolated PBMC were co-cultured with EBV supernatant for different time points (1, 5, 12, 14, 21 days). Equal amounts of cell lysate (5 or 20 µg) were incubated with the active site probe DCG-04 (A) to visualize cysteine proteases, Mars116 to visualize active CatG (B), or immunoblot with anti-HLA-DR antibody (CHAMP) (C). Two different experiments n=2 different donors. EBV:Epstein-Barr virus; HLA: human leukocyte antigens; PBMC: peripheral blood mononuclear cells.

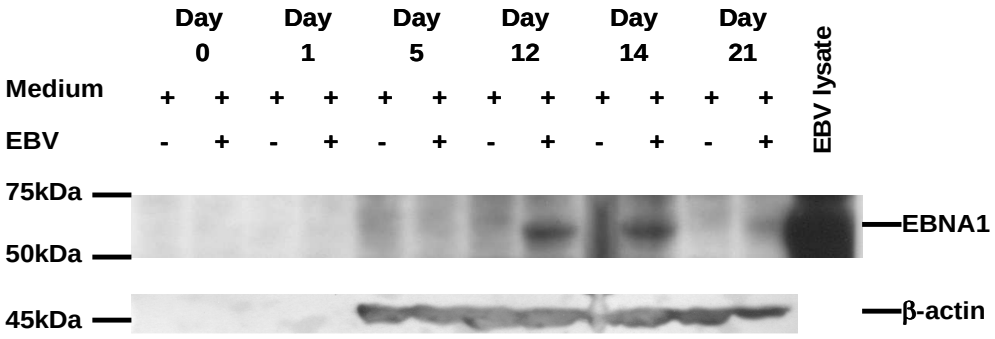


Figure 32: Detection of EBNA1 levels after EBV transformation. Freshly isolated PBMC were co-cultured with EBV supernatant for different time points (1, 5, 12, 14, 21 days). Equal amounts of cell lysate (40 µg) were immunobloted with anti-EBNA antibody (Antibodies-online GmbH, Aachen, Germany). Two different experiments n=2 different donors. EBNA: Epstein-Barr virus nuclear antigen; PBMC: peripheral blood mononuclear cells.

3.8. Processing of Ii with CatG

At pH 7.4, CatG converted p31 to p10, in a CatG concentration dependent manner indicated by more accumulation of p10 (Figure 33A), in contrast, when BLC cell lysate were incubated with CatG at pH 5.0, p31 was resistant to proteolysis (Figure 33B), indicating a pH dependent invariant chain (Ii) degradation by CatG. Active CatG can be detected obviously in preloaded BLC with a CatG concentration of 18 µg/ml (Figure 34A), however, no differences between CatG-preloaded BLC and control were observed identified by no Ii-derived intermediate accumulation (Figure 34B), indicating that Ii was not processed by CatG *in situ*.

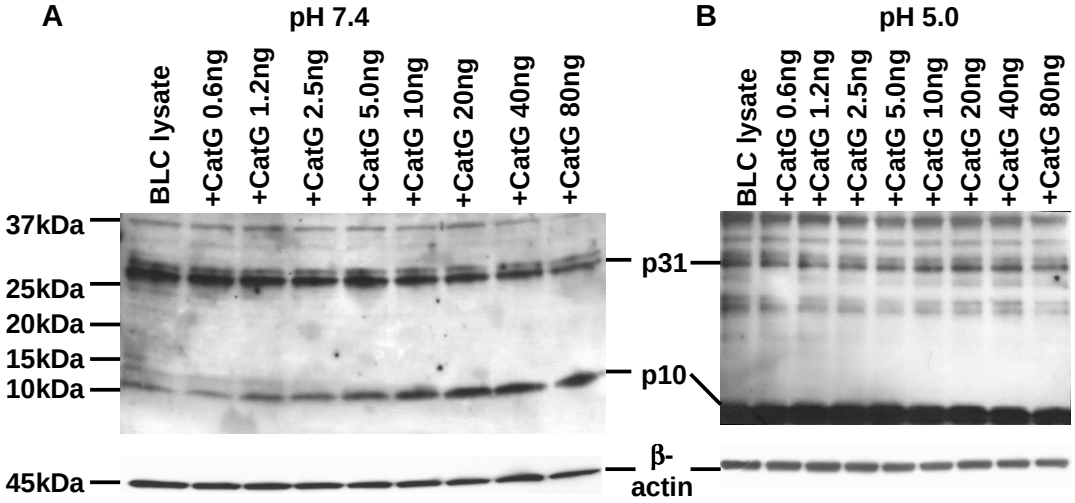


Figure 33: Digest of Ii with CatG *in vitro*. 20 µg of cell lysate from BLC were incubated with different amounts of human purified CatG (0.6, 1.2, 2.5, 5.0, 10, 20, 40, and 80 ng) at pH 7.4 (A) or pH 5.0 (B) for 1 h at 37℃ and immunoblotted with anti-CLIP. One representative western blot of three independent experiments is shown. BLC: B lymphoblastoid cells; CLIP: class-II associated invariant chain peptide; Ii: invariant chain.

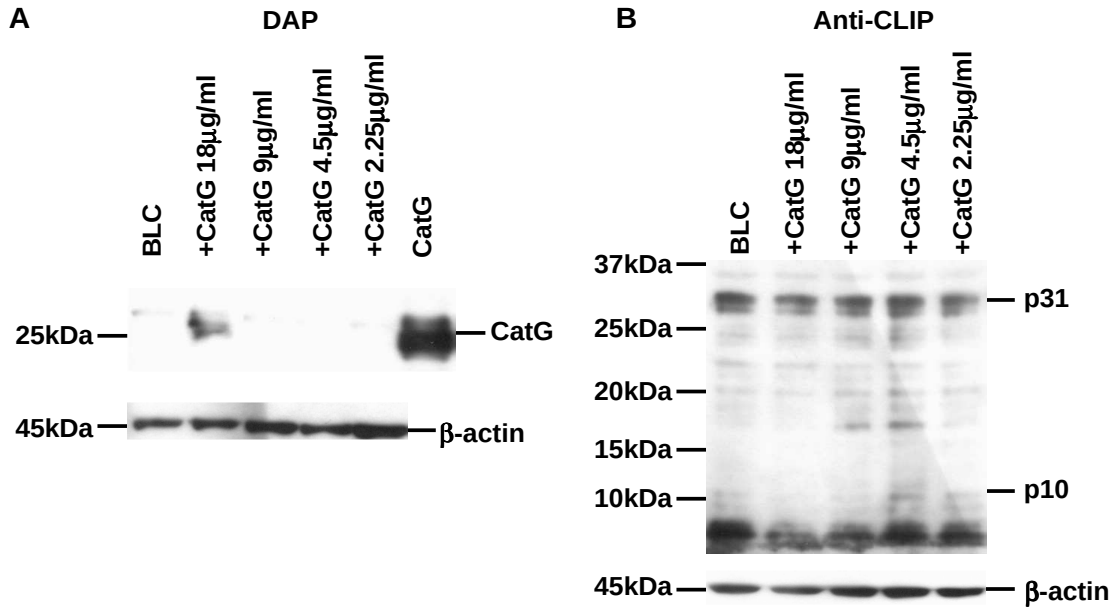


Figure 34: Preloading of BLC with CatG and observation of Ii processing within intact cells. A) BLC were pretreated with different amounts of purified CatG (18, 9, 4.5, and 2.25 µg/ml) for 4.5 h at 37℃. CatG uptake was confirmed in an independent experiment without containing CatG inhibitor in the lysis buffer. Active CatG was visualized by using DAP activity-based probe with 20 µg cell lysate. B) BLC were lysed by using lysis buffer containing CatG inhibitor (10 µM) to avoid post lysis degradation. Ii intermediates were detected by anti-CLIP immunoblot using 20 µg cell lysate. One representative of two experiments is shown. BLC: B lymphoblastoid cells; CLIP: class-II associated invariant chain peptide; Ii: invariant chain.

3.9. Inhibition of CatG did not alter Ii processing in B cells or mDC1

B cells, mDC1, or BLC treated with the broad-spectrum cysteine protease inhibitor, E64d, showed an increase of p22 and p10 as expected, but no accumulation of Ii degradation intermediates were observed when CatG inhibitors (CatGinh, SucVPF, and MeSucVPF) were used, indicating inhibition of CatG in intact cells does not alter Ii degradation in B cells or mDC1 (Figure 35). Short time (4 h) treatment of PBMC with the same inhibitors showed that an early accumulation of p22 and p10 only occurred when incubated with E64d (Figure 36).

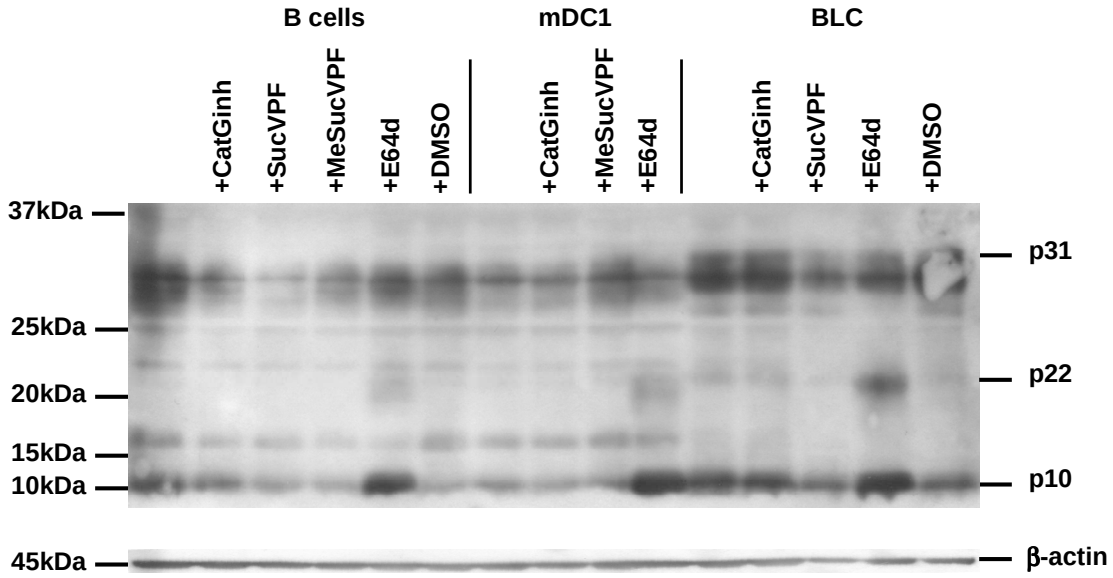


Figure 35: Inhibition of intracellular CatG using selected CatG inhibitors in B cells, mDC1, or BLC. Freshly isolated primary B cells and mDC1 from peripheral blood and BLC were treated with CatGinh, SucVPF, MeSucVPF, or E64d in a concentration of 10 µM for 72 h. Ii intermediates were detected by anti-CLIP immunoblot. Two independent experiments n=2 different donors. BLC: B lymphoblastoid cells; CLIP: class-II associated invariant chain peptide; Ii: invariant chain; mDC: myeloid dendritic cells.

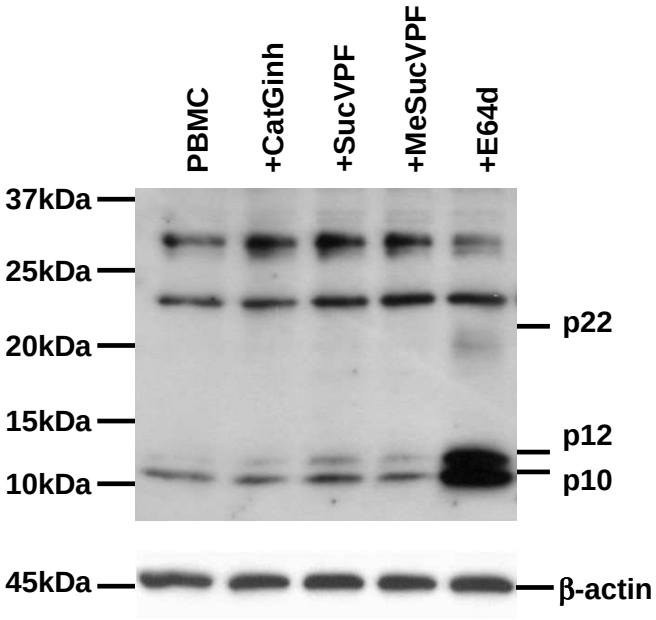


Figure 36: Short time inhibition of intracellular cathepsins using selected inhibitors in PBMC. Cell lysate from PBMC were incubated with CatGinh, SucVPF, MeSucVPF, or E64d (10 μM) for 4 h at 37℃. Ii intermediates were detected by anti-CLIP immunoblot. Two independent experiments n=2 different donors. CLIP: class-II associated invariant chain peptide; Ii: invariant chain; PBMC: peripheral blood mononuclear cells.

4. Discussion

4.1. Downregulation of proteases within APC leads to reduced proinsulin-reactive T cell activation

In the present study, it was found that inhibition of CatG by using a specific CatG inhibitor reduced proinsulin-reactive T cell activation in PBMC from T1D compared to controls. These data suggest that CatG is involved in the processing and presentation of proinsulin. This is not without precedent, since CatG is expressed in primary APC and has been identified to dominate the processing of antigens and autoantigens (Burster et al. 2004; Stoeckle et al. 2009; Burster et al. 2010b). In addition, proinsulin is one of the major autoantigens in T1D. To present proinsulin-peptides to T cells, proinsulin has to be internalized and processed to form a MHC class II-peptide complex.

Data here suggest that CatG might be directly associated with the development of T1D. Multiple studies, by using serine protease inhibitors, have been investigated in both mouse and human. For instance, α1-antitrypsin (AAT), the acute-phase serine protease (including CatG) inhibitor can reduce the gene expression of proinflammatory cytokines and restore euglycemia in new onset diabetic NOD mice (Koulmanda et al. 2008). Moreover, Kalis et al. reported that AAT can stimulate insulin secretion *via* the cAMP pathway and protect β cells against cytokine-induced apoptosis (Kalis et al. 2010). In a clinical trial, oral administration of frankincense extract inhibited CatG activity in human blood, and showed anti-inflammatory potential (Tausch et al. 2009). In summary, the data provided here demonstrated that effective processing and presentation of proinsulin is depending on the activity of CatG in PBMC from T1D. For future experiments, it might be interesting to investigate whether the reduction of CatG activity by, for example, inhaling/intake frankincense might be beneficial to prevent T1D.

It was observed that other protease inhibitors, especially cysteine protease inhibitor E64d, seem to lower cytokine secretion, when pulsed with proinsulin, however, these data did not reach significance, suggesting that cysteine proteases are not as

much important in proinsulin processing compared to CatG. Inhibition of cysteine proteases, specifically CatS, was shown to reduce Ii processing and therefore the maturation of MHC class II molecules (Riese et al. 1996). The small differences shown here by using E64d might account for the impaired maturation of MHC class II molecules and not proinsulin processing. On the other hand, the aspartic protease CatD and the serine protease CatG were found to be irresponsible for either Ii processing or maturation of MHC class II (Villadangos et al. 1997; Reich et al. 2011). Thus, the inhibition of cysteine proteases can lead to both impaired MHC class II maturation and antigen processing in contrast to CatG and CatD, which are only responsible for antigen processing within the endocytic compartment.

4.2. Comparison of T cell epitopes of type 1 diabetes *vs.* proinsulin-derived peptides after *in vitro* processing

Distinct proinsulin-derived fragments were found after *in vitro* digest, T cell stimulation was used as a biological test system to look for disease relevant epitopes. Therefore, the comparison between peptides from *in vitro* processed proinsulin and known T cell epitopes of T1D will be discussed in the following section.

B cell- or mDC1-derived lysosomal cathepsins did not generate the T cell epitopes like C18-A1 (P17) and A5-A21 (P21), which are two known human T1D T cell epitopes within the proinsulin sequence (Durinovic-Bello et al. 2002; Durinovic-Bello et al. 2006). Several proinsulin-derived peptides, DCins5, 11, and 12, generated by mDC1-derived cathepsins, were similar to B27-C15, C3-C26, and C25-A12 (Figure 37), which were determined by peptide elution from proinsulin pulsed with human BLC (Arif et al. 2004). In addition, the sequence of DCins15 obtained by proinsulin processing with mDC1- or B cell-derived lysosomal cathepsins, was similar to the T cell epitope A6-A21 found in PBMC (Durinovic-Bello et al. 2002). C17-A1, B9-B23, B11-B27, B24-C4, C13-C29, and A1-A15 were determined in PBMC to be CD4+ T cell epitopes (Zhang et al. 2008). However, these fragments were not obtained by processing of proinsulin with mDC1- or B

cell-derived lysosomal cathepsins. B1-B17 was also verified as a putative T cell epitope using PBMC (Durinovic-Bello et al. 2002), this peptide was not detected after processing of proinsulin using mDC1. Rather, it was discovered by using B cell-derived lysosomal cathepsins.

Proinsulin-derived peptides eluted from HLA-DR4 BLC	**Peptides obtained after proinsulin digest with mDC1-derived lysosomal cathepsins**
TPKTRREAEDLQVGQVELGGGP **(B27-C15)**	PKTRREAEDLQVGQVELGGG **(DCins11)**
EDLQVGQVELGGGPGAGSLQPLAL **(C3-C26)**	EDLQVGQVELGGGPGAGSLQPL **(DCins12)**
DLQVGQVELGGGPGAGSLQPLALE **(C4-C27)**	DLQVGQVELGGGPGAGSLQPL **(DCins13)**
ALEGSLQKRGIVEQCCTSICS **(C25-A12)**	ALEGSLQKRGIVEQCCT **(DCins5)**

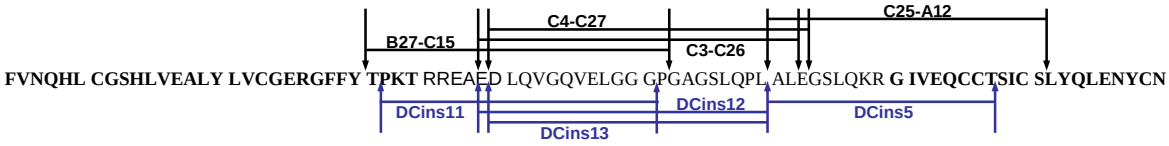


Figure 37: Sequence overview of identified proinsulin-derived peptides *vs.* DCins peptides. Those in black show the sequences of peptides eluted from human HLA-DRB1*0401/0401 BLC (Arif et al. 2004), those in blue indicate the sequences of DCins 11, 12, 13, and 5 peptides generated after proinsulin digest with mDC1-derived lysosomal cathepsins. The arrows indicate the cleavages sites, and the bars show the resulted fragments.

The fragmentation patterns obtained from purfied cathepsins were compared with the data obtained after proinsulin processing with B cell- or mDC1-derived lysosomal cathepsins as well as published data. The fragment, C33-A13, generated by purified CatG, is similar to the HLA-DRB*0401 restricted T cell clone epitope A1-A13, but missing one amino acid at the N-terminal end (Mannering et al. 2005). Within the β chain of insulin, CatG cleavage sites are similar to six out of seven previously demonstrated cleavage sites, ^{6}LC^{7}, ^{11}LV^{12}, ^{15}LY^{16}, ^{16}YL^{17}, ^{17}LV^{18}, ^{24}FF^{25}, and ^{25}FY^{26}, the exception is the cleavage site between ^{6}LC^{7} (Blow and Barrett 1977). This discrepancy might be accounted for the different conditions between the published data (oxidized β chain, at pH 8.5) and the assay used here (pH 7.2 and not oxidized proinsulin).

In summary, based on the results obtained after *in vitro* proinsulin digest compared

to those eluted from MHC class II molecules of a B cell line, indicates that the antigen processing machinery of mammal cells might not be able to generate previously described T cell epitopes. Additionally, B cell lines do lack CatG, but express abundant levels of AEP (Manoury et al. 2002; Burster et al. 2004; Watts et al. 2005). Therefore, the fragments from peptide elution and *in vitro* digests using cathepsins from primary APC did not match as expected.

4.3. T cells in PBMC from type 1 diabetes patients are activated by distinct antigenic peptides from *in vitro* processed proinsulin

It was found here that DCins6 and DCins12 induced distinct T cell activation from T1D donors (HLA-DRB1*0401/0701), compared to HLA-matched control donors, indicating novel T cell epitopes. These two peptides were generated after proinsulin digestion with human mDC1-derived lysosomal cathepsins or human purified CatG. In addition, elevated CatG activity in PBMC from T1D patients was found, which might account for the production of high amounts of DCins peptides. PBMC from controls were not differentially stimulated by DCins peptides, suggesting that control donors do not harbor the same T cell repertoire as T1D donors. Additionally, CD8+ T cells are also present in PBMC, therefore, the possibility that CD8+ T cells were activated can not be excluded.

Autoantigenic epitope recognition is a potentially critical element in antigen-specific immunotherapy of T1D (Roep 1996; Roep 2003). Therefore, T cell epitope discovery is an important component of T1D research. In the present study, DCins6 and DCins12 represented novel T cell epitopes in HLA-DRB1*0401/0701 T1D patients. Furthermore, the sequence of DCins10 was equal to the epitope (B1-B15) known to bind to the protective HLA allele HLA-DQB1*0602 (Ettinger and Kwok 1998). In the data provided, this peptide did not induce the secretion of TNF-α, but did bind to HLA-DRB1*0401. It can be speculated that DCins10 (B1-B15) is directly beneficial towards the suppression of autoaggressive T cells.

As shown here, DCins6 and DCins12 can induce high secretion of TNF-α. It is well defined that TNF-α plays a pro-apoptotic role in pancreatic β cells of T1D (Ortis et

al. 2008; Ortis et al. 2010). However, this effect was not observed in the surrogate cell line used in the herein study. One reason might be due to the different cell model used for the experiments. BON cells, which harbor similar genes and receptors compared to human β cells, do not fully resemble mature β cells. On the other hand, it was reported that diabetogenic T cells can trigger the expansion of Treg cells depending on TNF-α both in NOD mouse and *in vitro* (Grinberg-Bleyer et al. 2010). The data provided, addressing a relation between TNF-α and Treg cells, did not show any induction of Treg cells in the presence of T cell supernatants containing higher levels of TNF-α. This could partially attribute to the different cell types used, differences between mouse and human, or disparities within data from *in vitro* and *in vivo*. Since Bilate and Lafaille found that TNF-α produced by effector T cells can be effective in boosting Treg cell expansion only *in vivo* (Bilate and Lafaille 2010).

In summary, cleavage sites obtained by purified CatG were similar to those acquired by mDC1-derived lysosomal cathepsins, indicating processing of proinsulin by the combined action of proteases from mDC1 is mostly controlled by CatG *in vitro*. CatG-generated proinsulin peptides, DCins6 and DCins12, stimulated T cells from T1D patients only, suggesting novel T cell epitopes.

4.4. Regulation of cathepsins after exposure to cytokines or TLR ligands

The question remains, which factor might be responsible for the high levels of CatG activity in PBMC? None of the cytokines tested (IL-1β, TNF-α, IFN-γ, IL-4, IL-17, and TGF-β1) altered the activity of CatG or cysteine proteases in PBMC. Only IFN-γ was found to downregulate CatG and CatD activity in mDC1. The effect of LPS on protease activity was investigated, because LPS can promote the secretion of proinflammatory cytokines. Moreover, LPS together with its receptor, toll-like receptor 4 (TLR4), were found to be higher in T1D patients compared to healthy controls (Devaraj et al. 2008; Devaraj et al. 2009). Strikingly, LPS slightly, but not significantly, increased CatG activity in PBMC, and increased CatX, B, and S activity in CD14+ cells, while CatG activity was reduced after LPS exposure in

mDC1.

There are conflicting data about the regulation of cathepsins in different cells. Burster et al. found that IFN-γ downregulates CatG activity in microglia-derived lysosomes and LPS reduces CatG activity in mDC1 (Burster et al. 2007a; Burster et al. 2010a). In another study, LPS was found to decrease intracellular CatG expression in a human monocytic cell line THP-1 (Rivera-Marrero et al. 2004). Moreover, when murine microglia was treated with IFN-γ to mimic a proinflammatory cytokine milieu, CatG was downregulated, whereas the activities of CatS, CatB, CatL, CatD, and AEP did not change. However, cytokines were suggested to increase CatG activity in some cell types. For instance, TNF-α induced higher levels of CatG in HeLa cells and increased cell surface levels and secretion of CatG in polymorphonuclear leukocytes (Owen et al. 1995; Eliassen et al. 2000; McGettrick et al. 2001). Collectively, regulation of CatG depends not only on the activation stimuli, but also on cell type, which might explain the different results obtained here. Therefore, regulation of CatG activity in APC by cytokines and its relationship to antigen processing/presentation needs further investigation.

LPS was found to upregulate CatX, B, and S activity in CD14+ cells. Other TLR ligands might also influence the protease activity in APC. Therefore, their role was investigated. The study here showed that TLR ligands did not alter CatG activity in PBMC, whereas several TLR ligands, TLR1/TLR2, TLR3, and TLR5, slightly increased the activities of CatX, CatB, and CatS in these cells. Cysteine proteases (in particular AEP, CatL, and CatK) were found to be involved in the conversion of proTLR9 to mature TLR9 for proper signal transduction, indicating a link between TLR activation and cathepsins (Asagiri et al. 2008; Matsumoto et al. 2008; Park et al. 2008; Sepulveda et al. 2009; Ewald et al. 2011). Moreover, Enhanced activities of CatB, CatL, and CatS were also detected in human monocytic cell line THP-1 after exposure to LPS, TLR2, and TLR3 ligands (Creasy and McCoy 2011).

T1D is a proinflammatory state characterized by increased levels of circulating biomarkers of inflammation, such as IL-1β, IL-6, IFN-γ, and TNF-α (Devaraj et al.

2006). Loss of pancreatic β cells in T1D is associated with an increase in Th1 proinflammatory cytokines in the islet milieu, with a concomitant reduction in Th2 or Treg anti-inflammatory cytokines (Wachlin et al. 2003; Yoon and Jun 2005). Consistently, CatG secretion has been shown to be induced at the inflammatory site of synovial fluid in RA patients (Miyata et al. 2007). Based on this knowledge, the effect of cytokines on cathepsin activity in T1D was determined. However, CatG activity was not regulated by these cytokines or TLRs ligands tested. The activities of CatX, CatB and CatS were upregulated by TLR1/TLR2, TLR3, and TLR5 ligands in PBMC, suggesting a link between TLR signals and cathepsin upregulation.

4.5. Vitamin D reduces proinsulin presentation in type 1 diabetes and decreases CatG activity in mDC1 from healthy donors

The data provided here revealed that proinsulin induces a T cell response in T1D patients, which can be mitigated by $1\alpha,25(OH)_2D_3$, determined by reduced secretion of TNF-α, IFN-γ, IL-17, and IL-22. Indeed, it was found that T cell differentiation was skewed away from a Th1 or Th17 phenotype towards a Th2 phenotype by $1\alpha,25(OH)_2D_3$ (Borgogni et al. 2008; Daniel et al. 2008). In addition, vitamin D can stimulate Treg cells by increasing levels of CTLA-4 and FoxP3, in contrast to proinflammatory cytokines, particularly IFN-γ, IL-17, and IL-21, which were reduced by vitamin D treatment (Jeffery et al. 2009).

It was found here that vitamin D downregulated CatG activity only in mDC1, but not in B cells. The most convincing evidence of immunomodulatory effects of $1\alpha,25(OH)_2D_3$ are on DC. Multiple studies showed that $1\alpha,25(OH)_2D_3$ can inhibit DC differentiation from its precursors and maturation by different stimuli (Griffin et al. 2001; Gauzzi et al. 2005). Accordingly, vitamin D can only reduce CatG activity in mDC1, but not in B cells as demonstrated here.

The present study demonstrated that vitamin D had no effect on the activity of cysteine proteases in neither B cells nor mDC1. CatG activity was reduced in mDC1 only. However, vitamin D was identified to have the ability to regulate cysteine proteases and cystatins (such as cystatin D, E/M, and A) (Alvarez-Diaz et

al. 2010). In 1996, it was reported that 1α,25$(OH)_2D_3$ can regulate CatB in breast cancer cells (Simboli-Campbell et al. 1996). Recently, Alvarez-Diaz and colleagues demonstrated that 1α,25$(OH)_2D_3$ induces the expression of cystatin D which is an inhibitor of CatS, H, and L in human colon cancer cells (Alvarez-Diaz et al. 2009). Furthermore, 1α,25$(OH)_2D_3$ also modulates the activity of serine proteases. Studies analyzing the effect of 1α,25$(OH)_2D_3$ on the expression of serine proteases, urokinase and tissue plasminogen activators (uPA and tPA) as well as their inhibitors (PAIs) found that 1α,25$(OH)_2D_3$ decreases the secretion of uPA and tPA and increases PAI-1 secretion in breast cells (Koli and Keski-Oja 2000; Bao et al. 2006). There are many reasons for this disparity. First, the different cell types tested in the experiments which harbor variant cathepsins. Secondly, lower levels of CatG, found in control donors, might be explained by the presence of physiological levels of vitamin D.

Alternatively, mDC1 from T1D were resistant to CatG regulation by vitamin D. This could explain higher levels of CatG found in T1D. Indeed, It has been shown that vitamin D had no effect on residual pancreatic β cell function in new-onset T1D (Bizzarri et al. 2010; Walter et al. 2010). Here vitamin D can reduce the activity of CatG only in mDC1 from controls, but not in mDC1 from T1D. Therefore, it is most likely that the protective effect of vitamin D may be beneficial in the initiation stage of T1D, supplementation of vitamin D after disease onset might not have any favorable effects. In addition, It was found that serum levels of 1α,25$(OH)_2D_3$ in patients with recently diagnosed T1D are lower than in healthy controls (Borkar et al. 2010). Therefore, one might speculate that low serum levels of vitamin D in T1D might be insufficient to regulate CatG activity.

In conclusion, Vitamin D mitigates proinsulin-reactive T cell activation in T1D. CatG activity is downregulated by vitamin D in mDC1 from controls, while mDC1 from T1D patients are resistant to CatG regulation by vitamin D. Moreover, T1D patients harbor reduced levels of vitamin D (Pani et al. 2000). Therefore, the therapeutic use of vitamin D has been suggested as a promising tool useful for the prevention

and treatment of autoimmune diabetes (Mathieu and Badenhoop 2005; Mathieu et al. 2005; Mathieu 2010). In particular, supplementation of vitamin D in the initiation stage of T1D could be recommended as evidenced by the data shown here.

4.6. Regulation of T cell activation and cathepsin activity by EBV

In the present study, it was found that EBV can induce distinct proinsulin-experienced T cell activation, which can be reduced by a CatG inhibitor and, to a lesser extent, by the cysteine protease inhibitor E64d. This suggests EBV infection might trigger the activation of diabetogenic T cells and treatment with CatG inhibitors might be beneficial to prevent the activation of diabetogenic T cells, resulting in reduced incidence of T1D as speculated in 4.1. Previous studies have indicated EBV infection to be associated with autoimmune diseases, but no major conclusions have been drawn between EBV infection and T1D yet. Indeed, there is some evidence that EBV may be capable of triggering T1D by molecular mimicry. BOLF1, an 11 amino acid sequence of the EBV protein, was reported to be homologous to residues in the Asp-57 region of the HLA-DQw8 β chain peptide, which is considered to be strongly linked to T1D susceptibility (Sairenji et al. 1991). It was also found that a pentapeptide sequence in the Asp-57 region of the HLA-DQ β chain is successively repeated six times in the EBV-BERF4-encoded epitope (Horn et al. 1988; Parkkonen et al. 1994). On the contrary, there was epidemiological evidence for the protective role of EBV infection, supported by significant lower amount of antibodies against EBV in the sera of T1D patients when compared with healthy controls or their first-degree family members (Krause et al. 2009). Nevertheless, humans encounter more than one virus infection in their lifetime. Therefore, the initiation of autoimmune diseases should integrate more than just one possible environmental triggering factor.

The regulation of cathepsin activity after *in vitro* EBV exposure was addressed here. Surprisingly, EBV stimulation had no effect on cathepsin activity in human primary B cells, mDC1, or pDC. These data are in contrast to the findings that EBV downregulated CatG activity and upregulated CatX activity in B cells from some

healthy donors. One explanation might be that EBV failed to effectively infect these cells. Fiola et al. observed that EBV does not establish infection in freshly isolated pDC after exposure to EBV for 24, 48, and 72h (Fiola et al. 2010). Another explanation may be due to donor-specific characteristics, for example, previous infection or decreased EBV susceptibility due to a genetic variation. On the other hand, DC were demonstrated to be not relevant for EBV infection in EBV IgG+ donors, and EBV-exposed DC can not detect EBV viral antigens by RT-PCR until 12 days of incubation (Bickham et al. 2003). pDC, however, was found to be involved in anti-EBV immunity in humanized NOD-SCID mice by using PBMC from healthy EBV IgG+ donors (Lim et al. 2007).

Accordingly, EBNA1, a marker of EBV infection, was detectable here after 12 days of cell culture with EBV. Therefore, day 12 is the turning point where regulation of cathepsins by EBV occurs during B cell transformation. Initially CatX activity was upregulated and then downregulated after 12 days of culture. CatG activity was undetectable after cell culture for 12 days. The differences of cathepsin distribution and activity between primary B cells and EBV-transformed BLC might partially account for these results (Table 4) (Lautwein et al. 2004). While CatG is expressed in primary B cells, CatG is absent in BLC (Stoeckle et al. 2009). Indeed, some virues were reported to regulate cathepsins in APC, but no data are found with respect to EBV. For instance, CatB, C, S, and X RNA and protein were decreased in monocyte-derived DC after HIV infection, whereas CatL was increased. Additionally, decreased levels of mature MHC class II molecules were observed (Harman et al. 2009). Another study found that CatB activity in influenza A-infected DC was significantly upregulated (Burster et al. 2007b). Furthermore, HCMV infected monocyte-derived DC reduced the activities of CatL, H, S, X, and AEP, accompanied with decreased biosynthesis and maturation of MHC class II molecules (Kessler et al. 2008). These published findings, meanwhile, support that viruses affect the MHC class II antigen processing pathway.

Table 4: Cathepsin activity between primary human B cells and EBV-transformed BLC

Cell type	CatB	CatC	CatH	CatL	CatS	CatX	AEP	CatD	CatG
B cell (CD19+)	+	-	+	-	+	++	-	+	+
BLC	+	+	+	-	+	++	+++	+	-

Note: BLC: EBV-transformed B lymphoblastoid cells. +++ very high, ++ high, + modestly, − not found

The present study also indicated that the expression of MHC class II molecules in B cells was decreased by EBV, as expected that EBV has the ability to downregulate the expression of MHC class I and II molecules in proliferating B cells (Levitsky and Masucci 2002). However, this result does not explain the notion that activation of proinsulin-reactive T cells by EBV exposure was due to increased proinsulin presentation, since levels of MHC class II molecules were decreased. Hence, it seems that there is no relation between the activation of proinsulin-reactive T cells and increased proinsulin presentation.

In conclusion, a common feature of viruses is the ability to manipulate the host immune system and evade immune inspection. The findings here suggested that EBV evades the immune system by regulation of the antigen processing machinery, in order to induce EBV-driven immune escape.

4.7. CatG is dispensable in processing of Ii in B cells and mDC1

Whether the serine protease CatG is involved in the initial step of Ii degradation in primary APC is unknown. The data here indicated that Ii can be degraded to several intermediates by CatG *in vitro* and thereby generate CLIP. However, no differences between non-CatG expressing BLC and primary human B cells or mDC1 in the proteolysis of Ii were found, even by using different CatG inhibitors. Inhibition of CatG in intact cells did not alter Ii degradation in B cells or mDC1. These data obtained were not expected, since the cathepsin distribution differs between primary APC and cell lines (Dudziak et al. 2007). As part of the endocytic antigen processing pathway, proteolytic cleavage of the Ii is important for the generation of CLIP, which can prevent premature loading of antigenic peptides to

MHC class II molecules. Variations in antigen processing are cell type dependent. For instance, AEP dominates TTC and MBP processing in BLC but not in primary B cells (Watts et al. 2005; Burster et al. 2010b). Furthermore, cysteine proteases, such as CatS, CatL, or CatV, play a pivotal role in the final stage of Ii degradation (p10 to CLIP) depending on the cell type studied (Riese et al. 1996; Driessen et al. 1999). Therefore, one might expect differences in Ii processing regarding the cell type used, however, the present data showed no differences between BLC and primary human B cells or mDC1 in Ii processing.

In summary, the indicated results are in agreement with Maehr and colleague's statement that *in vitro* Ii digestion pattern observed when utilizing purified proteases cannot be extended to living cells (Maehr et al. 2005a). CatG degraded Ii only at pH 7.2 but not at pH 5.0 and no differences in Ii processing was found by using CatG inhibitor indicating that CatG does not process Ii in the endocytic compartments. Thus, CatG is dispensable in Ii processing in primary human B cells and mDC1.

4.8. Conclusions

Taken together (Figure 38), effective processing and presentation of proinsulin depended on the activity of CatG in PBMC from T1D. Cleavage sites obtained by purified CatG were similar to those acquired by mDC1-derived lysosomal cathepsins when proinsulin was processed *in vitro*, suggesting the critical role of CatG to control the processing of proinsulin. Furthermore, T cells from T1D patients were activated by CatG-generated proinsulin peptides, DCins6 and DCins12, indicating novel T cell epitopes. While vitamin D reduced CatG activity in mDC1 from healthy donors, mDC1 from T1D were resistant from downregulation of CatG. Inhibition of CatG *via* a physiological level of vitamin D or CatG inhibitor reduced proinsulin pulsed T cell activation. In addition, EBV exposure induced proinsulin-reactive T cell recall, which can be impaired by CatG inhibitor or E64d. However, CatG is dispensable in Ii processing in primary human B cells and mDC1. The data here are therefore recommended as the basis for the generation of specific immunomodulators, including CatG inhibitors, which are useful for

immunotherapy.

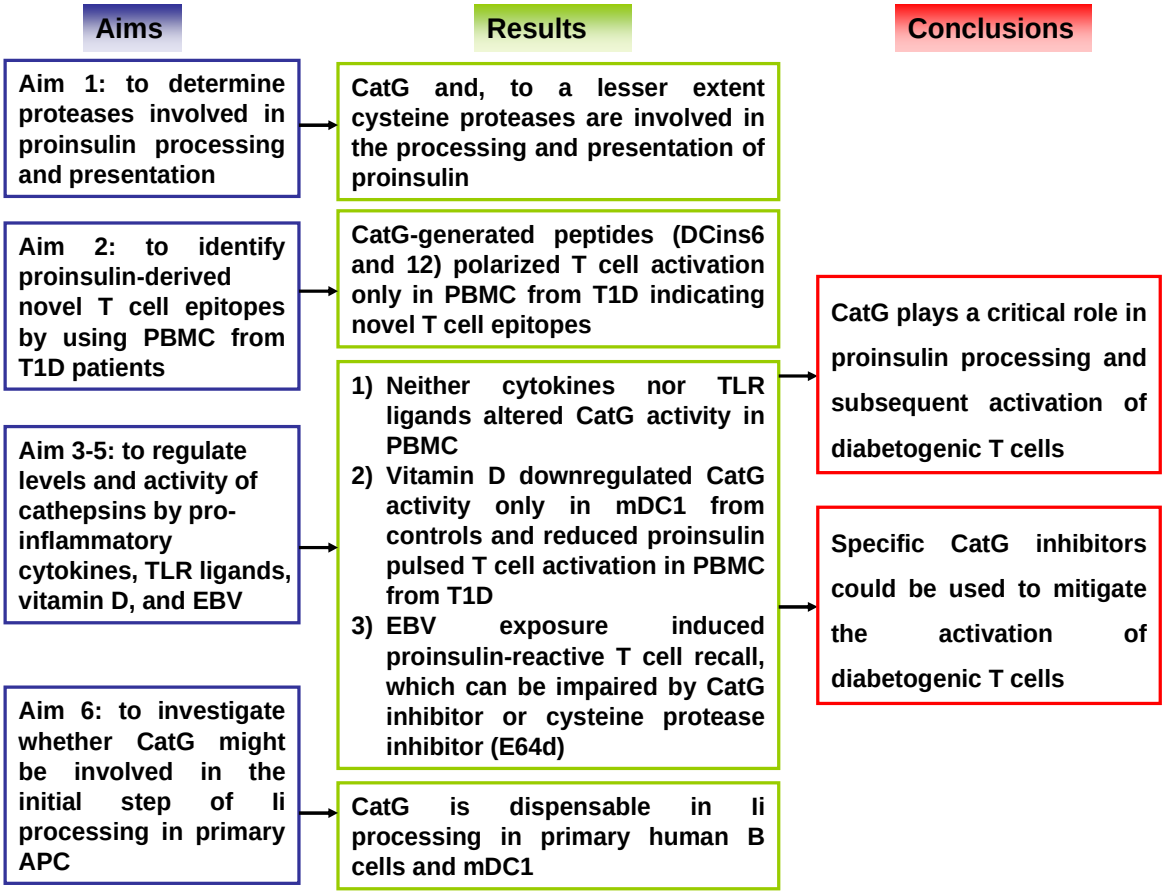


Figure 38: Summary of the present study. The left panel (blue boxes) shows the aims of the study, the major results represent in the middle panel (green box), and the right panel (red box) is the conclusions of the present study. APC: antigen presenting cells; CatG: cathepsin G; EBV: Epstein-Barr virus; mDC: myeloid dendritic cells; PBMC: peripheral blood mononuclear cells; T1D: type 1 diabetes; TLR: toll like receptor.

5. Summary

Autoantigenic peptides resulting from self-proteins such as proinsulin do play an important role in the development of type 1 diabetes mellitus (T1D). Self-proteins can be processed by cathepsins (Cats) within endocytic compartments and loaded to major histocompatibility complex class II molecules for CD4+ T cell inspection. However, the processing and presentation of proinsulin by antigen presenting cells in humans is only partially understood. It was demonstrated by using CatS, B, and L deficient mice that these proteases are important in the onset of autoimmune diabetes. The serine protease, CatG, dominates the processing of antigens and autoantigens. It was found that CatG activity was elevated in peripheral blood mononuclear cells (PBMC) from T1D patients compared to controls. Therefore, the present study determined the impact of cathepsins (in particular CatG) on the activation of proinsulin-reactive T cells in T1D. Proinsulin served as an autoantigen model, cathepsins involved in proinsulin processing were determined by using specific cathepsin inhibitors in the T cell assays. Regulators, such as proinflammatory cytokines, toll like receptor ligands, and vitamin D, were used to elucidate their potential to regulate the activity of cathepsins in the pathogenesis of T1D. Furthermore, EBV exposure, as a model of environmental challenge for T1D, was performed to investigate its role in proinsulin processing and cathepsin regulation. First, it was demonstrated that CatG and, to a lesser extent, cysteine proteases are involved in the proinsulin processing. Second, *in vitro* processing of proinsulin by primary human myeloid dendritic cell (mDC1)-derived lysosomal cathepsins resulted in several proinsulin-derived intermediates, which were similar to those obtained by using purified CatG, and some of the intermediates were generated by CatD and CatS. CatG-generated peptides (DCins6 and DCins12) polarized T cell activation only in PBMC harvested from T1D patients indicating novel T cell epitopes. Third, proinsulin-reactive T cells from T1D patients can be functionally inhibited by both a specific CatG inhibitor and vitamin D. Fourth, vitamin D reduced CatG activity specifically within mDC1 from healthy controls, compared

to mDC1 from T1D, which were resistant to CatG regulation by vitamin D, suggesting an protective effect of vitamin D in the development of T1D. Fifth, EBV induced proinsulin-reactive T cell activation, which can be downregulated by CatG inhibitor or E64d. Finally, CatG was found to be dispensable in Ii processing in primary human B cells and mDC1. Collectively, these data support the notion that CatG plays a critical role in proinsulin processing and subsequent activation of diabetogenic T cells. Therefore, specific CatG inhibitors could be used to mitigate the activation of diabetogenic T cells.

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8. Curriculum vitae

Curriculum vitae not included in the online version for reasons of privacy protection.