Characterization of preproinsulin (ppins)-specific CD8 T cell responses in novel models of autoimmune diabetes

Dissertation zur Erlangung des Doktorgrades der Humanbiologie (Dr. biol. hum.) der medizinischen Fakultät der Universität Ulm

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2010
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Tag der Promotion: 18.06.2010
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Abbreviations

General abbreviations

A  A-chain of insulin
aa  amino acid(s)
(m)Ab  (monoclonal) antibody
APC  allophyocyanin
APCs  antigen-presenting cells
B  B-chain of insulin
B6  C57BL/6J
β-ME  SS-mercaptoethanol
BFA  brefeldin A
BM  bone marrow
bp  basepair(s)
BSA  bovine serum albumin
C  C-peptide of insulin
cat.no.  catalog number
CD  Cluster of Differentiation
Ci  curie
CIP  calf intestinal alkaline phosphatase
CTLA-4  Cytotoxic T-Lymphocyte Antigen 4
Cy5.5  Cyanine Dye 5.5
DMEM  Dulbecco’s Modified Eagle Medium
DMSO  dimethyl sulfoxide
DNA  deoxyribonuclein acid
E. coli  Escherichia coli
EAD  experimental autoimmune diabetes
Abbreviations

EDTA ethylenediaminetetraacetic acid
ER endoplasmatic reticulum
FCM flow cytometry
FCS fetal calf serum
FITC fluorescein isothiocyanate
(e)GFP (enhanced) green fluorescent protein
H&E hematoxylin-eosin
HBS HEPES buffered saline
HBSS Hank's balanced salt solution
HEK293 human embryonic kidney 293
HeLa cervical cancer cells from Henrietta Lacks
HLA human leukocyte antigen
HRP horseradish peroxidase
IDDM insulin dependent diabetes mellitus
IFN interferon
Ig immunoglobulin
IP immunoprecipitation
kb kilobase(s)
kDa kiloDalton
L IGκ leader
LB luria bertani
M molar
MACS magnetic activated cell sorting
MHC major histocompatibility complex
NK-Zellen natural killer cells
NOD mouse Non Obese Diabetes mouse
OVA ovalbumin
PAGE Polyacrylamide gel electrophoresis
PAS Protein A sepharose
PBS phosphate-buffered saline
PE phycoerythrin
PD-1 Programmed Death 1
PD-L1 Programmed Death-Ligand-1
PFA paraformaldehyde
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>pins</td>
<td>proinsulin</td>
</tr>
<tr>
<td>ppins</td>
<td>preproinsulin</td>
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<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>RIP</td>
<td>rat insulin promotor</td>
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<tr>
<td>rpm</td>
<td>rounds per minute</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>S or HBsAg</td>
<td>hepatitis B virus small surface antigen</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SP</td>
<td>signal peptide</td>
</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>tg</td>
<td>transgenic</td>
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<tr>
<td>Th</td>
<td>T helper</td>
</tr>
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<td>U</td>
<td>units</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
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<tr>
<td>wt</td>
<td>wild type</td>
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**Abbreviations**

### Nucleotid abbreviations
- A adenine
- C cytosine
- G guanine
- T thymidine

### Amino acid abbreviations

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<td>A</td>
<td>Ala alanin</td>
<td>M</td>
<td>Met methionine</td>
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<tr>
<td>C</td>
<td>Cys cysteine</td>
<td>N</td>
<td>Asn asparagine</td>
</tr>
<tr>
<td>D</td>
<td>Asp aspartic acid</td>
<td>P</td>
<td>Pro proline</td>
</tr>
<tr>
<td>E</td>
<td>Glu glutamic acid</td>
<td>Q</td>
<td>Gln glutamine</td>
</tr>
<tr>
<td>F</td>
<td>Phe phenylalanin</td>
<td>R</td>
<td>Arg arginine</td>
</tr>
<tr>
<td>G</td>
<td>Gly glycine</td>
<td>S</td>
<td>Ser serine</td>
</tr>
<tr>
<td>H</td>
<td>His histidine</td>
<td>T</td>
<td>Thr threonine</td>
</tr>
<tr>
<td>I</td>
<td>Ile isoleucine</td>
<td>V</td>
<td>Val valine</td>
</tr>
<tr>
<td>K</td>
<td>Lys lysine</td>
<td>W</td>
<td>Trp tryptophan</td>
</tr>
<tr>
<td>L</td>
<td>Leu leucine</td>
<td>Y</td>
<td>Tyr tyrosine</td>
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1 Introduction

1.1 Diabetes mellitus

Diabetes mellitus is a metabolic disease which is spread all over the world. About six percent of the world population suffer from diabetes. With four million listed cases of death per year it has a similar incidence like HIV [33, 50]. Patients have a disordered glucose metabolism, either due to a decreased insulin production or an impaired response of the insulin producing pancreatic β cells, resulting in inappropriately high blood sugar called hyperglycemia. Frequent urination (polyuria) and resultant excessive thirst (polydipsia) are the first, rapidly developing symptoms of increased blood glucose levels. Also weight loss and fatigue can be observed within the first weeks of disease onset. Until now there is neither prevention or protection, nor cure known [55]. The only therapy consists of regular and lifelong injections of artificial insulin with an accurate measuring of blood glucose levels. The failure of therapy may have serious consequences from diabetic ketoacidosis to coma or death [21].

The pancreas is a gland organ in the digestive and endocrine system of vertebrates. Its major part, the endocrine part, produces digestive enzymes and pancreatic juice which are secreted into the small intestine. Whereas the exocrine pancreas, represents only two percent of the entire organ and consists of cell clusters, named islets of Langerhans. The islets are composed of four main cell types classified by their secretion: α cells secrete glucagon, β cells secrete insulin, δ cells secrete somatostatin, and PP cells secrete pancreatic polypeptide.

1.2 Type 1 Diabetes

Type 1 diabetes is known as insulin-dependent diabetes and develops most frequently in children and adolescents under the age of 20. It is marked by insulin insufficiency caused
by an autoreactive, T cell-mediated damage of insulin producing β cells \[35\]. Insulin is the only known target-antigen for those T cells \[11\], but other β cell specific antigens are under discussion \[31\]. The activator and background of the disease is still unclear. There is a strong hint of genetic inheritance of particular HLA genotypes which promotes diabetes type 1 \[10, 34\]. However, the inherited susceptibility is only one factor. It seems that type 1 diabetes requires an additional environmental trigger, for example viral infections or commonly stress \[43\].

### 1.3 Type 1 Diabetes in mice models

There is increasing evidence from patients with T1D and from murine models of diabetes that autoreactive CD8 T cells contribute significantly to the development of this autoimmune disease \[4, 30, 37, 53, 62\]. Studies have shown that the RIP-B7.1 mouse is an attractive model to investigate the CD8 T cell-mediated pathogenesis of experimental autoimmune diabetes (EAD). This mouse express the co-stimulator molecule B7.1 (CD80) under the control of the rat insulin promoter (RIP) in the β cells of islets of Langerhans \[18\]. DNA vectors encoding murine preproinsulin-II efficiently induced EAD in RIP-B7.1 mice within 3-4 weeks after immunization \[36, 38\]. Thus, RIP-B7.1 mice are used in this study to characterize the specificity and diabetogenic potential of CD8 T cell responses. Another mouse model to investigate autoimmune diabetes is the Non Obese Diabetes (NOD) mouse \[2\]. Makino and colleagues (Shionogi Research Laboratories in Aburahi, Japan) developed the NOD strain and in 1980 it was first referred to \[29\]. This mouse develops autoimmune insulin dependent diabetes mellitus (IDDM) spontaneously at 15 to 20 weeks of age. The incidence of spontaneous diabetes is sex dependent with approximately 60-80% females and 20-30% males developing the disease \[2\].

### 1.4 DNA immunization

DNA immunization is an attractive method to prime CD8 T cell responses in mice. As shown in Fig. 1, plasmid DNA (encoding preproinsulin under viral promoter control) is injected e.g. into the tibialis anterior muscle of mice. The DNA is taken up either by myocytes (1) or antigen presenting cells (2). The major advantage of this method is
that proteins (antigens) are produced by host cell machinery. To induce T cell responses, the protein has to be presented by professional antigen presenting cells (APC). The presentation of antigen occurs either through the exogenous (1, 3-4) or endogenous (2) pathway. In the classical exogenous pathway antigens are secreted from transfected cells and taken up by APCs, where they are proceeded and loaded onto MHC class II complexes (4). Antigens processed in non-APC can also be presented via cross presentation. The transfected cell undergoes apoptosis and the apoptotic or necrotic bodies containing the antigen are engulfed by APCs. Antigens derived from cross presentation are presented by MHC class I complexes (3), like in the endogenous pathway (2). In this pathway DNA is directly assimilated by APCs, translated and load on MHC class I molecules. The antigen-load APCs travel to the draining lymph node (5) and there present the antigens to T cells. Depending on the MHC class molecule, CD4 and B cells or CD8 T cells are activated. TCR- antigen-load MHC interaction prime the corresponding T cells (7) in combination with co-stimulatory signals. The activated lymphocytes migrate to the target tissue (8) where they can induce an immune response.

1.5 Co-stimulatory and co-inhibitory signals

T cell priming by professional antigen-presenting cells (APC) is modulated by co-stimulatory and co-inhibitory signals. The activation of T cells is a complex process that requires a multitude of interactions between APCs and T cells. The first essential signal is the binding of the T cell receptor to the MHC-bound antigen on the APC. Specificity of the immune response is defined by this interaction but it is not sufficient for activation. Further co-signals are required to trigger an immune response. These signals can be either co-stimulatory or co-inhibitory. The co-stimulatory B7/CD28 pathway is one of the best-known signaling pathways and provides critical signals for T cell activation and tolerance induction [13]. The B7.1 is expressed by the presenting β cells in RIP-B7.1 mice as well as effector CD8 T cells. Non-physiological expression of B7.1 on the surface of β cells could bind to either co-stimulator molecule CD28, or the co-inhibitor molecules CTLA-4 or PD-L1 (Fig:1.2) [8]. These mice developed EAD after immunization with ppins antigens [19, 38]. Thus, the non-physiological, co-stimulatory B7.1/CD28 interaction in RIP-B7.1 mice may allow more efficient effector function delivery by CD8 T cells that apparently cannot be overcome by co-inhibitory B7.1/PD-L1 and B7.1/CTLA-4 interactions.
Figure 1.1: Schematic presentation DNA immunization. Adapted from DNA vaccines. [25]
Co-inhibitory signals generated by ‘programmed death-1’ (PD-1)/‘programmed death ligand-1’ (PD-L1 or B7-H1) interactions down modulate T cell responses and maintain self-tolerance in autoimmune diabetes [24, 23]. Inducible or constitutive expression of PD-L1 is found in many peripheral tissues including the β cells of pancreatic islets [37]. Ligation of PD-1 (expressed on activated T cells) to PD-L1 (expressed by epitope-presenting cells) down-modulates T cell proliferation but can also co-stimulate T cell activation [20, 22, 53]. Furthermore, PD-L1 interacts specifically with the co-stimulatory B7.1 (CD80) molecule upregulated by activated T cells to inhibit their responses. PD-1/PD-L1 interaction facilitates establishment of self-tolerance thereby partially controlling diabetes development of NOD mice [36, 48, 57]. Selective, transgene-driven overexpression of PD-L1 by pancreatic β cells can result in autoimmunity and transplant rejection suggesting operation of a co-stimulatory PD-L1 pathway [52].

Figure 1.2: Schematic presentation of B7 Family Receptors on antigen presenting cells and CD28 Family Ligand on T cells.
1.6 Processing of diabetic epitopes for MHC-class I-restricted presentation

In RIP-B7.1 mice DNA vectors encoding murine preproinsulin-II (ppins) efficiently induce EAD [19, 20]. The phenotype of EAD is characterized by progressive islet invasion of T cells (insulitis), β cell destruction and hyperglycemia [20]. It is unknown how diabetic epitope(s) are processed and presented from the ppins antigen and why DNA-based immunization with ppins can readily break tolerance and efficiently induce EAD.

Proteins targeted to the ER by hydrophobic signal sequences or specific trans membrane domains present a major source of MHC class I-binding peptides. Most ER-targeted proteins undergo retrograde transport to the proteasome- and TAP-dependent processing pathway. This results in the release of entire proteins or their fragments from the ER to the cytosol, where they have access to the conventional MHC class I processing pathway [48]. It is assumed that proteasomes generate the final COOH-terminus of a MHC class I-binding peptide, whereas ER-associated amino peptidases trim off the NH₂-terminal extensions [6, 32, 49]. Direct ER-associated protein processing and subsequent MHC class I-loading of antigenic peptides have been described for MHC class I-binding epitopes that do not require proteolytic trimming of the COOH-termini. For example, some ER-targeting signal sequences contain a CD8 T cell epitope with a final COOH-terminus that is released by ER-resident signal peptidases [9, 51]. In vitro analysis showed that presentation of epitopes required neither proteasomes, nor TAP [9, 51]. Furthermore, this streamlined processing may increase the presentation efficiency of epitopes [15]. Two HLA-A*0201-restricted epitopes from the 24 residue human preproinsulin signal peptide (SP) localized at the extreme COOH-terminus have been identified previously [51]. CD8 T cells with these specificities were found in patients with type 1 diabetes (T1D), suggesting that they may be directly involved in this disease [51]. In the RIP-B7.1 model, ppins-specific DNA immunization efficiently induced insulin A-chain-specific CD8 T cells [20]. Because the COOH-terminus of the epitope corresponds to that of the ppins molecule, no further COOH-terminal proteolytic trimming is necessary.
1.7 Aim of the study

The RIP-B7.1 mouse model has been informative for characterizing the crosstalk between diabetogenic preproinsulin-specific CD8 T cells with their target organ (i.e. the pancreas). However, many aspects of the priming and effector phase of diabetogenic immune response are still unknown.

The main focal points of my thesis were to investigate:

- how preproinsulin-derived antigens are expressed and processed in order to prime diabetogenic, preproinsulin-specific CD8 T cells
- whether different preproinsulin-specific CD8 T cell epitopes are primed and how they are modulated by strong (e.g., viral) CD8 T cell responses
- whether altering of the PD-L1(B7-H1)/PD-1 co-inhibition on pancreatic β cells could reveal a diabetogenic potential of ppins-specific CD8 T cells
2 Material and Methods

2.1 Chemicals

Chemicals used for the experiments are listed in the appendix.

2.2 Media and Buffers

Media and buffers used for the experiments are listed in the appendix.

2.3 Mice

C57BL/6J (B6) mice, H2 class I deficient Aα mice, RIP-B7.1 mice, PD-L1 knockout (PD-L1−/−) mice, PD-1 knockout (PD-1−/−) mice and RIP-B7.1/RIP-OVA mice were bred and maintained under standard pathogen-free conditions in the animal colony of Ulm University (Ulm, Germany). Male and female mice were used in the experiments at 6–8 weeks of age. RIP-B7.1 and RIP-B7.1/RIP-OVA mice were backcrossed for >15 generations to the C57BL/6 (H-2b) background as described previously [18].

2.4 Synthetic peptides

The synthetic peptides used in this study were obtained from JPT Peptide Technologies GmbH (Berlin, Germany). Peptides were dissolved in DMSO at a concentration of 10–20 µg/ml.
2 Material and Methods

| Insulin   | A\textsubscript{12–21} | SLYQLENYYCN |
|          | A\textsubscript{12–N21A} | SLYQLENYCA   |
|          | B\textsubscript{22–29}   | RGFFYTPM    |
| Library  |                     |

| Ovalbumin | OVA\textsubscript{257–264} | SIINFEKL    |

| HBs Antigen | S\textsubscript{190–197} | VWLSVIWM   |
|            | S\textsubscript{208–215} | ILSPFLPL   |

2.5 Cell lines and Generation of stable cell lines

The human HeLa S3 cell line (CCL-2.2) and the human HEK293 cell line (CRL-1573) were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). The Rauscher virus-transformed T lymphoma line RBL5 [27] is derived from a C57BL/6 (H-2b) mouse. The RBL-5 derived mutant cell line RMA/S was kindly provided by Dr K. Kärre (Stockholm, Sweden). All cell lines except HEK293 cells were cultured in Clicks/RPMI. HEK293 cells were cultured in DMEM.

2.6 Generation of expression plasmids

Construction of expression plasmids

The antigenic sequences were synthesized by GeneArt (Regensburg, Germany) and cloned in the pCI vector (cat. no. E1731, Promega) using the NheI and NotI restriction sites. DNA batches were produced in *E. coli* (PlasmidFactory GmbH). For construction of plasmids containing an ER-targeting signal (L), DNA sequence (METDTLLLWVLLL-WVPGSTGD) derived from the murine Igκ chain was attached to the NH\textsubscript{2}-terminus DNA sequence of corresponding DNA sequences. An ER-retention signal (SKDEL) was introduced into COOH-terminus by PCR. PCR product was inserted into PvuII and NotI sites of the expression vector.
2 Material and Methods

Restriction

Restriction digests contained 10µg plasmid, 20U restriction enzymes and the appropriate reaction buffers in a total volume of 50µl. The reactions were incubated at 37°C for 1 hour. To avoid self-ligation the target vectors were additionally treated for 30 minutes at 37°C with 10U of alkaline phosphatase (CIP) removing 5'-phosphate groups. All enzymes and buffers were used from New England Biolabs.

Agarose Gel

The different fragments of the restriction digests were separated via agarose gel electrophoresis. According to the fragments size 1–3% agarose gels were made by dissolving the agarose in 1xTAE buffer by heating. For visualization under UV light the DNA intercalating Ethidium bromide was added. The gel was loaded with the specimen mixed with loading buffer. Additionally DNA fragments a 1kb (cat. no. N3232) and 100bp ladder (cat. no. N3231, both New England Biolabs) were loaded to determine the fragment size. The voltage and time differed depending on the samples.

Purification

The desired DNA fragments were resected from the gel and purified using the Qiaquick Gel Extraction Kit (cat. no. 28706, Qiagen) as prescribed by manufacturer’s protocol.

Ligation

Ligation reaction was performed in a total volume of 20µl containing adequate amounts of purified insert DNA (ratio insert : vector 5:1), 100ng vector, 400U T4 DNA ligase (cat. no. M0202, New England Biolabs) and 2µl 10x ligase buffer and incubated at room temperature for 2 hours.

Transformation in E.coli

E. coli DH5 were transformed via heat shock method. Therefore bacteria were incubated on ice for 30 minutes with 10µl of ligation mixture, subsequently heat-shocked for 45 seconds at 42°C and immediately put on ice. After adding 750µl S.O.C. medium, bacteria were incubated for 45 minutes at 37°C. 100µl and 200µl of the transformation reaction
was plated onto LB agar plates supplemented with 100µg/ml ampicillin. The plates were incubated at 37°C overnight.

**Plasmid purification**

For small-scale plasmid purification the Mini Prep Kit (cat. no. 12123, Qiagen) was used as prescribed by manufacturer’s protocol. On a larger scale approach, plasmids were purified using Midi-, Maxi- or Mega Prep Kit (cat. no. 12143/12163/12183, Qiagen) according to manufacturer’s protocol. Batches of DNA used for immunization were produced in E. coli by PlasmidFactory GmbH (Bielefeld, Germany).

**2.7 Characterization of antigen expression**

**Calcium phosphate transfection**

HEK293 cells were split a day before transfection to ensure an appropriate confluence of 60–70%. The transfection samples containing 10µg DNA and 62µl 2M CaCl₂ in a total volume of 500µl were dropped slowly into 500µl 2M HEPES buffered saline (HBS) while vortexing. The mixture was rested for 90 seconds at room temperature and then added drop wise to the HEK cells. To prevent toxicity the medium was changed after 24 hours and the expression was detected after 48 hours.

**Immunoprecipitation**

Cells were labeled with 100µCi ³⁵S-methionine/cysteine (cat. no. IS103, Hartmann Analytic GmbH) in 3ml methionine/cysteine-free RPMI-1640 (cat. no. P04-18056, PAN) between 36 and 48 hours post transfection. Thereafter, they were cleared by washing with PBS and lysed with pH 8.0 IP-lysis buffer B for 30 minutes on ice. Non-labeled HEK293 cells were directly lysed 48 hours post transfection with pH 8.0 IP-lysis buffer A supplemented with the protease inhibitors, leupeptin and aprotinin. Both lysates were precipitated by incubation with rabbit anti-insulin antibody (cat. no. sc-9168, Santa Cruz Biotechnology) for 1 hour on ice and protein A-sepharose (PAS, cat. no. 17-0780-01, Pharmacia) for 1 hour on ice. Precipitates were washed five times with IP-washing buffer and two times with PBS and 0.1x PBS. They were recovered from PAS by incubation for
1 hour at 37°C with pH 6.8 IP-elution buffer. The samples were then processed either for SDS-PAGE (10–15%) and subsequent gel fluorography or for Western Blot analysis. Alternatively, non-labeled cells were directly lysed with IP-lysis buffer B (pH 8.0) and the total cell extracts were processed for SDS-PAGE.

**SDS**

12.5–15% SDS-Gels were loaded with 10µl samples supplemented with SDS-loading buffer derived from immunoprecipitation. Additionally 5µl stained protein marker (cat. no. RPN800E, GE Healthcare) or [14C]-marker (cat. no. CFA756-1UCI, GE Healthcare) were loaded for size control. Electrophoresis carried out at 60V to allow the samples to reach the resolving gel slowly. Then, voltage was increased to 120V and electrophoresis was stopped shortly before the samples ran out.

**Western**

Gels were processed for Western Blot by incubation in SDS-equilibration buffer. Blotting was performed with iBlot Dry Blotting System (Invitrogen) and accordingly the membrane was washed with 50% isopropanol following washing steps with water and with SDS-buffe TBS. Over night the unspecific binding sites of the nitrocellulose membrane were blocked with SDS-blocking buffer and subsequently washed three times with SDS-buffer GT. First the membrane was incubated with rabbit anti-insulin (cat. no. sc-9168, Santa Cruz Biotechnology) antibody for 1 hour followed by 3 washing steps with SDS-buffer GT. Immune complexes were detected either with 0.5–1 µCi 35S-labeled anti-rabbit IgG (cat. no. SJ 43, Amersham Biosciences) or with HRP conjugated anti-rabbit antibody (cat. no. NA934V, GE Healthcare) by incubation for 1 hour. The membrane was cleared from unbound antibody by 3 washes with SDS-buffer GT. Bound 35S-labeled IgG was detected via gel fluorography and HRP conjugated antibody via Immobilon Western Chemoluminescent HRP Substrate (cat. no. WBKL S0100, Millipore).

**2.8 Immunization of mice**

Intramuscular (i.m.) DNA immunization was achieved by injecting 75–100µg plasmid DNA dissolved in PBS into both tibialis anterior muscles.
2 Material and Methods

2.9 Determination of blood glucose levels

Diabetes was diagnosed when two consecutive blood glucose values exceeded 250mg/dl (13.8 mmol/l) (Disetronic Freestyle).

2.10 Histology

Immunohistochemistry

Pancreatic cryosections were cut 3µm thick and pretreated with blocking goat serum (cat. no. 50-197Z, Zymed) for 1 hour in a wet chamber. Then, sections were incubated with a primary guinea pig antibody to insulin (cat. no. A0564, Dako) (1:100) and a PE anti-CD8 antibody (1:100) (clone 53-6.7, cat. no. 553032, BD Pharmingen) for 1 hour. The secondary antibody, FITC-labeled anti-guinea pig IgG (cat. no. F-6261, Sigma-Aldrich) (1:100), was used to detect insulin-bound immune complexes. Sections were covered and fixed with Cytoseal60 mounting medium (cat. no. 18006, EMS). The Olympus IX71 fluorescence microscope was used equipped with a digital camera (C4742, Hamamatsu).

Immunofluorescence

HeLa cells were grown in twin chambers (cat. no. 155380, Nunc) in 2ml of medium (cat. no. 31885, Gibco) supplemented with 10% FCS. Afterwards cells were transfected with DNA plasmids with the Nanofectin transfection reagent (cat. no. Q051/005, PAA). Cells were fixed with 4% PFA and washed with PBS. For permeabilisation cells were treated with 0.1% Triton for 10 minutes. Then cells were stained for insulin with a primary guinea pig antibody to insulin (cat. no. A0564, Dako) and secondary with FITC-labeled anti-guinea pig IgG (cat. no. F-6261, Sigma-Aldrich) as well as rabbit anti-calnexin Ab (cat. no. sc-11397, Santa Cruz Biotechnology) followed by TRIC-labeled anti-rabbit Ab (cat. no. ab50598, Abcam). All antibodies were diluted 1:200 in PBS. After staining, excess antibodies were removed by 2 washing steps with PBS. Images of cells were acquired with a fluorescence microscope (IX71, Olympus) equipped with a digital camera (C4742, Hamamatsu).
2.11 Preparation of Lymphocytes

Preparation of Lymphocytes from spleen.

Spleens were removed and minced by rubbing in 10ml PBS supplemented with 1%BSA through a metal sieve. Thereafter, erythrocytes were lysed with 10ml lysis buffer for 4-5 minutes and washed twice with PBS/1%BSA. Before the second wash step cell remains were removed by pipetting.

Preparation of pancreatic cells

Pancreata were perfused in situ with 2ml collagenase P (cat. no. 11213865001, Roche) dissolved in HBSS (1mg/ml) (cat. no. 11213865001, Roche). Thereafter, pancreata were removed, digested with collagenase P for 8 minutes at 37°C, washed with cold HBSS supplemented with 10%FCS and passed through a strainer. Pancreatic cells were dissolved in 10ml of cold HBSS, underlayered with 5ml Histopaque-1077 (cat. no. 10771, Sigma-Aldrich) and purified by centrifugation for at 2400 rpm without brake. The interphase was taken and washed with HBSS/10%FCS. Pellet included β cells and lymphocytes.

2.12 Generation of bone marrow chimeras

Femurs and tibiae of donor mice were flushed with RPMI-1640 supplemented with 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin and 0.1M β-ME, and accordingly the obtained bone marrow (BM) was washed twice with same medium. Acceptor mice were sublethally irradiated with 900 rad. 5x10^6 bone marrow cells from donor mice were transferred intravenously (i.v.) into the acceptor mice.

2.13 Transfers of Lymphocytes or purified CD8 T cells

Lymphocytes were gained from pancreata or spleens as described before. In case of CD8 T cells transfer, CD8 T cells were purified before via MACS method (cat. no. 130-090-859, Miltenyi Biotec) according to manufacturer’s protocol. Acceptor mice were sublethally...
irradiated with 900 rad and induced intravenously with $3 \times 10^7$ lymphocytes or $3 \times 10^6$ CD8 T cells per mouse.

2.14 Determination of $K^b$ binding affinities

RMA/S cells were cultured for 24 hours at 37°C in serum-free medium (Ultra Culture). Afterwards, cells were pulsed with 200 µg/ml $K^b/A_{12-21}$, $K^b/B_{22-29}$ and as control with $K^b/OVA_{257-264}$ peptide for 4 hours at 37°C. The binding affinity of the peptides to MHC class I molecules was determined by staining the cells with PE-labeled anti-mouse $K^b$-Ab (cat. no. 553570, BD Biosciences) because only peptide-bound MHC class I molecules are stable on the cell surface.

2.15 Determination of antigen-specific CD8 T cell frequencies

To detect ppins-specific CD8 T cell responses, we used a peptide variant with a $K^b/A_{12-21}$ epitope that had a substitution of N to A at position 21 (SLYQLENYCA). This epitope variant facilitated in vitro expansion of primed CD8 T cells. Briefly, pancreatic cells ($10^5/100$ µl) were incubated for 14 hours in Ultra Culture medium with 20 µg/ml of the indicated peptides in the presence of brefeldin A (BFA) (0.5 µg/ml) (cat. no. 15870, Sigma-Aldrich). Cells were harvested and washed with buffer A. Non-specific binding of antibodies to the Fc-receptor was blocked by pre-incubating cells with mAb 2.4G2 (cat. no. 01241D, BD Biosciences) directed against the FcgRII/II CD16/CD32 (0.5 µg mAb/10⁶ cells/100 µl) and the surface stained with APC-conjugated anti-CD8 antibody for 20 minutes (cat. no. 17-0081-83, BD Biosciences). Then, cells were fixed with 2% paraformaldehyde (PFA) in PBS. Fixed cells were resuspended in FACS B (permeabilisation buffer), stained with FITC-conjugated anti-IFN-γ antibody (cat. no. 554411, BD Biosciences) for 30 minutes at room temperature, and washed twice in buffer FACS B. Stained cells were resuspended in PBS-BSA buffer. Frequencies of IFN-γ⁺ CD8 T cells were determined by FCM analysis. The values were analyzed with GraphPad PRISM software, version 4.0 (GraphPad Software). Hepatitis B surface antigen (HBsAg)-specific, Ovalbumin (OVA_{257-264})-specific or B_{22-29} specific CD8 T cells were detected by tetramer staining. Cells were treated as described above and incubated for 30 minutes at 4°C with
differing fluorochrome-labeled anti-CD8 mAb and PE-conjugated K<sub>h</sub>/S<sub>190–197</sub> tetramers (cat. no. T0400, Beckman-Coulter), PE-conjugated K<sub>h</sub>/OVA<sub>257–264</sub> tetramers (cat.no. T0300, Beckman-Coulter) or APC-conjugated K<sub>h</sub>/B<sub>22–29</sub> tetramers (Glycotope Biotechnology). Subsequently, cells were washed and analyzed by FCM.

2.16 Statistics

For statistic analysis GraphPad Prism (Version 4, GraphPad Software Inc.) was used. Figures show mean values and standard error of the mean (SEM) and the data of at least three independent replicates of one presentable experiment. The statistical significance of differences in the mean CD8 T cell frequencies between groups was determined with the unpaired student’s t-test. A value of (*) $P < 0.05$ was considered significant.
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3.1 The B7.1 mouse model

RIP-B7.1 mice (on the C57BL/6 background) express under the control of the rat insulin promoter (RIP) the co-stimulatory molecule B7.1 (CD80) selectively in pancreatic β cells. These mice are a well-established model for studying de novo induction of diabetogenic CD8 T cells. A single intramuscularly injection of pCI/ppins plasmid DNA encoding murine preproinsulin-II (ppins) (Fig: 3.1) efficiently induced hyperglycemia in RIP-B7.1 mice with an onset of EAD after approximately 3–4 weeks (Fig: 3.2). RIP-B7.1 mice with non-coding pCI vector injections did not develop EAD (Fig: 3.2). The cumulative incidence was >95% at 4–5 weeks post immunization.

![Figure 3.1: Map of ppins. The signal peptide (SP), the B- and A-chains, the C-peptide and the sequence of A_{12−21} epitope are indicated. Ppins was cloned into the multiple cloning site of the pCI expression vector.](image)

Both male and female RIP-B7.1 mice developed EAD after immunization with pCI/ppins DNA (Fig: 3.3).

To identify the T cell subsets required to prime the diabetogenic response in the RIP-B7.1 mouse model, I immunized MHC class II-deficient (A_{α−/−}) RIP-B7.1 mice (RIP-B7.1/MHC-II−/−). EAD was efficiently induced by pCI/ppins DNA in these mice which express no conventional CD4 T cells. Equally, the depletion of CD4 T cells with mAb YTS-191 before ppins-specific immunization [20] or at early time points of diabetes (blood glucose levels 250-400mg/dl) had no effect on the progress of the disease (Fig: 3.4 B). In contrast, anti-CD8 mAb (YTS-169) treatment efficiently blocked diabetes induction [20].
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Figure 3.2: Induction of diabetes in RIP-B7.1 mice. RIP-B7.1 mice were immunized with non-coding pCI (n=5) or pCI/ppins (n=5) DNA. At indicated times after immunization, blood glucose levels (mg/ml) were determined. Each symbol represents one mouse.

Figure 3.3: Male and female RIP-B7.1 mice were immunized with pCI/ppins DNA at six weeks of age. At indicated times after immunization, blood glucose levels (mg/ml) and diabetes incidences (%) were determined.
and transiently cured pCI/ppins-immunized, early diabetic RIP-B7.1 mice (Fig: 3.4 B). CD8 T cell levels restored 2–4 weeks after anti-CD8 antibody treatment was discontinued. Concomitant with the re-emerging CD8 T cell levels disease reappeared. Thus, EAD was induced by a CD4 T cell-independent, diabetogenic CD8 T cell response to ppins in this model.

![Figure 3.4: (A) RIP-B7.1 and MHC-class II-deficient RIP-B7.1/MHC-II−/− mice were immunized with pCI/ppins DNA. At the indicated times after immunization, blood glucose levels (mg/ml) and cumulative diabetes incidences (%) were determined. (B) RIP-B7.1 mice were immunized with pCI/ppins DNA and in early stage of diabetes treated at 3 weeks post immunization with either anti-CD8 Ab (n=3) or anti-CD4 Ab (n=3).](image)

Figure 3.4: (A) RIP-B7.1 and MHC-class II-deficient RIP-B7.1/MHC-II−/− mice were immunized with pCI/ppins DNA. At the indicated times after immunization, blood glucose levels (mg/ml) and cumulative diabetes incidences (%) were determined. (B) RIP-B7.1 mice were immunized with pCI/ppins DNA and in early stage of diabetes treated at 3 weeks post immunization with either anti-CD8 Ab (n=3) or anti-CD4 Ab (n=3).

The development of EAD in pCI/ppins-immunized RIP-B7.1 mice is characterized by CD8 T cell infiltration into pancreatic islets and insulin deficiency. CD8 T cells infiltration and destruction of islet cells were detected in RIP-B7.1 mice by immunohistochemistry (Fig: 3.5). At day 2 post immunization, in pancreatic islets of healthy, normoglycemic mice no CD8 T cells were detectable and insulin expression was not affected. By day 12 after immunization, the mice were still normoglycemic, but CD8 T cell infiltration increased and insulin expression was reduced. Hyperglycemia and extensive insulitis accompanied by infiltration of CD8 T cells and reduced insulin expression was observed 20 days after immunization.
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**Figure 3.5**: RIP-B7.1 mice were immunized with pCI/ppins DNA and analyzed at day 2 (healthy), day 12 (pre-diabetic) and day 20 (diabetic) after immunization. Blood glucose levels (mg/ml) of representative mice are shown in brackets. At the indicated time points, pancreatic sections were analyzed for insulin expression and CD8 T cell infiltration by histology.

**Induction of EAD in RIP-B7.1 mice by A-chain K\(^b\)/A\(_{12-21}\)-monospecific CD8 T cells**

CD8 T cells isolated from immunized, diabetic RIP-B7.1 mice specifically recognized the K\(^b\) restricted A\(_{12-21}\) epitope (SLYQLENCN) of ppins\(^{19, 28}\). *Ex vivo* stimulation of ppins-primed, pancreas-infiltrating CD8 T cells with antigenic ppins\(_{101-110}\) (A\(_{12-21}\)) peptide but not with all other peptides of a ppins library revealed a CD8 T cell population with specifically inducible IFN\(^\gamma\) expression (Fig: 3.6).

The A\(_{12-21}\) epitope inefficiently stabilized K\(^b\) molecules on the surface of TAP-deficient RMA-S cells (Fig: 3.7). This suggested that the K\(^b\)/A\(_{12-21}\) epitope has only low avidity for K\(^b\).

The frequency of K\(^b\)/A\(_{12-21}\)-specific CD8 T cells in pancreatic CD8 T cell populations from DNA-immunized RIP-B7.1 mice was low (0.5–1.5% of pancreas-infiltrating CD8 T cells) (Fig: 3.6). I identified an epitope variant with an alanine (A) exchange for the COOH-terminal asparagine (N) at position 110 (A\(_{21}\)) that efficiently (cross)-reacted with CD8 T cells primed by natural ppins antigens *in vitro*. PCl/ppins\(_{N110A}\) (encoding the variant A\(_{12-N21A}\) sequence) induced EAD in RIP-B7.1 mice with comparable kinetics and efficacies as pCl/ppins. However, irrespective of the ppins antigen used, IFN\(^\gamma\)^+ CD8 T cells expanded 10-20 fold better after *in vitro* stimulation with the mutant A\(_{12-N21A}\)
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Figure 3.6: RIP-B7.1 mice were immunized with pCI/ppins DNA and analyzed at diabetic state. Pancreatic cells were re-stimulated \textit{ex vivo} with a peptide library of insulin composed of 9aa peptides. Specific IFN-γ\(^+\) CD8 T cells were identified by flow cytometry.

![Graph showing IFN-γ+ CD8 T cells](image)

**Figure 3.7**: TAP-deficient RMA-S cells were either not pulsed (-/-) or pulsed for 4 hours with high doses (200\(\mu\)g/ml) of \(\text{K}^b/\text{A}_{12-21}\) or \(\text{K}^b/\text{OVA}_{257-264}\) peptides, followed by surface staining of trimeric \(\text{K}^b\) molecules and FCM analysis.

RMA-S cells pulsed with:
-/-  \(\text{K}^b/\text{A}_{12-21}\)  \(\text{K}^b/\text{OVA}_{257-264}\)
peptide than with natural peptide (Fig. 3.8). It has been shown that amino acids at the extreme COOH-terminus of epitope influence the stability and antigenicity of MHC class I/peptide complexes [12, 56]. I thus identified an epitope variant that allows me to detect and quantitatively determine diabetogenic CD8 T cell responses. If not indicated otherwise all following immunization experiments were made with the pCI/ppins<sub>N110A</sub> DNA (defined as pCI/ppins) and all following expression constructs contain the A<sub>12−N21A</sub> sequence (defined as A<sub>12−21</sub>).

**Figure 3.8:** RIP-B7.1 mice were immunized with pCl/ppins or pCl/ppins<sub>N110A</sub>. Cumulative diabetes incidences (%) are shown. Pancreatic cells derived from diabetic mice were re-stimulated *ex vivo* with A<sub>12−21</sub>, A<sub>12−N21A</sub> or the control S<sub>208−215</sub> peptide. Specific IFN-γ<sup>+</sup> CD8 T cells were determined by flow cytometry.

The number of pancreas-infiltrating K<sub>b</sub>/A<sub>12−21</sub>-specific IFN-γ<sup>+</sup> CD8 T cells increased in the course of the disease. As shown in Fig. 3.5 at day 2 post immunization, in pancreatic islets of healthy, normoglycemic mice no CD8 T cells were detectable. *Ex vivo* re-stimulation confirmed that there were no K<sub>b</sub>/A<sub>12−21</sub>-specific IFN-γ<sup>+</sup> CD8 T cells infiltrating the pancreatic tissue. At day 12 after immunization, low levels of epitope-specific IFN-γ<sup>+</sup> CD8 T cells migrated in the pancreas. Hyperglycemia and infiltration of high numbers of CD8 T cells were observed 20 days after immunization. At this severe state of EAD, about 10-15% of all infiltrating CD8 T cells were epitope specific (Fig. 3.9).
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Figure 3.9: RIP-B7.1 mice were immunized with pCI/pps. At different time points of disease pancreatic cells were re-stimulated ex vivo with the K\(^b/A_{12-N_{21}A}\) peptide or with the control K\(^b/OVA_{257-264}\) peptide. Specific IFN\(\gamma^{+}\) CD8 T cells were identified by flow cytometry.
3.2 Processing of insulin

Expression of ppins in the ER allows priming of diabetic CD8 T cells

The murine preproinsulin II (ppins) contains 110 amino acid residues (Fig: 3.1). Naturally, in the pancreatic β cells, the ppins is translocated into the ER and processed to bioactive insulin [19]. When the entire ppins-encoding gene was introduced into non-pancreatic cells e.g. in transiently transfected HEK293 cells, ppins was expressed and translocated into the ER, where signal peptide was removed to generate proinsulin (pins) (Fig: 3.10, lane a). Only a single bond at 9–10kDa representing the pins was detectable. Further downstream processing of pins to insulin was not observed. ER-associated pins was thus expected to be the major substrate for generating diabetogenic epitopes in non-pancreatic cells.

![Figure 3.10: Expression of ppins in HEK293 cells. HEK293 cells were transiently transfected with pCI/ppins DNA and expression was observed via immunoprecipitation with anti-insulin Ab.](image)

Proteins can be directed to the ER by fusing them with a NH2-terminal signal sequence [60]. I constructed two vectors: the pCI/A12−21 vector that encodes only the insulin Kb/A12−N21k sequence and the pCI/L-A12−21 vector that encodes the insulin Kb/A12−N21k and the Igκ Leader (L) sequence (METDTLLLWVLLLWVPGSTGD). The signal sequence derived from the murine Igκ chain directs the 10 aa long peptide into the ER. The fusion
protein K\textsuperscript{b}/L-A\textsubscript{12–21} induced EAD in RIP-B7.1 mice. The induction of EAD was delayed at week 8-10 comparing to mice immunized with pCI/ppins (Fig: 3.11 A). Diabetogenic CD8 T cells levels in pancreata of pCI/ppins and pCI/L-A\textsubscript{12–21}-immunized mice were compared. The numbers of K\textsuperscript{b}/A\textsubscript{12–21}-specific IFN\textgamma\textsuperscript{+} CD8 T cells in pancreatic tissues of diabetic mice were similar (Fig: 3.11 B). At this stage of EAD, the epitope-specific CD8 T cells reached their maximal level of 8-13% of total pancreatic CD8 T cells. Thus, K\textsuperscript{b}/A\textsubscript{12–21}-monospecific CD8 T cells induce EAD in RIP-B7.1 mice.

![Figure 3.11](image)

**Figure 3.11:** (A) RIP-B7.1 mice were immunized with non-coding pCI, pCI/ppins and pCI/L-A\textsubscript{12–21} DNA. At the indicated times after immunization, blood glucose levels (mg/ml) and cumulative diabetes incidences (%) were determined. (B) Pancreatic cells were re-stimulated *ex vivo* with the K\textsuperscript{b}/A\textsubscript{12–21}A\textsubscript{12} peptide or with the control K\textsuperscript{b}/OVA\textsubscript{257–264} peptide. Specific IFN\textgamma\textsuperscript{+} CD8 T cells were identified by flow cytometry.

I further tested whether the localization of the epitope in the cytosol could lead to induction of K\textsuperscript{b}/A\textsubscript{12–21}-specific CD8 T cells and EAD in RIP-B7.1 mice. Mice immunized with the pCI/A\textsubscript{12–21} vector, encoding the antigenic K\textsuperscript{b}/A\textsubscript{12–21}A\textsubscript{12} sequence without the ER-targeting Ig\kappa leader (L), did not develop EAD within 12 weeks after immunization (Fig: 3.12 A). Also the ER-targeted L-A\textsubscript{1–21} but not the A\textsubscript{1–21} construct efficiently induced EAD in RIP-B7.1 mice (Fig: 3.12 B). An ER-retarding signal sequence (SEKDEL) was fused to the COOH-terminus (pCI/L-A\textsubscript{12–21}-ER and pCI/L-A\textsubscript{1–21}-ER) to enforce the importance of the epitope being processed in the ER (Fig: 3.12 A, B). Both, pCI/L-A\textsubscript{12–21}-ER and pCI/L-A\textsubscript{1–21}-ER vectors induced EAD in RIP-B7.1 mice with a comparable kinetic and incidence. Induction of diabetogenic, K\textsuperscript{b}/A\textsubscript{12–21}-specific CD8 T cells was
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observed when the antigenic K\(^b\)/A\(_{12-21}\) peptide or the A-chain was expressed in the ER.

![Schematic presentation of expression constructs.](image)

**Figure 3.12:** Schematic presentation of expression constructs. The position of the entire A\(_{1-21}\) chain, the A\(_{12-21}\) fragment and the murine Ig\(\kappa\)-leader sequence are indicated. A start methionine was cloned in the front of the A\(_{1-21}\) and A\(_{12-21}\) sequences encoded by the pCI/A\(_{1-21}\) and pCI/A\(_{12-21}\), respectively. RIP-B7.1 mice were immunized with pCI/A\(_{12-21}\), pCI/L-A\(_{12-21}\), pCI/L-A\(_{12-21}\)-ER, pCI/A\(_{1-21}\), pCI/L-A\(_{1-21}\) and pCI/L-A\(_{1-21}\)-ER DNA and cumulative diabetes incidences (%) were determined.

I further used a green fluorescent protein (eGFP)-based reporter system to exclude or localize the ppins from or to the ER. GFP was fused to either the NH\(_2\) (pCI/GFP-ppins) or the COOH-terminus (pCI/GFP-ER) of the ppins (Fig: 3.13). In the later construct, the ppins-GFP sequence was further fused COOH-terminally with the ER-retention signal (SEKDEL) to retard the ppins-GFP in the ER [58, 60]. The receptor for the SEKDEL retention signal reside in an early GOLGI compartment and function...
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through a recycling process that retrieves ER-proteins back from the GOLGI apparatus to the ER. The subcellular localization of the insulin/GFP fusion proteins was analyzed in HeLa cells transiently transfected with the pCI/GFP-ppins and pCI/ppins-GFP-ER.

**Figure 3.13:** Schematic presentation of eGFP-expression constructs. The position of the GFP (eGFP), the ppins (SP-B-C-A), the ER-retention sequence (SEKDEL) and the K\(^{b}\)/A\(_{12-21}\) epitope are indicated.

Imaging immunofluorescence microscopy detected the GFP-ppins fusion protein in the nucleus and cytosol (Fig: 3.14). Staining of calnexin, an ER-localized lectin, showed that there was no co-localization in the ER. In contrast, the signal peptide of ppins directed the ppins-GFP-ER protein into the ER and this fusion protein was co-localized in the ER but was not observed in the nucleus or cytosol. Hence, targeting ppins to the ER was shown for pCI/ppins-GFP-ER vector but not for pCI/GFP-ppins vector.

Insulin- and GFP-specific Western blot analyses showed the GFP-ppins and ppins-GFP-ER fusion proteins were efficiently expressed in transient transfected HEK cells, and no expression of intermediate products was detectable (Fig: 3.15). Notably, the expression levels of Insulin/GFP fusion antigens were >200-fold higher than of the ppins expressed by the pCI/ppins vector.

I immunized RIP-B7.1 mice with the respective vectors. The pCI/ppins-GFP-ER but not the pCI/GFP-ppins plasmid induced EAD in RIP-B7.1 mice (Fig: 3.16 B). High frequencies of K\(^{b}\)/A\(_{12-21}\)-specific IFN\(\gamma^{+}\) CD8 T cells were detected in pCI/ppins-GFP-ER-immunized, diabetic RIP-B7.1 mice but not in healthy, pCI/GFP-ppins-immunized RIP-B7.1 mice. These findings confirmed that this T cell specificity was efficiently primed by processing ER-associated but not cytosolic antigens. K\(^{b}\)/A\(_{12-21}\)-specific CD8 T cells were also induced by COOH-terminally extended ppins-specific polypeptides (e.g. ppins-GFP-ER) expressed in the ER, indicating that the epitope position at the COOH-terminus is less important for its diabetogenicity than is targeting the antigen to the ER.
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Figure 3.14: HeLa cells were transiently transfected with pCI/GFP-ppins and pCI/ppins-GFP-ER DNA. 24 hours after transfection cells were stained for either insulin (FITC) or the ER-located calnexin (TRITC) and supplied to immunofluorescence microscopy.

Figure 3.15: HEK cells were transiently transfected with pCI/GFP-ppins, pCI/ppins-GFP-ER and pCI/ppins DNA. 24 hours after transfection cells were lysed and analyzed by Western blot. Blots were stained for either insulin or GFP.
Results

Figure 3.16: RIP-B7.1 mice were immunized with pCl/GFP-ppins or pCl/ppins-GFP-ER and cumulative diabetes incidences (%) were determined. Pancreatic CD8 T cells derived from diabetic (pCl/ppins-GFP-ER) and healthy (pCl/GFP-ppins) RIP-B7.1 mice were re-stimulated ex vivo with K\(^b\)/A\(_{12-21}\)A peptide or with the control K\(^b\)/OV\(_{257-264}\) peptide. Specific IFN-γ\(^+\) CD8 T cells were identified by flow cytometry.

Processing to insulin is not required for priming of autoreactive CD8 T cells

Non-pancreatic APC expressed pins but no further downstream products. To ensure, that natural downstream processing of pins to insulin in the ER is not required for priming of autoreactive CD8 T cells, mutant insulin variants were generated. Alanine substitution of 3 arginine and 1 lysine (RR and RK) in the cationic processing sites (pCl/ppins-RK\(_{mut}\)) and of five of six cysteine sites (except the C in the A\(_{12-21}\) epitope) (pCl/ppins-C\(_{mut}\)) of ppins abolish the natural processing of pins to insulin. The mutations either block the insulin-specific processing or the disulfide-mediated insulin assembly (Fig: 3.17). The mutant vectors pCl/ppins-RK\(_{mut}\) and pCl/ppins-C\(_{mut}\) efficiency induced EAD with similar kinetics in immunized RIP-B7.1 mice (Fig: 3.18). Thus, processing of ppins to insulin is not required for T cell priming.
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Figure 3.17: Schematic presentation of mutated insulin constructs. The positions of the mutations, the ppins (SP-B-C-A) and of the K\textsuperscript{b}/A\textsubscript{12-21} epitope are indicated.

Figure 3.18: RIP-B7.1 mice were immunized with either pCI/ppins, pCI/ppins-RK\textsubscript{mut} or pCI/ppins-C\textsubscript{mut}. Cumulative diabetes incidences (%) were determined at the indicated time points.
3.3 Selective analysis of the effector phase of EAD

Adoptively transferred, ppins-specific CD8 T cells induce EAD in RIP-B7.1 hosts

To investigate the effector phase of pre-primed diabetogenic CD8 T cells, I used adoptive cell transfer experiments [38]. RIP-B7.1 mice were immunized with non-coding pCI, pCI/ppins or pCI/L-A_{12-21} DNA (Fig: 3.19 A). 2–3 weeks after immunization splenocytes were isolated and transferred into non-immunized RIP-B7.1 hosts. Mice which received cells from pCI/ppins or pCI/L-A_{12-21} immunized mice developed EAD with a similar outcome of disease at week 8 post transfer and a cumulative diabetes incidence of > 80% (Fig: 3.19 A, group 2 and 3). Transfer-EAD depends on pre-primed CD8 T cells since pCI primed RIP-B7.1 mice derived splenocytes or CD8 T cells did not induce EAD (Fig: 3.19 A, group 1). After transfer, high numbers of K^b/A_{12-21}-specific CD8 T cells were detected in pancreatic tissue in diabetic mice (Fig: 3.19 B). The effector phase of EAD model thus depends on autoreactive K^b/A_{12-21}-specific CD8 T cells.

Figure 3.19: (A) Splenocytes of RIP-B7.1 mice immunized with non-coding pCI, pCI/ppins and pCI/L-A_{12-21} DNA splenocytes were transferred into RIP-B7.1 hosts. At indicated times after transfer, cumulative diabetes incidences (%) were determined. (B) At diabetic state, pancreatic cells were re-stimulated ex vivo with the K^b/A_{12-21}A peptide or with the control K^b/OVA_{257-264} peptide. Specific IFN-γ^+ CD8 T cells were identified by flow cytometry.
3.4 Characterization of ppins-specific immunodominance hierarchies

Induction of EAD in RIP-B7.1 mice by ppins\(\Delta A_{12-21}\)-encoding DNA

To investigate if there are further subdominant epitopes (except the K\(^b\)/A\(_{12-21}\)) primed by ppins, a ppins-encoding vector lacking the K\(^b\)/A\(_{12-21}\) sequence was cloned (pCl/ppins\(\Delta A_{12-21}\)) (Fig: 3.20).

![Figure 3.20](map of ppins\(\Delta A_{12-21}\). The signal peptide (SP), the B- and A-chains and the C-peptide are indicated. The C-terminal A\(_{12-21}\) sequence is deleted in this expression construct.)

Unexpectedly, RIP-B7.1 mice immunized with pCl/ppins\(\Delta A_{12-21}\) DNA developed EAD within 4 weeks after immunization (Fig: 3.21A). The immunized RIP-B7.1 mice showed a similar disease progress like control, pCl/ppins-immunized mice, with an equal cumulative diabetes incidence of >95% (Fig: 3.21B). Hence, I was able to induce diabetes in RIP-B7.1 mice with ppins-encoding DNA lacking the diabetogenic K\(^b\)/A\(_{12-21}\) sequence.

B-chain-specific CD8 T cells induce EAD in RIP-B7.1 mice

pCl/ppins\(\Delta A_{12-21}\)-immunized RIP-B7.1 mice were screened for the antigenic peptide sequence using the insulin-peptide library, described above (Fig: 3.22). After re-stimulation, two peptides B\(_{21-30}\) and B\(_{23-32}\) (ERGFFYTPMS and GFFYTPMSRR) were observed to induce IFN\(\gamma\) production in the primed pancreatic CD8 T cells. Fine mapping of the antigenic sequence revealed the optimal B\(_{22-29}\) sequence (RGFFYTPM). This epitope contains two anchor motifs for K\(^b\) molecules at position Y5 and M8 [39].

The new epitope was tested for stabilization of K\(^b\) molecules on the surface of TAP-deficient RMA-S cells (Fig: 3.23). In contrast to the A\(_{12-21}\) peptide the B\(_{22-29}\) peptide could efficiently stabilize K\(^b\) molecules on the cell surface (compare Fig: 3.7). The avidity was comparable to the well defined K\(^b\)/OVA\(_{257-264}\) epitope.
3 Results

**Figure 3.21:** B6 and RIP-B7.1 mice were immunized with the epitope A_{12-21} lacking pCI/ppinsΔA_{12-21} DNA and cumulative diabetes incidences (%) were determined.

**Figure 3.22:** RIP-B7.1 mice were immunized with pCI/ppinsΔA_{12-21} DNA and analyzed at diabetic state. Pancreatic cells were re-stimulated *ex vivo* with a peptide library of insulin composed of 9aa peptides. Specific IFN-γ⁺ CD8 T cells were identified by flow cytometry.
RMA-S cells pulsed with:

\(-/-\)  \(K^b/OVA_{257-264}\)  \(K^b/B_{22-29}\)

**Figure 3.23:** TAP-deficient RMA-S cells were either not pulsed (-/-) or pulsed for 4 hours with high doses (200µg/ml) of \(K^b/B_{22-29}\) or \(K^b/OVA_{257-264}\) peptides, followed by surface staining of trimeric \(K^b\) molecules and FCM analysis.

Tetramers could be generated against the \(K^b/B_{22-29}\) epitope but not against the \(K^b/A_{12-21}\) epitope. In RIP-B7.1 mice immunized with the non-coding pCI vector no \(B_{22-29}\) tetramer\(^+\) CD8 T cells were detected (Fig: 3.24B). In contrast, in pCI/ppins\(\Delta A_{12-21}\) immunized RIP-B7.1 mice high numbers of \(B_{22-29}\) tetramer\(^+\) but no control tetramer\(^+\) (\(OVA_{257-264}\) tetramer) CD8 T cells infiltrated the pancreatic tissue. Frequencies correlated with the numbers of IFN\(\gamma^+\) CD8 T cells after ex vivo stimulation with the \(K^b/B_{22-29}\) peptide. Specific CD8 T cells accumulated in the pancreatic tissue whereas they were barely detectable in spleen (Fig: 3.24A).

**Figure 3.24:** RIP-B7.1 were immunized with pCI/ppins\(\Delta A_{12-21}\) (A,C) or with non-coding pCI vector. Pancreatic \(B_{22-29}\)-specific CD8 T cell levels were determined at day 21 post-priming by \(K^b/B_{22-29}\)-specific tetramers. Specific IFN\(\gamma^+\) CD8 T cells were identified by flow cytometry.
3 Results

The $K^b/B_{22-29}$-epitope is suppressed by the $K^b/A_{12-21}$ epitope

Priming of $K^b/A_{12-21}$ (but not $K^b/B_{22-29}$)-specific CD8 T cells by pCl/ppins suggested that the later response is suppressed by the $A_{12-21}$-specific immune response. Immunization of RIP-B7.1 mice with pCl/ppins or with pCl/ppins$\Delta A_{12-21}$ DNA induced EAD and specific IFN$\gamma^+$ CD8 T cells either against the $K^b/A_{12-21}$ or the $K^b/B_{22-29}$ epitope (Fig: 3.21B), demonstrating that both epitopes are presented on the pancreatic $\beta$ cells. To further investigate whether priming of $K^b/B_{22-29}$-specific, autoreactive CD8 T cells is suppressed by $K^b/A_{12-21}$-restricted CD8 T cells responses, I established in vivo immune-competition experiments. The co-immunization of pCl/ppins DNA into different muscles induced EAD (Fig: 3.25 A) and $K^b/A_{12-21}$-specific as well as $K^b/B_{22-29}$-specific CD8 T cell responses (Fig: 3.25 B). Thus, co-priming of $K^b/A_{12-21}$- and $K^b/B_{22-29}$-specific CD8 T cells was obtained when both vectors were delivered into different sites. But immunization with pCl/ppins only induced $K^b/A_{12-21}$- (but not $K^b/B_{22-29}$)-specific CD8 T cells. The antigen presenting cells are limited in expressing both epitopes. Thus, the dominance effect occurred during the priming phase of autoreactive CD8 T cells on APC not on pancreatic $\beta$ cell level.

Figure 3.25: (A) RIP-B7.1 mice were immunized with pClement, pClement$\Delta A_{12-21}$ DNA or both DNA in the left or right tibialis, respectively. Cumulative diabetes incidences (%) were determined and (B) pancreatic cells were re-stimulated ex vivo with $K^b/A_{12-21}$ peptide, $K^b/B_{22-29}$ peptide or with the control $K^b/S_{208-215}$ peptide. Specific IFN$\gamma^+$ CD8 T cells were identified by flow cytometry.

Priming of diabetogenic, $K^b/A_{12-21}$-specific CD8 T cell responses were suppressed when
‘strong’ $K^b$-restricted CD8 T cells responses to unrelated epitope were co-primed at the same site of antigen delivery. The hepatitis B surface antigen (HBsAg or S) encoded by the pCI/S plasmid contains a well-defined $K^b/S_{190-197}$ epitope [46]. Injection of a mixture of the pCI/ppins and pCI/S plasmids into the same muscle of RIP-B7.1 mice efficiently induced $K^b/S_{190-197}$-specific CD8 T cell responses but did not induce EAD (Fig. 3.26). Co-injection of pCI/ppins and non-coding pCI plasmids efficiently induced EAD and no HBsAg-specific CD8 T cells. Anti-viral $K^b/S_{190-197}$-specific CD8 T cell responses and ppins-specific EAD were induced when pCI/ppins and pCI/S plasmids were injected into different muscles of RIP-B7.1 mice. Hence, local co-priming of ‘strong’ HBsAg-specific CD8 T cells suppressed induction of the diabetogenic T cell response. Epitope competition for $K^b$ molecules may suppress ppins-specific CD8 T cell priming.

![Figure 3.26](image)

**Figure 3.26:** RIP-B7.1 mice were immunized once with a mixture of either pCI/ppins and non-coding pCI DNA or pCI/ppins and hepatitis B surface antigen (HBsAg) encoding pCI/S DNA. Furthermore, I injected pCI/ppins into the right and pCI/S DNA into the left tibialis anterior muscle of RIP-B7.1. Cumulative diabetes incidences (%) were determined. Splenic HBsAg-specific CD8 T cell levels were determined at day 12 post-priming by $K^b/S_{190-197}$-specific tetramers.

I further generated an in frame fusion construct of the ppins and the $K^b/OVA_{257-264}$ epitope (pCI/ppins-OVA$_{257-264}$). A nine residue linker sequence was positioned between
the ppins and OVA sequence. In RIP-B7.1 mice, this construct did not elicit diabetogenic K\textsubscript{b}/A\textsubscript{12−21}-specific CD8 T cell responses and EAD but efficiently induced OVA-specific CD8 T cell responses (Fig: 3.27 A, B). Hence, priming of OVA-specific CD8 T cells suppress the ppins-specific T cell response. RIP-B7.1 mice expressing OVA in pancreatic β cells under RIP promoter control [5] (RIP-B7.1/RIP-OVA mice) were immunized with pCI/ppins-OVA\textsubscript{257−264} or pCI/ppins. Immunization with pCI/ppins and pCI/ppins-OVA\textsubscript{257−264} efficiently induced EAD in these mice (Fig: 3.27 A). In pancreatic tissue of pCI/ppins-immunized, diabetic RIP-B7.1/RIP-OVA mice high numbers of K\textsubscript{b}/A\textsubscript{12−21}-specific CD8 T cell were detectable (Fig: 3.27 C). Whereas, pCI/ppins-OVA\textsubscript{257−264}-immunized, diabetic RIP-B7.1/RIP-OVA mice generated high levels of OVA-tetramer specific CD8 T cells but no K\textsubscript{b}/B\textsubscript{22−29} or K\textsubscript{b}/A\textsubscript{12−21}-specific CD8 T cells (Fig: 3.27 B). Thus, the pancreatic β cells of RIP-B7.1/RIP-OVA mice present both OVA and ppins epitopes, indicating that the ppins-specific epitopes are suppressed from the K\textsubscript{b} restricted OVA epitope on the APCs but not on the pancreatic β cells.

**Figure 3.27:** (A) RIP-B7.1 mice and RIP-B7.1/RIP-OVA mice were immunized with pCI/ppins-OVA\textsubscript{257−264} and pCI/ppins and cumulative incidences (%) were determined. (B, C) Pancreatic cells were re-stimulated ex vivo K\textsubscript{b}/A\textsubscript{12−21}, K\textsubscript{b}/B\textsubscript{22−29} or the control K\textsubscript{b}/S\textsubscript{208−215} peptide and K\textsubscript{b}/OVA\textsubscript{257−264}-specific CD8 T cells were stained with tetramers. Tetramer\textsuperscript{+} CD8 T cells and specific IFN-γ\textsuperscript{+} CD8 T cells were identified by flow cytometry.
3.5 The B6 mouse model

B6 is a common inbred strain of laboratory mouse. In contrast to the RIP-B7.1, the B6 mice do not express the co-stimulatory CD80 on pancreatic β cells. Injection of pCI/ppins into B6 mice did not induce diabetes within 15 weeks of observation (Fig: 3.28). Thus, the lack of co-stimulatory CD80 prevented the autoimmune attack of β cells in B6 mice.

Figure 3.28: B6 and RIP-B7.1 mice were immunized with pCI/ppins (n=5) DNA. At indicated times after immunization, blood glucose levels (mg/ml) were determined. Each symbol represents one mouse.

I used adoptive transfer experiments to test whether functional ppins-specific CD8 T cells were primed in B6 mice. Purified CD8 T cells from non-diabetic B6 mice immunized with pCI/ppins were transferred in RIP-B7.1 host and blood glucose levels were determined (Fig: 3.29 A). RIP-B7.1 hosts efficiently developed EAD after transfer with primed but not with non-primed B6-derived CD8 T cells. Ppins-specific CD8 T cells accumulated in the pancreata of transplanted, diabetic RIP-B7.1 hosts (Fig: 3.29 B). Thus, immunization of B6 mice with ppins DNA efficiently triggered an epitope K\(^b\)/A\(_{12−21}\)-specific CD8 T cell response.
3 Results

Figure 3.29: (A) CD8 T cells of non- or pCl/ppins-immunized B6 mice were isolated and transferred into RIP-B7.1 hosts. At indicated times after transfer, blood glucose levels (mg/ml) and cumulative diabetes incidences (%) were determined. (B) At diabetic state, pancreatic cells were re-stimulated ex vivo with the K\(^\beta\)/\(A_{12-21,1}\) peptide or with the control K\(^\beta\)/OVA\(_{257-264}\) peptide. Specific IFN-\(\gamma\)^+ CD8 T cells were identified by flow cytometry.
PD-L1 protects islets from B6 mice

Low levels of PD-L1 are expressed on non-lymphoid tissues including pancreatic islet β cells [24, 23]. As shown previously, blocking PD-L1 accelerates the manifestation of T cell-mediated diabetes in NOD mice [3, 57]. This suggests that PD-L1-mediated co-inhibition in the pancreas protects islets from immune attack. I tested if EAD can be triggered in ppins-immune B6 mice by injection of a blocking anti-PD-L1 antibody. B6 mice injected with either pCI or pCI/ppins were treated with anti-PD-L1 antibody or control antibody at day 12 and 15 post immunization. PCI/ppins-immunized B6 mice rapidly developed hyperglycemia after injection of anti-PD-L1 but not control antibody (Fig: 3.30 B, C). Control group (injected with pCI and treated with anti-PD-L1 antibody) did not develop EAD (Fig: 3.30 A). PD-L1 blockade thus specifically abrogated the protection of β cells from ppins-specific CD8 attack.

![Figure 3.30](image)

**Figure 3.30:** B6 mice were immunized with either (A) control vector pCI or (B) pCI/ppins DNA and at day 12 and 15 post immunization anti-PD-L1 Ab was injected. (C) B6 mice were immunized with pCI/ppins and as control treated with non-relevant mAb. Blood glucose levels (mg/dl) were determined at the indicated time points.

Treating pCI/ppins-immunized B6 mice with anti-PD-L1 did not induce a severe disease in all mice. Degrees of EAD differed within the group. Most the mice developed a severe insulitis (blood glucose levels ≥550mg/dl) within 1-2 weeks after treatment. An influx of CD8 T cells into the pancreatic islets and reduced insulin production resulting from islet destruction were observed (Fig: 3.31). High levels of K^{b}/A_{12-21}-specific CD8 IFN-γ^{+} CD8 T cells infiltrated the pancreas. However, some mice developed a moderate
Results

and transient hyperglycemia (blood glucose levels 260-380mg/dl) and subsequently became normoglycemic. In the hyperglycemic phase, significant lower levels of CD8 T cells infiltrated into the pancreas and insulin production was reduced but still intact. When blood glucose levels were restored and mice became normoglycemic, CD8 T cells disappeared from pancreas. K$^b$/A$^{12-21}$-specific CD8 T cells were barely detectable at this phase. Hence, blocking PD-L1 co-inhibition on pancreatic β cells enhances the predisposition to autoimmune destruction by ppins-specific CD8 T cells. Notably, induction of severe EAD correlated with the levels of pancreas-infiltrating K$^b$/A$^{12-21}$-specific CD8 T cells.

Figure 3.31: B6 mice were immunized with pCI/ppins DNA and treated with anti-PD-L1 Ab. Invasion of CD8 T cells and insulin expression were detected by Immunohistochemistry. Pancreatic cells were re-stimulated ex vivo K$^b$/A$^{12-N21A}$ peptide or with the control K$^b$/S$^{208-215}$ peptide and specific IFN-γ$^+$ CD8 T cells were identified by flow cytometry.
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To confirm the dependence of ppins-specific immunization on PD-1/PD-L1 inhibitor signals, PD-L1 knockout mice (PD-L1\(^{-/-}\)) were immunized with pCI/ppins. Immunized PD-L1\(^{-/-}\) mice developed hyperglycemia within 4-5 weeks post immunization. Injection of pCI/ppins induced EAD in these mice (Fig: 3.32) with a cumulative diabetes incidence of >80%. PD-L1\(^{-/-}\) mice with non-coding control vector injections did not develop EAD within the first 18 weeks of life.

![Figure 3.32: Induction of diabetes in PD-L1\(^{-/-}\) mice. PD-L1\(^{-/-}\) mice were immunized with non-coding pCI or pCI/ppins DNA. At indicated times after immunization, blood glucose levels (mg/ml) were determined.](image)

Furthermore, the EAD in PD-L1\(^{-/-}\) mice was mediated exclusively by autoreactive CD8 T cells. Depletion of CD8 T cells cured early diabetes in PD-L1\(^{-/-}\) mice but not depletion of CD4 T cells (Fig: 3.33).

To determine the epitope specificity of the diabetogenic CD8 T cells, pancreatic cells of diabetic PD-L1\(^{-/-}\) mice were re-stimulated \textit{ex vivo} with the peptide library of insulin (Fig: 3.34). I only detected K\(^b\)/A\(_{12-21}\)-specific IFN\(\gamma^+\) CD8 T cells in the pancreata of immunized and diabetic PD-L1\(^{-/-}\) mice. Thus, CD8 T cells with this specificity also play a prominent role in the destructive autoimmune response in PD-L1\(^{-/-}\) mice.
Figure 3.33: PD-L1\(^{-/-}\) mice were immunized with pCl/ppins DNA and in early stage of diabetes treated with either anti-CD8 Ab (n=3) or anti-CD4 Ab (n=3). Blood glucose levels (mg/ml) were determined at the indicated time points.

Figure 3.34: PD-L1\(^{-/-}\) mice were immunized with pCl/ppins DNA and analyzed at diabetic state. Pancreatic cells were re-stimulated ex vivo with a peptide library of insulin composed of 9aa peptides. Specific IFN\(^{\gamma}\)\(^{+}\) CD8 T cells were identified by flow cytometry.

Co-inhibitory signals of PD-L1 are generated by binding to its receptor PD-1. To determine if this co-inhibition regulates ppins-induced EAD, we used the PD-1 knockout mouse (PD-1\(^{-/-}\)). A single injection of the pCl/ppins (but not of pCl vector DNA) into PD-1\(^{-/-}\) mice induced epitope-specific CD8 T cell response and EAD (Fig. 3.35). EAD developed in PD-1\(^{-/-}\) mice with a median onset of 4-5 weeks post immunization and
3 Results

a cumulative diabetes incidence of 30-50% by week 5-7. EAD development was thus attenuated in immunized PD-1−/− mice as compared to EAD development in immunized RIP-B7.1 or PD-L1−/− mice.

Figure 3.35: Induction of diabetes in PD-1−/− mice. PD-1−/− mice were immunized with non-coding pCI or pCI/ppins DNA. At indicated times after immunization, cumulative diabetes incidences (%) were determined.

PD-1/PD-L1 co-inhibition controls diabetogenic CD8 T cells

I generated bone marrow chimeras to test whether PD-L1 and/or PD-1 expression by lymphohematopoietic cells or stromal cells (including islet β cells) is critical to induce EAD in ppins-immune mice. To distinguish between donor- and host-derived T cells in bone marrow (BM) chimeras, I used wt CD45.1+ B6 mice in this set of experiments. BM cells (PD-L1+ PD-1+) from CD45.1+ donor mice were transferred into lethally irradiated, congeneric PD-L1−/− or PD-1−/− (CD45.2+) host mice. Furthermore, BM cells from PD-L1−/− or PD-1−/− donor mice were transferred into lethally irradiated wt CD45.1+ host mice. At 6–7 weeks post transplantation, chimeric mice contained >90% of donor T cells. Chimeric mice were immunized 7 weeks post transplantation with the pCI/ppins (or control pCI DNA). Wt/PD-L1−/− chimeras (group 1) but not wt/PD-1−/− chimeras (group 3) developed EAD after pCI/ppins DNA immunization. Furthermore, PD-1−/−/wt
chimeras (group 7) but not PD-L1−/−/wt chimeras (group 5) developed EAD after immunization with pCl/ppins. EAD manifestation in the groups 1 and 7 correlated with an influx of A12−21-specific IFN-γ+ CD8 T cells into the target tissue. Hence, either the selective deficiency of PD-L1 on target cells (group 1) or the deficiency of PD-1 on T cells (group 7) triggered ppins-specific, CD8 T cell-mediated EAD. Binding of PD-1 expressed by activated CD8 T cells to PD-L1 expressed by pancreatic β cells thus seem to control the diabetogenic potential of autoaggressive T cells.

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4 Discussion

4.1 Efficient priming of epitope-specific CD8 T cells requires ER-associated processing

There is increasing evidence from patients with type 1 diabetes that autoreactive CD8 T cells specific for the insulin are involved in this disease. I used the RIP-B7.1 mouse model to selectively study EAD and the pathogenic crosstalk between insulin A-chain-specific CD8 T cells with their target organ (i.e., the pancreas). Immunization with preproinsulin-encoding vectors efficiently induce Kb/A12–21-specific CD8 T cells and EAD in RIP-B7.1 mice in a CD4 T cell independent manner [37]. Transgene-driven B7.1 (CD80) expression in the pancreatic β cells of RIP-B7.1 mice makes them susceptible to CD8 T cell-mediated immune attack. The B7.1 is expressed by the presenting β cells in RIP-B7.1 mice as well as effector CD8 T cells. B7.1 on the surface of β cells could bind to either the co-stimulator molecule CD28, or the co-inhibitor molecules CTLA-4 or PD-L1 on the surface of T cells [8, 14]. The non-physiological, co-stimulatory B7.1/CD28 interaction in RIP-B7.1 mice may allow more efficient effector function delivery by CD8 T cells that apparently cannot be overcome by co-inhibitory B7.1/PD-L1 and B7.1/CTLA-4 interactions. The progression of EAD, destruction of pancreatic islets and increasing insulin deficiency in RIP-B7.1 mice were associated with an invasion of Kb/A12–21-specific CD8 T cells into the pancreatic islets. Screening of diabetic RIP-B7.1 mice with a peptide library of insulin showed that exclusively Kb/A12–21-specific CD8 T cells were generated after immunization with ppins-encoding DNA.

I used this model to investigate how preproinsulin-derived antigens are expressed and processed in non-pancreatic antigen-presenting cells in order to generate the antigenic Kb/A12–21 peptide. To develop EAD professional, non-pancreatic APCs and pancreatic β cells of immunized RIP-B7.1 mice have to present the Kb/A12–21 epitope. EAD can be triggered in RIP-B7.1 mice by intramuscular injection of a high dose of minimal epitope
A\textsubscript{12–21}-encoding DNA with a NH\textsubscript{2}-terminally fused Ig\textsubscript{\kappa} leader, confirming that K\textsuperscript{\beta}/A\textsubscript{12–21} epitope is sufficient to induce EAD.

I investigated \textit{in vivo} if ER-associated processing of ppins-derived antigens was essential to prime K\textsuperscript{\beta}/A\textsubscript{12–21}-specific CD\textsubscript{8} T cells and lead to EAD. This unusual epitope contains a final COOH-terminus, thus does not require further COOH-terminal, proteolytic trimming. Small ER-targeted, antigenic peptides triggered CD\textsubscript{8} T cells in RIP-B7.1 mice whereas the corresponding cytosolic antigens did not induce EAD. Thus, for the induction of diabetes by transfection with ppins-encoding DNA, non-pancreatic APCs have to express ppins and direct it into the ER where further processing to pins occurs. The L-A\textsubscript{12–21} fusion peptide was expressed at high enough levels that sufficient amounts of the K\textsuperscript{\beta}/A\textsubscript{12–21} epitope were released within the ER (by signal peptidases) to load a critical number of nascent K\textsuperscript{\beta} molecules in order to prime CD\textsubscript{8} T cell responses [1]. These peptide concentrations may not be reached when the A\textsubscript{12–21} peptide was expressed in the cytosol and must compete with the bulk of high affinity, antigenic peptides for TAP-dependent transport into the ER [32]. Processing and loading of the entire, ER-targeted insulin A-chain (L-A\textsubscript{1–21}) to the K\textsuperscript{\beta} molecules may also proceed in the ER. When epitopes with a final COOH-terminus are released from ER-targeting signal peptides in the ER, the epitopes are trimmed by ER-resident amino peptidases. Presentation of these epitopes requires neither proteasomes, nor TAP [9, 51]. Targeting the vector-encoded ppins to different compartments by fusing GFP significantly enhances expression levels of the autoantigen but did not generate K\textsuperscript{\beta}/A\textsubscript{12–21}-specific CD\textsubscript{8} T cells responses and EAD in immunized RIP-B7.1 mice. Thus, the diabetogenic epitope was inefficient or not at all presented if the antigen is excluded from the ER. Additionally, the precise conformation forming of ppins and the processing to insulin is not proved necessary to prime diabetogenic T cell responses. Alanine substitution of 3 arginine and 1 lysine (RR and KR) in the cationic processing site and of five of six cysteine sites of ppins abolish the natural processing of pins to insulin. Either processing was blocked by inhibiting the excision of C-peptide or the conformational disulfide-mediated insulin assembly. These mutations significantly reduced the expression levels of ER-associated pins in transiently transfected cells. However, the mutant vectors triggered comparable levels of K\textsuperscript{\beta}/A\textsubscript{12–21}-specific CD\textsubscript{8} T cells and induced similar EAD incidences as those observed with ppins-encoded DNA. Thus, these findings suggest that the expression of antigens in the ER, rather than antigen conformation or expression levels, is required for efficient priming of diabetogenic CD\textsubscript{8} T cell responses.
I figured out that priming of autoreactive CD8 T cells in RIP-B7.1 mice efficiently occurs when antigens were targeted to the ER. K<sup>b</sup>/A<sub>12−21</sub>-specific CD8 T cells were induced in RIP-B7.1 mice by injection of high doses of ppins-encoding vectors. This confirmed that plasmid DNA immunization potently stimulates CD8 T cell responses in mice [16]. DNA immunization induces Th1-biased immune responses to most antigens [17]. It was shown that DNA immunization of low amounts of plasmid DNA with gene gun facilitated Th2-biased immunity [45] and failed to induce EAD in RIP-B7.1 mice [38]. Thus, induction of autoreactive CD8 T cell responses requires co-stimulation of the Th1-biased immune system. Alternatively, plasmid DNA-induced innate immune responses may directly facilitate β cell susceptibility for T cell-mediated destruction [26]. This is unlikely because K<sup>b</sup>/A<sub>12−21</sub>-monospecific CD8 T cells isolated from diabetic RIP-B7.1 mice efficiently triggered EAD after adoptive cell transfer into RIP-B7.1 host [38]. Thus, DNA immunization does not directly influence presentation of the K<sup>b</sup>/A<sub>12−21</sub> epitope by β cells.

4.2 EAD induction is suppressed by strong antigens

Long CD8 T cell epitopes have been described [7] but the K<sup>b</sup>-restricted 10 mer A<sub>12−21</sub> epitope of insulin contains only an auxiliary anchor motif at position Y3 [39]. Specific ex vivo re-stimulation of primed CD8 T cells requires high doses of the antigenic peptide. Direct biochemical binding assays indicate a very low avidity of this peptide for K<sup>b</sup>. The antigenic A<sub>12−21</sub> peptide does not stabilize surface K<sup>b</sup> class I molecules on the surface of TAP-deficient RMA-s cells. Hence, the A<sub>12−21</sub> epitope is a low affine epitope. The classification of the ppins K<sup>b</sup>/A<sub>12−21</sub> epitope as ‘weak’ is further supported by immune-competition experiments. Co-priming of ppins-specific (pCI/ppins) and K<sup>b</sup> restricted HBs-specific (pCI/S) CD8 T cells at the same site of injection suppressed induction of ppins-specific, pathogenic CD8 T cell responses in RIP-B7.1 mice. Indicating that the induction of K<sup>b</sup>/OVA<sub>257−264</sub>-specific CD8 T cells inhibited triggering K<sup>b</sup>/A<sub>12−21</sub>-specific CD8 T cells. These results suggest that epitope competition for K<sup>b</sup> molecules [44, 47, 54] may suppress ppins-specific T cell priming. Likewise, immunization of RIP-B7.1 mice with an in frame fusion construct of the ppins and the K<sup>b</sup>/OVA<sub>257−264</sub> epitope (pCI/ppins-OVA<sub>257−264</sub>) did not lead to EAD and induction of ppins-specific CD8 T cells. Thus, the OVA epitope suppress the low affine ppins-specific epitopes. In RIP-B7.1/RIP-Ova mice, expressing
both insulin and ovalbumin in pancreatic \( \beta \) cells, immunization with either pCI/ppins or pCI/ppins-OVA\(_{257-264} \) efficiently induced EAD and triggered either \( K^b/A_{12-21} \)-specific or \( K^b/OVA_{257-264} \)-specific CD8 T cells. \( K^b/A_{12-21} \)-specific and \( K^b/OVA_{257-264} \)-specific CD8 T cells were not induced simultaneously. Indicating that the epitope suppression occurs in the priming phase on the APC level but not on the \( \beta \) cells of RIP-B7.1/RIP-OVA.

Unexpectedly, a ppins-encoded vector lacking the epitope \( A_{12-21} \) sequence, efficiently induced EAD in RIP-B7.1 mice. I characterized a new diabetogenic epitope (\( K^b/B_{22-29} \)). CD8 T cell responses to this epitope were suppressed when the \( A_{12-21} \) sequence was present on the antigen (pCI/ppins) but efficiently primed in the absence of the \( A_{12-21} \). This was not expected because the \( B_{22-29} \) epitope exhibited a much higher binding affinity to \( K^b \) molecules than the \( A_{12-21} \) epitope. Co-immunization of both DNAs into different muscles triggered \( K^b/A_{12-21} \)-specific as well as \( K^b/B_{22-29} \)-specific T cells, indicating that the dominance effects occurred on APCs during the priming phase (i.e. when priming proceed at the same site [47, 59]). Thus, the low affinity \( K^b/A_{12-21} \) epitope dominates the high affinity \( K^b/B_{22-29} \) epitope. The molecular mechanisms of this immune suppression are yet not understood. It is assumed that T cells with low avidity for tissue-restricted antigen evade central and peripheral tolerance and cause autoimmunity [40, 61]. This may also be the case for \( K^b/A_{12-21} \)-specific CD8 T cells. These T cells may escape from negative selection in the thymus or regulatory T cells easier than CD8 T cells primed from a 'stronger' epitope [61].

4.3 PD-1/PD-L1 co-inhibition regulates EAD

I investigate the induction of ppins-specific CD8 T cells (and EAD) in inbred B6 mice. I showed that priming of ppins-reactive IFN\( \gamma^+ \) CD8 T cell responses is readily achieved in wt B6 mice. It was unexpectedly easy to prime ppins (\( K^b/A_{12-21} \))-specific CD8 T cells in B6 mice by DNA-based immunization. The primed autoreactive CD8 T cells revealed their diabetogenic potential after adoptive transfer in congenic RIP-B7.1 host. However, these CD8 T cells could not induce EAD in B6 mice. The exact mechanisms for protection from diabetes in B6 mice are still unknown. Control could operate at different levels: suppression of generated specific CD8 T cells populations, epitope-presentation of target tissue, down regulation of autoreactive CD8 T cells by co-inhibitory signals from target tissue. The down regulation by co-inhibitory signals from target tissue is a possibility.
to inhibit autoimmune attack [57]. The expression of co-stimulatory CD80 molecule in RIP-B7.1 mice and a possible suppression of inhibitory signals by β cells may enhance the sensitivity for specific CD8 T cell destruction [8, 41]. Hence, a critical checkpoint that prevents such self-reactive T cells to become diabetogenic is the susceptibility of the target cells in the pancreas, the β cells. RIP-B7.1 and two well defined (PD-L1−/−, PD-1−/−) mouse lines allowed me to identify critical checkpoints for the control of diabetogenic, ppins-specific CD8 T cells: (i) the co-stimulator molecule B7.1 (CD80) expressed on β cells (RIP-B7.1 mice); (ii) the co-inhibitor molecule PD-L1 (B7-H1) expressed by β cells (PD-L1−/− mice); or (iii) the co-inhibitor PD-1 molecule expressed by CD8 T effector cells (PD-1−/− mice). I assumed that the mechanism to control the pathogenic potential of autoreactive CD8 T cells in B6 mice might be a down regulation by co-inhibitory signals from pancreatic tissue [23]. The co-inhibitory PD-1/PD-L1 pathway is described to precipitate diabetes progress in NOD mice [3, 57]. With regard to these studies, PD-L1/PD-1 interactions could generate such an inhibitory, EAD-protective signal in the B6 model. Immunization of B6 mice with the ppins-encoding DNA combined with antibody-mediated PD-L1 blockade also revealed the diabetogenic potential of ppins-specific CD8 T cells in these mice. I thus considered it likely that the regulation of target β cell susceptibility is critical for the manifestation or suppression of diabetogenic potential of CD8 T cells. It has been shown that PD-1 and its ligands, PD-L1 and PD-L2, deliver inhibitory signals that regulate the balance between T cell activation, tolerance, and immunopathology [37, 42]. PD-L1 is expressed on presenting β cells as well as effector CD8 T cells, whereas PD-1 is expressed on T cells. PD-L1 expressed by pancreatic β cells can interact with either PD-1, or B7.1 expressed by activated CD8 T cells to inhibit the diabetogenic response [8]. There are thus many molecular interactions that are potentially involved in CD8 T cell/β cell contact. Using bone marrow chimeric mice, I showed that the deficiency of PD-L1 on target cells or PD-1 on T cells was essential to trigger ppins-specific, CD8 T cell-mediated EAD. Binding PD-1 on activated T cells to PD-L1 expressed by pancreatic β cells may hence downmodulate the diabetogenic potential of ppins-specific CD8 T cells.
5 Summary

RIP-B7.1 mice expressing the co-stimulator molecule B7.1 (CD80) on pancreatic β cells are a well-established model to characterize de novo induction of autoreactive, insulin-specific CD8 T cells and experimental autoimmune diabetes (EAD). Preproinsulin-encoding vectors efficiently induced EAD in RIP-B7.1 mice. Destruction of pancreatic islets was associated with an influx of insulin A-chain K<sub>b</sub>/A<sub>12−21</sub>-specific CD8 T cells.

In this study, RIP-B7.1 mice were used to explore the priming requirements of diabetogenic, insulin A-chain (K<sub>b</sub>/A<sub>12−21</sub>)-specific CD8 T cells. The COOH-terminus of the K<sub>b</sub>/A<sub>12−21</sub> epitope corresponds to that of the ppins molecule and thus does not require further COOH-terminal proteolytic trimming. Targeting the K<sub>b</sub>/A<sub>12−21</sub> epitope, the insulin A-chain or the ppins into the ER was a prerequisite to efficiently prime diabetogenic CD8 T cells. Stable expression of ppins in the nucleus or cytosol prevented priming of autoreactive CD8 T cells and EAD. Thus, direct loading of the A<sub>12−21</sub> epitope on newly synthesized K<sub>b</sub>-molecules in the ER may be an essential step for enabling presentation of this low-affine epitope to diabetogenic CD8 T cells.

I found evidence that immunodominance hierarchies operated in professional antigen presenting cells but not on insulin-producing target β cells. Co-priming of K<sub>b</sub> restricted viral or OVA-specific CD8 T cell responses at the same site of antigen delivery prevented priming of ppins-specific, diabetogenic CD8 T cells. CD8 T cell-mediated immunodominance hierarchies also operated within the ppins antigen. CD8 T cells isolated from ppins-immune, diabetic RIP-B7.1 mice specifically recognized the K<sub>b</sub>-restricted A<sub>12−21</sub> epitope of ppins. Ex vivo stimulation of ppins-primed, pancreas-infiltrating CD8 T cells with the antigenic A<sub>12−21</sub> peptide but not with all other peptides of a ppins-specific library revealed a CD8 T cell population with specifically inducible IFN-γ expression. However, a mutant ppinsΔA<sub>12−21</sub> antigen (pCI/ppinsΔA<sub>12−21</sub>) efficiently induced insulin B-chain (K<sub>b</sub>/B<sub>22−29</sub>)-specific CD8 T cells and EAD in immunized RIP-B7.1 mice. Thus, K<sub>b</sub>/B<sub>22−29</sub>
-specific CD8 T cells were suppressed by co-primed immunodominant $A_{12-21}$ CD8 T cell responses but efficiently presented by insulin-producing $\beta$ cells.

I established novel mouse models to selectively study the pathogenic crosstalk between insulin A-chain ($K^b/A_{12-21}$)-specific CD8 T cells with pancreatic target $\beta$ cells. It has been shown that PD-1 (programmed death-1) and its ligands, PD-L1 and PD-L2, deliver inhibitory signals that regulate the balance between T cell activation, tolerance, and immunopathology. I thus considered it likely that the regulation of target $\beta$ cell susceptibility is critical for the manifestation or suppression of CD8 T cell-mediated diabetes. It was unexpectedly easy to prime ppins-specific CD8 T cells in B6 mice by DNA-based immunization but these CD8 T cells revealed their diabetogenic potential after adoptive transfer into congenic RIP-B7.1 hosts, or after antibody-mediated PD-L1 blockade. Thus, immunization had induced ppins-specific CD8 T cells in B6 mice but pancreatic $\beta$ cells were protected from immune attack by these cells. Using PD-L1$^{-/-}$, PD-1$^{-/-}$ knockout and bone marrow chimeric mice I showed that the deficiency of PD-L1 on target cells or PD-1 on T cells was essential to trigger ppins-specific, CD8 T cell-mediated EAD. Binding of PD-1 on activated T cells to PD-L1 expressed by pancreatic $\beta$ cells may hence down modulate the diabetogenic potential of ppins-specific CD8 T cells. Co-stimulator and co-inhibitor molecule expression by epitope-presenting cells can hence modulate their susceptibility to be attacked by autoaggressive CD8 T cells and thereby ultimately determine the course of autoimmune disease.
Bibliography


### Appendix

### Chemicals and Reagents

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## Buffer and Media

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### Appendix

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List of publications


*contributed equally
Acknowledgements

I want to thank Prof. Dr. Reinhold Schirmbeck for being a great boss, for his leadership and good advices.

I would like to thank Prof. Dr. Jörg Reimann for the chance being a member in his group.

Especially I want to thank Conny Schuster for working with me on this topic - even though it was just a short time- and for all the constructive discussions.

I also want to thank my other PhD colleagues Michael Reiser and Andreas Wieland, and of course the other group and 'non-group' members Dr. Petra Riedl, Kati Ölberger, Claudi Heilig, Dr. Petra Birk and Dr. Mona Wegert for 3 awesome years and a really fantastic time in the lab.

A special thank goes to my love, Alex, for his support in the last 3 years and for being always by my side.

Last but not least, I want to thank my parents to whom I owe everything.