Regulation of CD8 T cell-mediated diabetes induction by co-inhibitory and co-stimulatory signals delivered by pancreatic β cells

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Tag der Promotion: 04.05.2012
For my parents
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### General abbreviations

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<tbody>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>ag</td>
<td>antigen</td>
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<tr>
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<td>ß-ME</td>
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<tr>
<td>BFA</td>
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<tr>
<td>bp</td>
<td>basepair(s)</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>cat.no.</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>Ci</td>
<td>curie</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>EAD</td>
<td>experimental autoimmune diabetes</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>ER</td>
<td>endoplasmatic reticulum</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>FCM</td>
<td>flow cytometry</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>g</td>
<td>acceleration due to gravity</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>Hek293</td>
<td>human embryonic kidney 293</td>
</tr>
<tr>
<td>HIV</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<td>kilobase(s)</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>luria bertani</td>
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<tr>
<td>LN</td>
<td>lymph node</td>
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<tr>
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<td>major histocompatibility complex</td>
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</tr>
<tr>
<td>ODN</td>
<td>oligodeoxynucleotide</td>
</tr>
<tr>
<td>Ova</td>
<td>ovalbumin</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamid gel electrophoresis</td>
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<tr>
<td>PCR</td>
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<td>pLN</td>
<td>pancreatic lymph node</td>
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<td>preproinsulin</td>
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<td>pins</td>
<td>proinsulin</td>
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<td>PPO</td>
<td>2,5-diphenyloxazole</td>
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<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
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<td>T1D</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td>tg</td>
<td>transgenic</td>
</tr>
<tr>
<td>T_{reg}cell</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>V</td>
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<td>wild type</td>
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### Nucleotide abbreviations

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<tr>
<td>C</td>
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<td>G</td>
<td>guanine</td>
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<td>T</td>
<td>thymine</td>
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### Aminoacid abbreviations

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<td>Ala  alanine</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>
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<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  phenylalanine</td>
<td>R</td>
<td>Arg  arginine</td>
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<td>G</td>
<td>Gly  glycine</td>
<td>S</td>
<td>Ser  serine</td>
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<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>A</td>
<td>Ala  alanine</td>
<td>M</td>
<td>Met  methionine</td>
</tr>
<tr>
<td>C</td>
<td>Cys  cysteine</td>
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<td>Asn  asparagine</td>
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<td>Pro  proline</td>
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<tr>
<td>E</td>
<td>Glu  glutamic acid</td>
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<td>Gln  glutamine</td>
</tr>
<tr>
<td>K</td>
<td>Lys  leucine</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. Autoimmunity

The discrimination of an organism, between self and non-self, results in the balance of immunodeficiency and autoimmunity. To maintain this balance, the T cell repertoire should be able to respond to potential foreign antigens but must not respond to self antigens expressed in various tissues. Therefore, T lymphocytes undergo random rearrangement of their antigen receptors during development. This process occurs within the thymus and results in only few immunological useful prothymocytes that are able to bind antigen: major histocompatibility complexes (MHC) via their T cell receptors (TCRs) with low avidity. This activates a positive selection mechanism in which cells survive, that are able to react against these antigens. Otherwise, random TCR rearrangement affords T cells that bind self-antigens with high avidity. Such autoreactive lymphocytes are deleted through negative selection [40].

The immune system has developed intricate ways for maintenance of self tolerance and prevention of autoimmune diseases [91]. Tolerance resembles the discrimination between self and non-self in the context of immunologic balance. Functional tolerance can be divided into two main parts: central tolerance describes the process of elimination of autoreactive lymphocytes during maturation in the thymus until these cells reach maturity, while peripheral tolerance is the suppression of autoreactive lymphocytes that have escaped thymic negative selection [40].

Negative selection in the thymus, also called clonal deletion, describes the process by which the ligation of TCR:self-peptide-MHC triggers cell death. Although this negative selection was thought to occur in the thymic cortex together with positive selection, recent studies showed that apoptosis following clonal deletion seems to occur in the medulla [106, 128, 149].
Introduction

Figure 1: Scheme of the T cell development in the thymus

The cortex can be separated into two main parts, the cortex and the medulla. Via the blood stream, early thymic seeding progenitors (TSPs) enter the thymus at the corticomedullary junction (CMJ). T cells (double negative (DN) 1 through DN2 cells) move from the CMJ to the subcapsular zone (SCZ) during early development, thereby interacting with cortical thymic epithelial cells (cTECs) and macrophages (M∅). After reaching the SCZ, cells first differentiate into DN3 and then into DN4 cells. Subsequently, they move back towards the medulla where they undergo proliferation and maturation into double positive (DP) cells. Differentiation into CD4 single positive (SP) or CD8 SP cell populations followed by negative selection occurs in the medulla. Thereafter, CD4 and CD8 SP cells leave the thymus and reach peripheral lymphoid organs via the blood stream. mTEC (medullary thymic epithelial cell). Adapted to Koch [66].

Since several tissue-specific protein expression levels are to low for inducing tolerance in the thymus, there are additional mechanisms of peripheral tolerance. A key molecule acting during peripheral tolerance is the antigen-presenting cell (APC). Such APCs, for example dendritic cells (DCs), catch self-antigens and present them to autoreactive T cells, a process that is called cross-presentation, thereby mediating T cell tolerance by deletion or anergy [49]. Furthermore, this process depends on the concentration of self-antigens [72, 86]. If the expression levels are very low, there is only minimal cross-presentation resulting in remaining self-reactive T cells. Therefore, processes like tissue necrosis or tumour development, which alter self-antigen expression levels, are able to influence the
extent of cross-presentation. Elevated cross-presentation of self-antigens in turn may lead to their detection by ignorant autoreactive T cells. It is crucial for the fate of autoreactive T cells, whether the process that leads to increased cross-presentation is linked to events that promote APC maturation. The incidence of increased self-antigen levels in the absence of APC maturation triggering signals leads to tolerance, while detection of self-antigen together with pro-inflammatory signals breaks tolerance and enables autoimmunity. Hence, breaking tolerance by activated ignorant T cells can occur if cross-presentation is coupled to APC maturation [91]. A further important group of lymphocytes in the context of autoimmunity are B cells that are responsible for production of autoantibodies, which are basically produced at low levels. This is on the one hand due to missing T cell help, but on the other hand there are specific B cell tolerance mechanisms [91]. Immature B cells displaying a self-reactive receptor in the bone marrow activate a series of cellular events: Excess of a certain threshold by the strength of receptor cross-linking and intracellular signaling, leads to B cell receptor (BCR) internalization and halting maturation [32, 48, 89]. This involves several cellular processes that finally allow BCR editing by rearrangement of the BCR light chain [55, 89]. This process in which highly reactive B cells, that are generated in the bone marrow, show an arrest in development and continue of V(D)J recombination of their immunoglobulin (Ig) genes, thereby changing the BCR antigen specificity [88], is called receptor editing. Such edited immature B cells sustain maturation and leave the bone marrow together with B cells that have no or low self-reactivity. Self-reactive B cells reaching the periphery are deleted or rendered anergic, while weakly self-reactive B cells exist in an ignorant state [91]. Moreover, there are several additional cell types that play also a role in controlling autoimmunity. Studies revealed that subsets of regulatory cells, suppressor cells, natural killer (NK) cells and natural killer T cells (NKT) have an influence on tolerance and autoimmunity either via APC/DC-dependent or independent processes [12, 19, 52, 115, 152].
1.2. Diabetes mellitus

Diabetes mellitus is a worldwide prevalent metabolic disease. 284 million people globally suffered from diabetes in 2010. This value corresponds approximately to 6.4% of the world population. Recent projections estimated that in 2030 about 439 million individuals (i.e. around 7.7% of the world population) are concerned [30]. The disease is characterized by a rapidly increasing incidence caused by diverse factors like population growth, aging, urbanization and elevated prevalence of obesity and physical inactivity [142].

There exist two main forms of diabetes [1]: type 1 and type 2 diabetes. Type 1 diabetes (T1D) is an autoimmune disease characterized by the T cell-mediated destruction of pancreatic β cells resulting in insulin deficiency. On this account, type 1 diabetes patients need the application of exogenous insulin for survival [151]. The most common type of diabetes mellitus, type 2 diabetes, is distinguished by insulin resistance combined with a reduced insulin secretion. People that suffer from type 2 diabetes are independent of exogenous insulin, although it is often required to control blood glucose levels beside alternative therapeutic approaches. The frequency of type 1 diabetes is low, compared to type 2 diabetes that concerns about 90% of diabetes patients [151]. However the main problem with type 1 diabetes is that until now there are no prevention or curing approaches available. Since hyperglycemia can lead to long term complications affecting the nervous or cardiovascular system, the main focus in type 1 diabetes therapy is to keep constant normal blood glucose levels. The only possible treatment are replacement therapies, otherwise severe complications like coma resulting from diabetic ketoacidosis can occur [65].

1.3. The pancreas and insulin biosynthesis

The organ primary affected in diabetes is the pancreas. It is a gland organ consisting of distinct functional units for digestion and glucose metabolism. It is
composed of an endocrine and an exocrine part. The latter is built of acinar and duct cells. These acinar cells produce digestive enzymes and are arranged in grape-like clusters. The ducts form a network including main and accessory pancreatic ducts that empty into the duodenum [9]. The enzymes secreted by the exocrine gland are needed for degrading carbohydrates, fats, proteins and acids, and flow down the pancreatic duct into the bile duct. The endocrine pancreas secretes several hormones including insulin, glucagon and somatostatin. It is built of cell clusters called Islets of Langerhans and consists of four cell types: $\alpha$ and $\beta$ cells regulate the glucose metabolism by producing glucagon and insulin respectively. PP and $\delta$ cells produce pancreatic polypeptide and somatostatin and are able to modulate the function of the other pancreatic cell types [9].

**Figure 2: Anatomy of the pancreas**

The pancreas can be divided into two functional parts; the exocrine part contains acinar and duct cells. Acinar cells are responsible for production of digestive enzymes and are arranged in grape-like clusters while the network of ducts ends up in the pancreatic ducts finally directed to the duodenum. The endocrine part, that secretes hormones, contains four cell types that are organized in islets. The glucose homeostasis is regulated by $\alpha$ and $\beta$ cells producing glucagon and insulin respectively. These cells are regulated by pancreatic polypeptide and somatostatin produced by PP and $\delta$ cells. a) Extract of the intestinal tract; b) Exocrine pancreas; c) One single acinus; d) Pancreatic islet. Adapted to Bardeesy [9].
The biosynthesis of insulin occurs in the pancreatic β cells. The precursor preproinsulin is initially processed to proinsulin by release of the signal peptide in the endoplasmic reticulum (ER), while the remaining polypeptide, consisting of the B-, C- and A-chain is folded. The molecular structure is stabilized by disulfide bonds connecting the A- and B-chain [125]. After leaving the ER, the molecule is targeted to the Golgi apparatus where it is packed into secretory vesicles [122]. Proinsulin is then further processed to mature insulin by the action of specific proteolytic enzymes, the so called prohormone convertases (PC1/3 and PC2) together with the exoprotease carboxypeptidase E. During this process, the connecting C-peptide is released [122].

Although several nutrients can trigger insulin secretion [77], glucose is the most potent stimulus. Glucose mediated insulin secretion occurs in two phases: Upon stimulation, there is an initial insulin release that diminishes afterwards. During the second phase, insulin secretion increases gradually within a longer period [126]. The sensor for insulin secretion is an elevated glucose level. Upon entering the β cell via a glucose transporter (GLUT2 in rodents and GLUT1 in humans), glucose is phosphorylated and enters the glycolytic pathway finally enabling ATP production [80]. The resulting increase of the ATP:ADP ratio in the cytoplasm ends up in an influx of Ca\textsuperscript{2+} into the cell, thereby triggering exocytosis of insulin granules [7, 57, 78]. While this pathway depends on ATP-sensitive potassium channels, there exists also a glucose-stimulated insulin secretion that acts independently of ATP-sensitive potassium channel activity. The former mechanism is supposed to trigger insulin secretion during the first phase, while the latter might act during the longer lasting second phase [57].

1.4. Type 1 diabetes

Type 1 diabetes also called juvenile diabetes or insulin-dependent diabetes, is a common autoimmune disease that occurs in individuals genetically susceptible by environmental factors [132]. It results from failures in central and peripheral tolerance causing progressive destruction of insulin-producing pancreatic β cells.
mediated by autoreactive T cells [23]. While the other cell types of pancreatic islets that produce glucagon, somatostatin and pancreatic polypeptide are unconcerned, selective destruction of the β cells leads to a complete deficiency in insulin production [99]. Reaching 80-90% of β cell destruction, neighboring α cells show an increased glucagon production. This insulin insufficiency and glucagon excess lead to hyperglycemia and ketoacidosis. The main features for distinguishing type 1 from type 2 diabetes are: the presence of autoantibodies, the genetic link, the insulin dependence and insulitis [132].

The autoimmune destruction of β cells is mediated by T cells recognizing several autoantigens [75, 131]. Recent studies indicate that both, CD4 and CD8 T cells, are required for type 1 diabetes initiation. It was shown that autoreactive T cells can differentiate into effector cells by β cell antigen engagement on APCs: Moreover it could be demonstrated that CD4 T cells are insulin-specific in the initial phase and CD8 T cells play a key role in β cell destruction [131].

Current studies suggest that among several disease relevant autoantigens, insulin plays a major role in initiating type 1 diabetes in both, mice and humans [105]. Although insulin has a key role in the initiation of type 1 diabetes, most islet-associated T cells display different antigen specificities during disease onset in mice. However the functional role of these bystander T cells is not yet understood [85]. In this context therapeutic approaches targeting insulin may have only affects on the initial phase before disease onset. Insulin-specific autoreactive CD4 T cells are also described in type 1 diabetes patients [120], where high-avidity insulin-reactive thymocytes are supposed to escape central tolerance. Recently, it has been shown that autoreactive CD8 T cells have a key role in diabetogenesis [131], but it is still not defined in which order events contributing to disease initiation and progression occur. It is yet unknown, whether autoreactive CD8 T cells are recruited before CD4 T cells, concurrently or even afterwards. During type 1 diabetes progression autoreactive CD8 T cells are selected by an avidity-regulation process controlled by tolerance and competition. The process in which high-avidity CD8 T cells compete with their low-avidity counterparts and replace them, is of importance since replaced low-avidity cells are supposed to have a strong impact on the progression of type 1 diabetes [2, 45, 46].
Another T cell subset that plays an important role during diabetes development are regulatory T cells (T\(_{\text{reg}}\)). Studies revealed that T\(_{\text{regs}}\) are able to suppress diabetogenic effector T cells [111]. On the one hand they can act by targeting DCs [111], but on the other hand it has also been shown that effector T cell suppression can occur APC-independent. Therefore a defect in regulatory cells, classically FoxP3\(^+\) CD4\(^+\) CD25\(^+\) T cells, is supposed to cause resistance of effector T cells against regulatory processes in type 1 diabetes [25, 39, 109, 148].

**Figure 3: Pathways in type 1 diabetes development**

After escape from thymic negative selection, high-avidity (HA) autoreactive CD4 and CD8 T cells migrate to peripheral lymphoid organs. For yet unknown reasons, β cell autoantigen is released and up-taken by antigen-presenting cells (APCs, e.g. dendritic cells (DCs) or B cells). Subsequently these cells migrate to the pancreatic lymph nodes (LNs) for priming naïve HA-autoreactive CD4 and CD8 T cells. Upon MHC class I engagement on APCs, CD8 T cells are activated mediated by HA autoreactive CD4 T helper cells and other signals like proinflammatory cytokines. After expansion and differentiation, autoreactive CD8 or CD4 T cells (CTLs) travel to the pancreatic islets via the blood and mediate β cell destruction. In order to suppress autoantigen presentation and T cell activation, FoxP3\(^+\) CD4\(^+\) CD25\(^+\) T cells (T\(_{\text{reg}}\)) are recruited to the pLN. Chronic stimulation via MHC class I leads to differentiation of autoreactive CD8 T cells into memory-like autoregulatory CD8 T cells thereby mediating suppression of autoantigen-presentation. Molecules (labeled in red) encoded by T1D associated genes are supposed to play a role in these pathways. Adapted to Santamaria [105].
1.5. Mouse models for studying type 1 diabetes

1.5.1. An overview of type 1 diabetes mouse models

Spontaneous models
The NOD mouse is a model that develops diabetes spontaneously. It is a very popular mouse model due to its similarities with human autoimmune diabetes [116]. Disease incidence is 60-80% in females and 20-30% in males [8, 64]. Diabetes onset occurs between 12-14 weeks of age in female mice and slightly later in male mice [3]. In the initial phase leucocytic aggregates at the perimeter of the islets and lymphocytic infiltrations into the islets can be detected. The initial predominant cells are CD4 T cells followed by CD8 T cells and macrophages. B cells can also be detected to a lower extend [150]. Islet infiltrating T cells isolated from diabetic NOD mice are either insulin-reactive [139] or show other specificities [134]. Recently CD8 T cells specific for the low-avidity insulin B-chain peptide K\textsuperscript{d}/B\textsubscript{15-23} could be identified [143]. Cells with this antigen-specificity were found in early islet infiltrates in the NOD mouse [143, 144]. It has been reported that these CD8 T cells, specific for the low-avidity K\textsuperscript{d}/B\textsubscript{15-23} epitope, can escape thymic negative selection and are able to cause diabetes upon activation [144]. Although the development of type 1 diabetes in the NOD mouse is primarily T cell-dependent, there are also autoantibodies detectable, which are not direct responsible for inducing diabetes, but contribute to the progression of the disease [37, 58].

Further mouse models that develop spontaneous diabetes are double transgenic (tg) models: In such mice a target antigen is transgenically expressed on pancreatic β cells via an insulin promoter, combined with the expression of a TCR specific for the same antigen. Available double tg models exist for the antigens ovalbumin (Ova), influenza hemagglutinin (HA) or lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) [132]. One example for double-transgenic mice is the DO11.10 TCR-Tg x Rip-mOva model [21]. These mice are characterized by the expression of the membrane-bound form of Ova in the pancreas and the
DO11.10 TCR. This constitution enables the development of autoreactive antigen-specific CD4 T cells causing diabetes [132].

**Pathogen-induced models**

LCMV infection of RIP-GP mice either on C57Bl/6, BALB/c or NOD background is a common model for virus-induced diabetes. RIP-GP mice express the LCMV-GP under the rat insulin promoter (RIP) in pancreatic β cells [92, 95]. Naïve RIP-GP mice show no diabetes induction. However LCMV infection triggers T cells able to recognize virus infected cells and GP expressing β cells. This leads to an immune mediated β cell destruction resulting in type 1 diabetes development within 1-2 weeks [92, 137].

Recent studies using the LCMV RIP-GP model showed that highly active CD8 T cells can persist in pancreatic β cells without displaying a destructive character. For converting the autoreactivity potential of such CD8 T cells into overt diabetes, toll-like-receptor (TLR) engagement was required [74]. Alternatively, it is supposed that autoimmunity might be promoted by local inflammation, thereby causing MHC class I molecule up-regulation in the pancreas [74].

**Transfer models**

Transfer of splenocytes from pre-diabetic or diabetic NOD females induces diabetes in NOD.CB17-Prkdcscid (NOD-scid) immunodeficient mice [103, 132], demonstrating that both, CD4 and CD8 T cells, are critical for diabetes development [83]. While inducing T1D by LCMV infection (mentioned above) allows characterization of virus or antigen-specific responses, the tracking of a manipulated or transgenically altered cell population is not possible. Therefore, another approach uses transfer of P14 (CD8 T cells specific for LCMV gp33-41 on H-2Dd [73]) or SMARTA (CD4 T cells specific for the LCMV gp61-80 on I-Aβ [97]) cells one day before RIP-GP mice are infected with LCMV. These cells respond to LCMV and migrate into the pancreas. Breeding in of allelic markers (e.g. CD45.1/2) or fluorochromes (e.g. GFP) or labeling with fluorescent dyes (e.g. CFSE) enables tracing of the transferred cells [132].
A well defined transfer model for studying type 1 diabetes is based on RIP-Ova-transgenic mice. There exist three types: RIP-Ova\textsuperscript{low}, RIP-Ova\textsuperscript{Hi} [72] and RIP-mOva [68] mice that are characterized by expression of ovalbumin (Ova) under the rat insulin promoter (RIP). They display either different expression levels of Ova (Ova\textsuperscript{low} or Ova\textsuperscript{Hi}) or have a membrane-bound form of Ova (mOva) [72]. Performing adoptive transfers using OT-I cells, that transgenically express a T cell receptor for the MHC class I-restricted K\textsuperscript{b}/Ova\textsubscript{257-264} (SIINFEKL) epitope, as donors and RIP-Ova-transgenic mice as hosts, offers a model for studying CD8 T cell tolerance. Studies revealed that OT-I cells were deleted in RIP-Ova\textsuperscript{Hi} and RIP-mOva but not in RIP-Ova\textsuperscript{low} mice [72]. Thus, adoptive transfer of OT-I-specific cells can be used for induction of Ova-specific autoimmune diabetes in RIP-Ova\textsuperscript{low} mice.

1.5.2. The RIP-B7.1 mouse model
RIP-B7.1 mice that express the co-stimulatory molecule B7.1 (CD80) in pancreatic β cells under control of the rat insulin promoter (RIP) [47] are an attractive model to characterize experimental autoimmune diabetes (EAD). Immunization of RIP-B7.1 mice with ppins-encoding plasmid DNA efficiently induced CD8 T cell-mediated EAD within 3-4 weeks, characterized by insulitis, insulin deficiency and hyperglycemia [101]. Studies revealed that EAD is induced by a CD4 T cell-independent, diabetogenic CD8 T cell response to ppins in this model [60]. Expression of IFN\textgamma, but not of perforin or type 1 interferon receptor signaling, was critical for the diabetogenic character of ppins-specific CD8 T cells in this model [60]. Two levels of diabetes development can be analyzed in the RIP-B7.1 diabetes model: The \textit{de novo} induction and expansion of autoreactive CD8 T cells by e.g., ppins-specific DNA immunization (priming phase), and the specific cross-talk of pancreatic β cells with the invading CD8 T cells (effector phase) [101].

Using the RIP-B7.1 mouse model for characterization of EAD provides several advantages: First, EAD occurs in both female and male mice displaying equal diabetes incidences and kinetics (in contrast to NOD mice). Second, as EAD development is equal in homozygous and heterozygous (RIP-B7.1/wt) animals,
they can be crossed with different genetically modified mice. Third, after initiation, EAD development is characterized by a short and predictable time course, and finally this mouse model is based on the common C57BL/6, H-2^b background. Such well-defined mice facilitate planning and performing of experiments, as several research tools are available [101].

1.6. MHC class I-restricted antigen processing and presentation

During its synthesis the MHC class I heavy chain binds to the endoplasmic reticulum (ER) chaperone calnexin. After dissociation of the heavy chain from calnexin, it binds to β₂-microglobulin followed by assembly in the peptide-loading complex. This complex is composed of two transporter associated with antigen processing (TAP1 and TAP2) subunits, the transmembrane protein tapasin, the ER chaperon calreticulin and the thiol oxidoreductase ERp57 [22]. Usually MHC class I molecules present peptides (containing 8-10 residues) from intracellular proteins to CD8 T cells. Such peptides are mainly derived from proteasomal protein degradation and are delivered to newly synthesized, ER-associated MHC class I molecules by TAP [114]. These peptides bind to MHC class I β₂-microglobulin heterodimers followed by release from the peptide-loading complex. Finally, complete assembled MHC class I:peptide complexes travel to the plasma membrane via the Golgi apparatus, where they are accessible to CD8 T cells [22].
Introduction

Figure 4: Processing and presentation of MHC class I-restricted antigens

Vaccines targeting CD8 T cells are introduced into the antigen presentation pathway to form HLA-class-I-peptide complexes on antigen-presenting cells. After vaccination protein antigens are generated in the cytosol. Proteasomal degradation of these proteins results in antigenic peptides targeted to the ER. Within the ER peptides bind to newly synthesized MHC class I molecules. The peptide-MHC class I complexes are then transported to the cell surface to mediate CD8 T cell stimulation. β2-m: β2-microglobulin; HLA: human leukocyte antigen; ER: endoplasmic reticulum; TAP: transporter for antigen processing; TCR: T cell receptor. Adapted to McMichael [82].

The processing of diabetogenic epitopes from ppins and the underlying mechanisms are not yet fully understood. Using the RIP-B7.1 model for development of EAD it can be presumed, that after immunization of the mice with ppins-encoding DNA, APCs process this ppins to proinsulin (pins) [60], while pancreatic β cells are able to further process pins to bioactive mature insulin [36, 123, 124]. However, both, the APC and the β cell, must present the same ppins epitope(s) to induce EAD. It has already been reported that proinsulin can be further processed in non-pancreatic cells to bioactive insulin [117, 133, 147]. This was supposed to be mainly due to the action of the Golgi-associated protease
furin [10, 35, 96]. This enzyme specifically recognizes R-X-(R/K)R motifs [110]. It could be shown that human proinsulin is further cleaved to insulin in Hek293 cells by introduction of furin recognition sequences at the B-C and C-A junction of proinsulin [41]. Studies investigated how ppins-derived antigens are expressed and processed to prime diabetogenic, K\(^b\)/A\(_{12-21}\)-specific CD8 T cells in the RIP-B7.1 model. After translocation into the ER, ppins is further processed to pins by cleaving the signal peptide (SP). In contrast to cytosolic peptides, targeting the K\(^b\)/A\(_{12-21}\) epitope, the insulin A-chain, or the ppins to the ER, efficiently elicited K\(^b\)/A\(_{12-21}\)-specific CD8 T cell responses. Moreover, it has been shown that proteasome inhibitors increased expression levels of cytosolic proinsulin (pins) but not of ppins-derived ER-associated pins [17]. Immunization of RIP-B7.1 mice with ppins-encoding DNA triggers autoreactive CD8 T cells specific for the K\(^b\)-restricted A\(_{12-21}\) epitope of the insulin A-chain [60]. Current studies revealed that deletion of the A\(_{12-21}\) epitope sequence on the ppins encoding plasmid DNA enables detection of autoreactive CD8 T cells with different epitope specificity: these cells recognize the K\(^b\)-restricted B\(_{22-29}\) epitope of the insulin B-chain. Since this epitope specificity was not detectable in ppins-immunized diabetic RIP-B7.1 mice, it has been assumed that immunodominance mechanisms are operating in this system [16].

1.7. The role of co-inhibitory and co-stimulatory signals in type 1 diabetes

T cell activation occurs through various interactions between antigen presenting cells (APCs) and T cells. Primarily an antigen presented via MHC on the APC binds to a TCR. This initial signal defines the specificity of an immune response, but for triggering an effective immune response additional co-stimulatory or co-inhibitory signals are required to stimulate or inhibit immune responses. It has been shown that co-stimulatory molecules of the B7/CD28 family deliver signals that are critical for T cell activation [20, 38]. RIP-B7.1 mice that express the co-stimulatory molecule B7.1 in pancreatic β cells developed CD8 T cell mediated EAD after DNA immunization with preproinsulin-encoding vectors [17, 47, 59].
Thus, pancreatic \(\beta\) cells that transgenically express the co-stimulatory molecule B7.1 are susceptible for T cell mediated autoimmune attack [102].

Co-inhibitory signals provided by the PD-1/PD-L1 interaction enable down-modulation of T cell responses and are important for maintaining self-tolerance in autoimmune diabetes [42, 113, 145]. Several peripheral tissues including pancreatic \(\beta\) cells show inducible or constitutive expression of PD-L1 [79, 93]. The interaction of APCs expressing PD-L1 with activated T cells expressing PD-1, leads to down-regulation of T cell proliferation and IFN\(\gamma\) production [61]. Moreover, PD-L1 can also interact with the co-stimulator B7.1 up-regulated on activated T cells thereby inhibiting the T cell response [18].

While the PD-1/PD-L1 interaction alleviates self-tolerance by partial control of diabetes development in NOD mice [4, 33, 42, 63], transgenic over-expression of PD-L1 on pancreatic \(\beta\) cells leads to EAD presuming a co-stimulatory pathway for PD-L1 [127]. Recent studies analyzed the influence of inhibitory molecules (PD-1 and PD-L1) on the specificity and diabetogenic potential of ppins-specific CD8 T cell responses in the RIP-B7.1 model. These experiments revealed that priming of ppins-specific CD8 T cells in B6 mice and transferring them in RIP-B7.1 hosts efficiently induced diabetes. Moreover, ppins-DNA immunization of either B6 mice treated with anti-PD-L1 antibody or PD-1\(^{-/-}\) or PD-L1\(^{-/-}\) mice induced EAD. Therefore, \(\beta\) cell destruction by autoreactive ppins (\(K^b/A_{12-21}\))-specific CD8 T cells is supposed to be induced by a mismatch in co-inhibitory (PD-L1) and co-stimulatory (B7.1) signals. Thus, specific activation of CD8 T cells and the \(\beta\) cell susceptibility to an autoimmune attack might be critical for diabetes development [102].
1.8. Aim of the study

RIP-B7.1 mice expressing the co-stimulatory molecule B7.1 (CD80) in pancreatic β cells, are an attractive model to characterize de novo induced preproinsulin (ppins)-specific CD8 T cells. This mouse model is suitable for studying the pathogenic cross-talk between autoreactive CD8 T cells and their target cells, the pancreatic β cells. Only insulin A-chain-(K<sub>b</sub>/A<sub>12-21</sub>)-specific CD8 T cells are found in ppins-immunized and diabetic RIP-B7.1 mice. However, a ppinsΔA<sub>12-21</sub>-antigen (encoding a truncated ppins lacking the COOH-terminal A<sub>12-21</sub>-epitope) efficiently induces insulin B-chain-(K<sub>b</sub>/B<sub>22-29</sub>)-specific CD8 T cells and EAD in RIP-B7.1 mice.

In the first part of my thesis I characterized the selective priming conditions and the diabetogenic potential of both ppins-(K<sub>b</sub>/A<sub>12-21</sub>- and K<sub>b</sub>/B<sub>22-29</sub>-) specific CD8 T cell responses in RIP-B7.1 mice. Three main strategies were applied: (i) genetic alteration of the ppinsΔA<sub>12-21</sub>-antigen by inserting artificial processing sites for either increasing or decreasing the generation of the K<sub>b</sub>/B<sub>22-29</sub>-epitope; (ii) modification of the wild type ppins-antigen, to allow processing and presentation of the K<sub>b</sub>/B<sub>22-29</sub>-epitope; and (iii) formation of truncated ppins-antigens, characterized by a stepwise deletion of COOH-terminal sequences up to the B-chain, to identify the minimal antigen determinant required for an efficient of K<sub>b</sub>/B<sub>22-29</sub>-specific CD8 T cells.

Both, distinct activation of CD8 T cells and β cell susceptibility to an autoimmune attack, are required for diabetes development. Pancreatic β cells are able to alter the epitope specificity of their interaction with autoaggressive CD8 T cells by co-stimulatory and co-inhibitory signals. In this context, I investigated whether inhibitory (PD-1/PD-L1) signaling has an impact on the priming and/or effector phase of autoreactive CD8 T cells. For this purpose, beside ppins-specific, also Ova-specific EAD was analyzed using the well-defined RIP-Ova mouse model that is characterized by the presentation of the K<sub>b</sub>/Ova<sub>257-264</sub>-epitope on pancreatic β cells. The RIP-Ova diabetes model was applied, as it offers experimental advantages compared to ppins-specific diabetes models, for example the possibility to use K<sub>b</sub>/Ova<sub>257-264</sub>-T cell receptor-transgenic OT-I mice.
2. Material and methods

2.1. Chemicals and reagents

All chemicals and reagents not separately mentioned in the text are listed in the appendix.

2.2. Media and buffers

All media and buffers not separately mentioned in the text are listed in the appendix.

2.3. Oligonucleotides, peptides and vectors

Oligonucleotides and synthetic peptides were purchased from Thermo Fisher Scientific (Ulm, Germany). Oligonucleotides were dissolved in water yielding a concentration of 100 pM. Table 1 contains the oligonucleotides used for cloning of plasmids during this thesis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-B-C-fow</td>
<td>aaaaaaagctagcgcaccatggccctgtgattcggg</td>
</tr>
<tr>
<td>SP-B-C-rev</td>
<td>aaaaaagcggccgcttatcactgtggtgccccactccag</td>
</tr>
<tr>
<td>SP-B-fow</td>
<td>aaaaaaagctagcgcaccatggccctgtgattcggg</td>
</tr>
<tr>
<td>SP-B-rev</td>
<td>aaaaaaagctagcgcaccatggccctgtgattcggg</td>
</tr>
<tr>
<td>pCI-Seq Analys-01-fow</td>
<td>cactcccagttcaattacagctc</td>
</tr>
<tr>
<td>pCI-Seq Analys-01-rev</td>
<td>gttggtttgtccaaacctcatc</td>
</tr>
</tbody>
</table>
Table 2 comprises the synthetic peptides used for \textit{in vitro} restimulation of CD8 T cells.

\begin{table}[h]
\centering
\caption{List of synthetic peptides}
\begin{tabular}{|l|l|}
\hline
Epitope & Peptide sequence \\
\hline
H2-K\textsuperscript{b}/S\textsubscript{208-215} & ILSPFLPL \\
H2-K\textsuperscript{b}/ppinsA\textsubscript{12-21} & SLYQLENYCN \\
H2-K\textsuperscript{b}/ppinsB\textsubscript{22-29} & RGFFYTPM \\
H2-K\textsuperscript{b}/Ova\textsubscript{257-264} & SIINFEKL \\
\hline
\end{tabular}
\end{table}

Peptides were dissolved in DMSO at a concentration of 10 mg/ml and diluted with culture medium prior to use.

Table 3 contains a list of all plasmids used during this thesis.

\begin{table}[h]
\centering
\caption{List of plasmids}
\begin{tabular}{|l|l|}
\hline
Plasmid & Insert \\
\hline
pCI/ppins & preproinsulin \\
pCI/ppins\textsubscript{ΔA12-21} & Preproinsulin lacking the ppins A\textsubscript{12-21} epitope sequence \\
pCI/SP-B-C & Signal peptide, B-and C-chain of preproinsulin \\
pCI/SP-B & B-and C-chain of preproinsulin \\
pCI/ppins-furin & preproinsulin with engineered furin sites at the B/C and C/A junctions \\
pCI/ppins-furin\textsubscript{ΔA12-21} & preproinsulin lacking the ppins A\textsubscript{12-21} epitope with engineered furin sites at the B/C and C/A junctions \\
pCI/ppins-minus-furin\textsubscript{ΔA12-21} & preproinsulin lacking the ppins A\textsubscript{12-21} epitope with alanine substitutions at the pancreas-specific cleavage sites \\
pCI/ppins-Ova & K\textsuperscript{b}/Ova\textsubscript{257-264} (SIINFEKL) COOH-terminally fused to preproinsulin \\
pCI/Ova & Ovalbumin \\
\hline
\end{tabular}
\end{table}
Material and methods

An adeno-associated vector encoding ppins (wt) or ppins-furin was kindly provided by Prof. Dr. Bosch (University of Barcelona, Spain).

2.4. Mice

C57BL/6J (B6) mice (Janvier), RIP-B7.1 mice [47], RIP-B7.1/B7.1<sup>−/−</sup> mice, PD-L1<sup>−/−</sup> mice [29], PD-1<sup>−/−</sup> mice [90], Rip-Ova<sub>low</sub> mice [13], PD-L1<sup>−/−</sup>/Rip-Ova<sub>low</sub> mice, RIP-B7.1/RIP-Ova<sub>low</sub> mice, OT-I mice, PD-L1<sup>−/−</sup>/OT-I mice, PD-1<sup>−/−</sup>/OT-I, B7.1<sup>−/−</sup>/OT-I mice, RIP-Ova<sub>low</sub> x OT-I mice, B6.Cg-Tg(CAg) mice (Jackson) and B6.Cg-Tg(CAg)/OT-I mice were bred and kept under standard pathogen-free conditions in the animal colony of the University of Ulm (Ulm, Germany). Male and female mice were used at 8-12 weeks of age, if not otherwise indicated. All animal experiments were performed according to the guidelines of the local Animal Use and Care Committee and the National Animal Welfare Law.

2.5. General molecular biology techniques

2.5.1. Polymerase chain reaction (PCR)

PCR was performed using the ProofStart DNA polymerase (cat.no F-531L, New England Biolabs (NEB)) in a total volume of 50 µl containing 1xPCR buffer, 2 µM dNTP, 10 pmol of forward and reverse primers, 100 ng template DNA and 2.5 U polymerase. The general PCR conditions are listed in table 4.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration cycles</th>
<th>Repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>94°C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>62°C</td>
<td>30 s</td>
<td>2-3 35x</td>
</tr>
<tr>
<td>72°C</td>
<td>60 s</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>4 min</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Standard PCR protocol
2.5.2. Restriction digests

Restriction digests were performed in a total volume of 50 µl containing 10 µg plasmid and at least 20 U restriction enzyme. The reactions were incubated at 37°C for 1.5 h. The target vector preparations were subsequently treated for 30 min at 37°C with 10 U of alkaline phosphatase (CIP) (cat.no. M0290L, NEB) to remove 5’-phosphate groups avoiding self-ligation of the target vector. All enzymes were purchased from NEB and the appropriate reaction buffers were chosen according to the manufacturer’s protocol.

2.5.3. Agarose gel electrophoresis

PCR and restriction digest products were processed using agarose gel electrophoresis to separate the generated fragments. Depending on the size of the generated fragments 1-2% agarose gels were used. The appropriate amount of agarose was dissolved in 1xTAE buffer in a microwave. Ethidium bromide was added directly for later visualization of DNA fragments. Samples were loaded with 6x loading buffer. To estimate the size of the DNA fragments, a 1 kb (cat.no. N3232L, NEB) and a 100 bp ladder (cat.no. SM0321, Fermentas) were loaded along with the samples. The electrophoresis was carried out at 100-140 V for 45 min. The DNA fragments were visualized using UV light and acquired using the GeneGenius system (Syngene).

For analyses of ODN on agarose gels, increasing amounts of peptides were formulated with ODN and the samples were visualized after agarose gel electrophoresis using the GeneGenius system (Syngene).

2.5.4. Purification of DNA fragments from agarose gels

DNA fragments visualized using UV light were excised. DNA fragments were purified using the Qiaquick Gel Extraction Kit (cat.no. 28706, Qiagen) according to the manufacturer’s instructions.
2.5.5. DNA ligation

Purified DNA fragments were ligated in a total volume of 20 µl containing 2 µl 10× ligase buffer, 100 ng vector, 400 U T4 DNA ligase (cat.no. M0202, NEB) and the appropriate amount of insert resulting in a molar ratio of 1 (vector) to 5 (insert). The reaction mixture was incubated at room temperature for at least 2 h prior to transformation.

2.5.6. Transformation of E. coli

10 µl of the ligation reaction were added to 100 µl of competent E. coli DH5α and incubated on ice for 30 min. The bacteria were heat-shocked for 45 s at 42°C and immediately placed on ice for 3 min. 750 µl S.O.C. medium, without antibiotics (cat.no. 15544-034, Invitrogen) were added and the bacteria were allowed to grow for 45 min at 37°C. Varying amounts of the transformation reaction (50-300 µl) were plated onto LB agar plates (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) agar) containing 100 µg/ml ampicillin for selection of successfully transformed bacteria. The plates were incubated at 37°C overnight.

2.5.7. Screening for positive bacteria clones

To identify successfully transformed clones of E. coli a PCR reaction was performed using primer pCI-Seq Analys-01(+) and pCI-Seq Analys-01(-) (Tab. 1) and the Qiagen Hot Start polymerase kit (cat.no. 203645; Qiagen) according to manufacturer’s instructions. The primers recognize the pCI vector backbone within the CMV promoter and the SV40 polyadenylation signal. PCR products were analyzed by agarose gel electrophoresis as described above.

2.5.8. Plasmid purification

For plasmid purification a single colony of transformed E. coli was picked and inoculated in 3-5 ml LB medium (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl) with the appropriate antibiotic for 4-6 h resulting in a preparatory
Material and methods

culture. Afterwards the culture was incubated at 37°C while shaking at 220 rpm overnight in appropriate volume of LB medium (28 ml for midi-preparation; 500 ml for mega-preparation). For vaccination and cell-transfection purposes, the plasmids were purified using the Qiagen Plasmid Midi-/Mega- Kit (cat.no. 12145/12183, Qiagen) according to the manufacturer’s instructions. Purified plasmids were dissolved in ddH₂O. Large scale plasmid DNA was produced and purified by PlasmidFactory GmbH (Bielefeld, Germany).

2.6. Cloning of plasmid vectors

All plasmid vectors used for transient expression and vaccination experiments are based on pCI vector (cat.no. E1731, Promega).

pCI/SP-B-C was cloned using pCI/ppinsΔA₁₂₋₂₁ as template for PCR with the corresponding primers (Tab. 1). The PCR fragment and the target vector pCI/ppinsΔA₁₂₋₂₁ were digested with NheI/NotI and ligated.

pCI/SP-B was cloned using pCI/ppinsΔA₁₂₋₂₁ as template for PCR with the corresponding primers (Tab. 1). The PCR fragment and the target vector pCI/ppinsΔA₁₂₋₂₁ were digested with NheI/NotI and ligated.

pCI/ppins-furin, pCI/ppins-furinΔA₁₂₋₂₁ and pCI/ppins-minus-furinΔA₁₂₋₂₁ were cloned using purchased plasmids (GeneArt®). The templates and the target vector pCI/ppins were digested with NheI/NotI and ligated.
2.7. Cell culture techniques

2.7.1. Cell lines and general cell culturing

The RBL-5 derived mutant cell line RMA-S was kindly provided by Dr K. Kärre (Stockholm, Sweden). RMA-S cells were cultured in Clicks/RPMI supplemented with 5% fetal calf serum (FCS), 1% L-glutamine, 1% penicillin/streptomycin and 0.1 M β-mercaptoethanol (β-ME). The human Hek293 cell line (CRL-1573) was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Hek293 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 0.1 M β-ME. All used cell culture reagents are listed in table 5.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Provider</th>
<th>Cat.no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-ME</td>
<td>Sigma</td>
<td>M-7522</td>
</tr>
<tr>
<td>Clicks/RPMI</td>
<td>Applichem</td>
<td>A2044</td>
</tr>
<tr>
<td>DMEM</td>
<td>Gibco</td>
<td>31855-023</td>
</tr>
<tr>
<td>FCS</td>
<td>Invitrogen</td>
<td>72400-021</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>PAA Laboratories</td>
<td>M11-004</td>
</tr>
<tr>
<td>penicillin/streptomycin</td>
<td>PAA Laboratories</td>
<td>P11-010</td>
</tr>
</tbody>
</table>

2.7.2. Calcium phosphate transfection

Hek293 cells were transiently transfected with plasmids using the calcium phosphate method. Summarized, Hek293 cells were grown to 70% confluency and the medium was exchanged about 30 minutes prior to transfection. For transfection, 62 µl 2 M CaCl₂ and 10 µg plasmid DNA were added to a total volume of 500 µl ddH₂O and mixed. The solution was added drop by drop to 500 µl of a 2x HBS buffer under slightly vortexing. Thereafter the solution was incubated for 90 s at room temperature and added drop by drop to the cells. The medium was exchanged 16 h after
transfection to avoid toxic effects of the complexes. The maximum of expression was reached after 36-48 h. Transfection efficacy was estimated using a pEGFP N1 encoding plasmid, transfection was esteemed successful and was further processed, if more than 80% of cells showed fluorescence.

### 2.7.3. Treatment of cells with proteasome inhibitors

Cells were transiently transfected as described above (2.7.2.). 24 h after transfection media was exchanged and the respective inhibitor diluted in media was added. All used proteasome inhibitors and their final concentrations are listed in table 6.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Provider</th>
<th>Cat.no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epoxomicin</td>
<td>1 µM</td>
<td>Enzo Life Sciences</td>
<td>BML-PI127-0100</td>
</tr>
<tr>
<td>Lactacystin</td>
<td>5 µM</td>
<td>Enzo Life Sciences</td>
<td>BML-PI104-0200</td>
</tr>
</tbody>
</table>

### 2.8. Biochemical methods

#### 2.8.1. Immunoprecipitation

24 h after transfection, Hek293 cells were labeled overnight using 100 µCi $[^{35}\text{S}]$-methionine/cysteine (cat.no. IS103, Hartmann Analytic GmbH) in 3 ml methionine/cysteine-free RPMI-1640 (cat.no. P04-18056, PAN) supplemented with 5% dialyzed FCS (cat.no. 10500-064, Invitrogen), 1% penicillin/streptomycin and 1% L-glutamine. Labeled cells were washed once with PBS and subsequently lysed with lysis buffer. Complete lysis of cells was achieved by shaking for 30 min on ice. Extracts were cleared by centrifugation (30 min, 20,000 x g, 4°C) and incubated for 1 h at 4°C with a polyclonal insulin antibody (rabbit α-insulin (H-86) polyclonal antibody, cat.no. sc-9168, Santa Cruz or guinean pig α-insulin polyclonal antibody, cat.no.
Material and methods

A0564, Dako). Thereafter, the immune complexes were incubated 1 h at 4°C with protein G sepharose (PGS) (cat.no. 17-0618-01, GE Healthcare). Incubation was followed by five washes with washing buffer and two washes using PBS and 0.1 x PBS. Antigens were recovered from sepharose beads by incubation for 1 h at 37°C with elution buffer. Eluates were lyophilized for SDS-PAGE.

2.8.2. SDS-PAGE for autoradiography

Lyophilized samples derived from immunoprecipitation were dissolved in loading buffer. Along with the samples a $^{14}$C-marker (cat.no. CFA645, GE Healthcare) diluted in loading buffer was applied. The samples were boiled for 5 min at 95°C and 10 µl of each sample were loaded onto SDS-polyacrylamide gels (15% acrylamide). The electrophoresis was performed at 60 V until the samples reached the resolving gel. The voltage was subsequently increased up to 140 V. The gel was stained for 10 min using CoomassieBlue staining solution (1.2 g CoomassieBlue, 450 ml methanol, 90 ml acetic acid, 460 ml ddH$_2$O). Gels were destained for at least 15 min with destaining solution (10% methanol, 10% isopropanol, 80% ddH$_2$O). Gels processed for later fluorography were dehydrated for 10 min in acetic acid and subsequently incubated for 10 min in enhancer solution (10% (w/v) PPO in acetic acid). PPO was precipitated by incubating the gel for 15 min in ddH$_2$O. The gel was subsequently dried by vacuum at 80°C for 2 h. The dried gel was placed in a cassette with a radiography film (cat.no. 28906846, GE Healthcare). The fluorography time was between 3 days and 2 weeks dependent on the signal intensity.

2.8.3. SDS-PAGE for western blot detection

Samples from SAM lysates were diluted in loading buffer and boiled for 5 min at 95°C. 10 µl of each sample were loaded onto SDS-polyacrylamide gels (15% acrylamide) or urea-containing SDS-polyacrylamide gels (16% acrylamide) using the Tricin-SDS-PAGE system [107]. Electrophoresis was performed at 60 V until the samples reached the resolving gel. The voltage was subsequently increased up to 140 V. After resolving proteins by SDS-PAGE they were transferred to a nitrocellulose membrane using the iBlot® system (cat.no. IB3010-01, Invitrogen).
Protein transfer was carried out according to the manufacturer’s instructions. Afterwards the membrane was blocked for 20 min at RT in TBS-Tween + 0.1% gelatin (w/v) + 3% milk powder (w/v) followed by incubation with the primary antibody (rabbit α-insulin (H-86) polyclonal antibody, cat.no. sc-9168, Santa Cruz) in TBS-Tween + 0.1% gelatin (w/v) over night at 4°C. The membrane was then washed three times with TBS-Tween + 0.1% gelatin (w/v) and afterwards incubated with the secondary antibody (α-rabbit IgG-HRP, cat.no. NA9340, GE Healthcare) for 45 min at RT. Finally the membrane was washed and detection was carried out as described in the protocol for the chemiluminescence kit (Immobilon™ Western Chemiluminescent HRP Substrate, cat.no. WBKLS0100, Millipore) followed by exposure to an ECL hyperfilm (Amersham Hyperfilm™ MP, cat.no. 92004, GE Healthcare).

2.9. Vaccination

Unless otherwise noted, mice were immunized into the tibialis anterior muscles (50 µl per muscle) with 100 µg plasmid DNA in PBS or the indicated amount of cationic peptides as described [108]. Where indicated, mice were treated with anti-CD4 antibody (YTS-191), anti-CD8 antibody (cat.no. YTS-169) or anti-PD-L1 antibody (cat.no. 16598285, eBioscience). Vaccination with indicated amount of recombinant proteins (10-20 µg) was performed with AbISCO®-100 (AbISCO) (cat.no. 20-0100-10, Isconova) as adjuvant, while 12.5 µg AbISCO per mouse were used as recommended by the manufacturer. Booster vaccinations were set 3 weeks after first injection with the same dose of plasmid DNA or protein respectively.

2.10. Detection of blood glucose levels

Mice were diagnosed as diabetic, when two consecutive blood glucose values exceeded 250mg/dl (13.8 mmol/l) (Disetronic Freestyle).
2.11. Determination of antigen-specific CD8 T cells

2.11.1. Preparation of splenocytes

Mice were sacrificed and spleens were removed. Splenocyte suspensions were prepared by rubbing spleens in 10 ml PBS+1% BSA through a sterile mesh. Erythrocytes were removed by lysis buffer (144 mM NH$_4$Cl, 17 mM Tris, pH 7.2). Splenocytes were washed twice with PBS+1% BSA and cell clumps were removed by pipetting. Afterwards splenocytes were resuspended in UltraCulture (cat.no. 12-725F, Cambrex) supplemented with 1% L-glutamine and 1% penicillin/streptomycin.

2.11.2. Preparation of pancreatic lymphocytes

Mice were sacrificed and 2 ml of a collagenase solution (Collagenase P, cat.no. 11213857001, Roche) were used at a concentration of 1 mg/ml for in situ injection into the pancreas. After excision the pancreas was digested for 8 min at 37°C. The reaction was stopped by adding 5 ml Hanks BSS (1x) (H15-009, PAA Laboratories) + 10% FCS (v/v). Subsequently the cell suspension was rubbed through a 100 µm sterile mesh and cells were pelleted by centrifugation (1400 rpm, 4 min, 4°C). After discarding the supernatant, the cell pellet was resuspended in 10 ml Hanks BSS and underlayed with 5 ml Histopaque®-1077 (cat.no. 10771-500ML, Sigma-Aldrich). After centrifugation (2400 rpm, 15 min, 4°C, without break) the interphase between supernatant and pellet was collected and washed with Hanks BSS + 10% FCS (v/v). Finally the cell suspension was centrifuged (1400 rpm, 4 min, 4°C) and cells were resuspended in UltraCulture (cat.no. 12-725F, Cambrex) supplemented with 1% L-glutamine and 1% penicillin/streptomycin.

2.11.3. Preparation of pancreatic lymph nodes

Mice were sacrificed and pancreatic lymph nodes (pLN) were removed. Cell suspensions were prepared by rubbing pLNs in 10 ml PBS+1% BSA through a sterile mesh. Erythrocytes were removed by adding lysis buffer (144 mM NH$_4$Cl, 17 mM Tris, pH 7.2).
Tris, pH 7.2). Cells were washed twice with PBS+1% BSA and cell clumps were removed by pipetting. Cells were resuspended in UltraCulture (cat.no. 12-725F, Cambrex) supplemented with 1% L-glutamine and 1% penicillin/streptomycin.

2.11.4. Determination of antigen-specific CD8 T cell frequencies

$2 \times 10^6$ splenocytes or pLN/pancreatic lymphocytes were washed once using FACS buffer A and incubated for 15 min at 4°C with anti-CD16/CD32 to block unspecific binding of anti-CD8 mAb to Fc receptors. Cells were washed once and incubated for 30 min at 4°C with FITC-conjugated anti-CD8 mAb (diluted 1/200) and APC/PE-conjugated MHC-I:peptide tetramers (diluted 1/200). After three washes, stained cells were fixed with 2% PFA and frequencies of tetramer$^+$ CD8 T cells were determined by FCM (LSR II, BD Biosciences). Used MHC-I:peptide tetramers are listed in table 7.

Table 7: List of MHC class I-tetramers

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Conjugation</th>
<th>Provider</th>
<th>Cat.no.</th>
<th>Lot.no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2-K$^b$/ppins$^{46-53}$</td>
<td>APC</td>
<td>Glycotope</td>
<td>KH294</td>
<td></td>
</tr>
<tr>
<td>H2-K$^b$/Ova$^{257-264}$</td>
<td>PE</td>
<td>Beckman Coulter</td>
<td>T20073</td>
<td>M007106</td>
</tr>
</tbody>
</table>

2.11.5. Determination of antigen-specific IFN$\gamma$-producing CD8 T cells

Splenocytes ($1 \times 10^7$/ml) or pLN/pancreatic lymphocytes were incubated with 2.5 µg/ml antigenic peptide in supplemented UltraCulture (cat.no. 12-725F, Cambrex) for 4 h at 37°C. Additionally, 5 µg/ml brefeldin A (BFA; cat.no. 15870, Sigma) were added to block anterograde protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus, thereby leading to intracellular accumulation of produced IFN$\gamma$. After 4 h restimulation, cells were washed once using FACS buffer A and incubated for 15 min at 4°C with anti-CD16/CD32 to block unspecific binding of anti-CD8 mAb to Fc receptors. Cells were washed once and incubated for 30 min at 4°C with PE-conjugated anti-CD8 mAb (diluted 1/200). After three washes, surface stained cells were fixed with 2% PFA for 15 min at RT. Thereafter, the fixed cells were washed once and prepared for intracellular staining by incubation with permeabilization buffer
(FACS buffer B) for 15 min at RT. Permeabilized cells were incubated with FITC-conjugated anti-IFN$\gamma$ mAb (diluted 1/200) for 30 min at RT in the dark to detect intracellular accumulated IFN$\gamma$. After three subsequent washes with permeabilization buffer, stained cells were resuspended in FACS buffer A, IFN$\gamma^+$ CD8 T cells were determined by FCM (LSR II, BD Biosciences). Table 8 comprises a list of antibodies used for FACS analysis.

Table 8: List of FACS antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conjugation</th>
<th>Provider</th>
<th>Cat.no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-CD8</td>
<td>FITC</td>
<td>eBioscience</td>
<td>11-0081-85</td>
</tr>
<tr>
<td>$\alpha$-CD8</td>
<td>PE</td>
<td>eBioscience</td>
<td>12-0081-83</td>
</tr>
<tr>
<td>$\alpha$-CD8</td>
<td>APC</td>
<td>eBioscience</td>
<td>17-0081-83</td>
</tr>
<tr>
<td>$\alpha$-IFN$\gamma$</td>
<td>FITC</td>
<td>eBioscience</td>
<td>11-7311-82</td>
</tr>
</tbody>
</table>

2.12. Adoptive transfer

2.12.1. CD8 T cell purification using MACS

Splenocytes were prepared as described above (2.12.1) and resuspended in 40 µl cold PBS + 1% FCS (v/v) per $10^7$ cells. Subsequently 10 µl per $1x10^7$ cells of a biotin-antibody cocktail (CD8a$^+$ T cell Isolation Kit, cat.no. 130-095-236, Miltenyi Biotec) were added and the mixture was incubated for 10 min at 4°C. Afterwards cells were treated with 10 µl $\alpha$-biotin-microbeads (CD8a$^+$ T cell Isolation Kit, cat.no. 130-095-236, Miltenyi Biotec) per $1x10^7$ cells, incubated for 15 min at 4°C and washed twice with cold PBS + 1% FCS (v/v). Cells were resuspended in 500 µl cold PBS + 1% FCS (v/v) and loaded onto a LS column (MACS® Separation Columns, cat.no. 130-042-401, Miltenyi Biotec). Once the cell suspension has passed through, the column was washed three times with 3 ml cold PBS + 1% FCS (v/v). The cells were counted for further applications.
2.12.2. CFSE labeling

CFSE staining of splenocytes was performed using a CFSE Cell Proliferation Kit (cat.no. C34554, Invitrogen). A 1 mM stock solution of CFSE was prepared by adding 90 µl DMSO to one CFSE vial. Cells were resuspended in warm PBS + 0.1% BSA at a concentration of $2 \times 10^6$/ml. The CFSE stock solution was diluted in PBS + 0.1% BSA to a final concentration of 10 µM. Next an equal volume of the CFSE staining solution was added to the splenocytes followed by incubation at 37°C for 10 min. After that the staining was quenched by adding 5 volumes of ice-cold PBS + 5% FCS and incubated on ice for 5 min. Finally cells were centrifuged and washed twice with PBS + 5% FCS before they were resuspended for further analysis.

2.13. Cocultivation of OT-I blasts with pancreatic cells

Splenocytes of OT-I mice were prepared as described above (see 2.11.1). OT-I blasts were in vitro generated by stimulation with K$^b$/Ova$^{257-264}$ for 3 d. Cells were purified using MACS as described above (see 2.12.1). Afterwards targets (1x10$^4$/well), that had been left untreated or pulsed for 2 h with K$^b$/Ova$^{257-264}$, were cocultured with blasts (1x10$^5$/well) for 1-3 d in 96-well U-bottom plates in UltraCulture (cat.no. 12-725F, Cambrex), followed by determination of IFNγ by ELISA as described below (2.15).

2.14. Determination of K$^b$-binding affinities

Surface stabilization of MHC class I, binding K$^b$/A$^{12-21}$, K$^b$/Ova$^{257-264}$ and K$^b$/B$^{22-29}$ peptides was determined in TAP deficient RMA/S cells. Briefly, 5x10$^5$ cells were cultured for 16 h at 37°C in UltraCulture medium (cat.no. BE 12-725F, Lonza, Belgium) supplied with 100 µg/ml K$^b$/A$^{12-21}$-, K$^b$/Ova$^{257-264}$- and K$^b$/B$^{22-29}$-peptide.
Material and methods

Afterwards cells were fixed with 2% PFA and H-2Kb surface expression of RMA-S was assessed with an anti-mouse H2-Kb antibody (cat. no. 553570, BD Biosciences) by FCM. 5x10^5 unpulsed RMA-S cells served as control.

2.15. Detection of IFNγ by ELISA

IFNγ was detected by conventional double-sandwich enzyme-linked immunosorbent assay (ELISA). MicroELISA plates (cat.no. 442404, Nunc) were coated overnight with 50 ng anti-IFNγ antibody (cat.no. 551216, BD Biosciences) diluted in coating buffer (1 M Na₂HPO₄ x 2H₂O pH 9.0) at 4°C. The plates were blocked for 1 h at room temperature with blocking buffer (PBS, 3% (w/v) BSA) in order to avoid unspecific binding. After two washes using washing buffer (PBS, 0.05% Tween 20), samples and IFNγ standards (cat.no. 554587, BD Biosciences) were added to the wells, incubated for 3 h at room temperature and washed four times with washing buffer. Bound IFNγ was detected by addition of 50 ng biotinylated anti-IFNγ antibody (cat.no. 554410, BD Biosciences) diluted in blocking buffer and incubation for 1 h at room temperature. After six washes with washing buffer, 50 ng streptavidin-conjugated alkaline phosphatase (SAP) (cat.no. 016-050-084, Jackson ImmunoResearch) were added for 30 min at room temperature. After eight washes with washing buffer, the SAP substrate nitrophenyl phosphated disodium salt (cat.no. 71768, Fluka) dissolved in diethanolamine buffer (10.3% (v/v) diethanolamine, 3.9 mM MgCl₂ x 6H₂O, 3 mM NaN₃) was added to the plates. The enzymatic reaction was stopped after 5-15 min by addition of 0.5 mM EDTA (pH 8.0). Extinction was analyzed at 405/490 nm using a TECAN micro plate reader (TECAN, Crailsheim, Germany) and EasyWin software (TECAN).
2.16. Histology

2.16.1. Hemalaun and Eosin staining (H&E staining)

Pancreatic cryosections were cut (2 µm) and pre-treated in distilled water. Sections were stained with hemalaun (cat.no. 1092490500, Merck) for 5 min. Subsequently, sections were cleared under running tap water. After that hemalaun staining was fixed in acid ethanol. After washing with distilled water, sections were counter-stained with eosin (cat.no. 2C-140, Waldeck) for 3 min. After washing (3 min in distilled water) several dehydration steps were performed in 70%, 80%, 90%, 96%, 100% ethanol. Finally, sections were preserved in xylene, covered and mounted with Cytoseal60 mounting medium (cat.no. 18006, EMS). Sections were acquired on a light microscope (Leica, Germany) equipped with a digital camera and Leica Application Suite software (Leica Microsystems, Switzerland).

2.16.2. Immunohistochemistry

Pancreatic cryosections were cut (2 µm) and pre-treated with blocking goat serum (cat.no. 50-197Z, Zymed) for 1 h in a wet chamber. Then, sections were incubated with primary antibodies for 1 h in a wet chamber. After two washing steps with PBS, sections were incubated with secondary antibodies for 1 h in a dark, wet chamber. All antibodies used for immunohistochemistry were diluted 1:100 and are listed in table 9. Finally, sections were covered and mounted with Cytoseal60 mounting medium (cat.no. 18006, EMS). A fluorescence microscope (Zeiss) equipped with a digital camera (C4742, Hamamatsu) was used to analyze sections.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Provider</th>
<th>Cat.no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>guinea-pig polyclonal α-insulin</td>
<td>Dako</td>
<td>A0564</td>
</tr>
<tr>
<td>rat α-CD8</td>
<td>AbD Serotec</td>
<td>MCA2694</td>
</tr>
<tr>
<td>α- guinea pig-FITC</td>
<td>Sigma</td>
<td>F-6261</td>
</tr>
<tr>
<td>α- rat-TRITC</td>
<td>Sigma</td>
<td>T 4280</td>
</tr>
</tbody>
</table>
2.17. Statistics

Statistic analyses were performed using GraphPad Prism (Version 4, GraphPad Software Inc.). Figures show mean values and SEM (standard error of the mean). If not indicated otherwise, figures show data of one representative experiment (of at least three independent replicates).
3. Results

3.1. Selective induction of K\textsuperscript{b}/A\textsubscript{12-21}-and K\textsuperscript{b}/B\textsubscript{22-29}-specific CD8 T cells by different ppins-encoding vectors

3.1.1. Induction of EAD in RIP-B7.1 mice by ppins- and ppins\textsuperscript{ΔA\textsubscript{12-21}}-encoding vectors

The RIP-B7.1 mouse model was used for studying K\textsuperscript{b}/A\textsubscript{12-21}-and K\textsuperscript{b}/B\textsubscript{22-29}-specific CD8 T cell responses during the course of EAD. For immunization studies I used the pCI/ppins (encoding the murine wt ppins-II sequence i.e., the signal peptide (SP), the B-, C- and A-chain) and the pCI/ppins\textsuperscript{ΔA\textsubscript{12-21}} (lacking the ppins-A\textsubscript{12-21}-sequence at the extreme COOH terminus of ppins) DNA construct (Fig. 5A). Thus, ppins encodes both K\textsuperscript{b}-restricted ppins-epitopes (K\textsuperscript{b}/B\textsubscript{22-29} and K\textsuperscript{b}/A\textsubscript{12-21}), whereas ppins\textsuperscript{ΔA\textsubscript{12-21}} encodes only the K\textsuperscript{b}/B\textsubscript{22-29}-epitope (Fig. 5A).

First, both epitopes were analyzed \textit{in vitro} for their binding/stabilizing capacity of empty MHC class I K\textsuperscript{b}-molecules on the surface of TAP-deficient RMA-S cells, a widely used assay to measure the avidity of an antigenic peptide for its restriction element. The B\textsubscript{22-29}-epitope contains an optimal K\textsuperscript{b}-binding motif, i.e., Y at anchor position P5 and M at anchor position P8, and efficiently stabilized K\textsuperscript{b}-molecules on the surface of RMA-S cells (Fig. 5B). They were more efficiently stabilized by the B\textsubscript{22-29}-epitope than by the A\textsubscript{12-21}-epitope (Fig. 5B), and it was possible to generate stable K\textsuperscript{b}/B\textsubscript{22-29} (but not K\textsuperscript{b}/A\textsubscript{12-21}) dimers or tetramers (see below). This indicates that the K\textsuperscript{b}/A\textsubscript{12-21}-epitope has a very low avidity for K\textsuperscript{b}-restricted MHC class I molecules.
A single injection of pCI/ppins plasmid DNA efficiently induced CD8 T cell-dependent EAD in RIP-B7.1 mice (Fig. 6A). Transgene-driven expression of the co-stimulator B7.1 (CD80) in pancreatic β cells, but not B7.1 expression by other cells in RIP-B7.1/B7.1−/− mice, was required to elicit ppins-specific CD8 T cells and EAD (Fig. 6B). Similarly, immunization with pCI/ppinsΔA12-21 plasmid DNA also induced diabetes in RIP-B7.1 and RIP-B7.1/B7.1−/− mice (Fig. 6). Injection of empty pCI vector DNA into RIP-B7.1 or RIP-B7.1/B7.1−/− mice did not cause EAD (Fig. 6). Thus, only β cell associated expression of the co-stimulatory molecule B7.1 is critical for induction of EAD.
**Results**

Figure 6: Selective expression of the co-stimulatory molecule B7.1

RIP-B7.1 (A) or RIP-B7.1/B7.1⁻/⁻ (B) mice (expressing the B7.1 molecule selectively in pancreatic β cells) were immunized with pCI (n=5), pCI/ppins (n=5) or pCI/ppinsΔA₁₂₋₂₁ (n=5) DNA. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

### 3.1.2. Characterization of ppins and ppinsΔA₁₂₋₂₁ in Hek293 cells

Next, expression studies comparing pCI/ppins and pCI/ppinsΔA₁₂₋₂₁ were performed. Hek293 cells were transiently transfected with the respective plasmids and radioactively labeled with a [³⁵S] methionine/cysteine mix. Cells were lysed 40 h post transfection, and proteins were immunoprecipitated from cell lysates or cell culture supernatants either with the H-86-α-insulin (Fig. 7A, lanes 1-6) or the guinea pig-α-insulin antibodies (Fig. 7A, lanes 7-10). Samples were analyzed on a 15% Tris-SDS-PAGE followed by fluorography. Using this approach a specific band migrating at the expected size for proinsulin (pins) could be detected with both insulin-specific antibodies in ppins-transfected as well as in ppinsΔA₁₂₋₂₁-transfected cells. Pins was expressed at higher levels than pinsΔA₁₂₋₂₁ (Fig. 7A). In order to characterize steady state levels of the different pins proteins I performed western blot analyses. Hek293 cells were transiently transfected with both DNA constructs. Whole cell lysates were harvested 24 h or 40 h post transfection and analyzed on a 15% Tris-SDS-PAGE followed by western blotting with the H-86-α-insulin antibody.
Results

Figure 7: Expression and stability analyses of ppins-encoding DNA constructs
A) Hek293 cells were transiently transfected with pCI (lanes 1-2), pCI/ppins (lanes 3, 4, 7, 8) or pCI/ppinsΔA12-21 (lanes 5, 6, 9, 10). Cells were labeled with [35S]-methionine/cystein, lysed and both, cell lysates and cell culture supernatants immunoprecipitated with either the H-86-α-insulin (lane 1-6) or the guinea pig α-insulin (lane 7-10) antibody and protein A sepharose. Immunoprecipitates were processed for 15% Tris-SDS-PAGE and analyzed by autoradiography. The position of proinsulin is indicated.

B) Hek293 cells were transiently transfected with pCI/ppins (lanes 1, 3, 5, 7) or pCI/ppinsΔA12-21 (lanes 2, 4, 6, 8). Cells were harvested 24 h (lanes 1, 2, 5, 6) or 40 h (lanes 3, 4, 7, 8) post transfection. Cell lysates were processed for either 15% Tris-SDS-PAGE (left panel) or 16% Tris-Tricine-urea-SDS-PAGE (right panel) followed by western blot analysis using the H-86-α-insulin antibody. The positions of pins and pins isomer forms are indicated.

D) Hek293 cells were transiently transfected with pCI (lane 1), pCI/ppins (lanes 2-4) or pCI/ppinsΔA12-21 (lanes 5-7). 34 h after transfection cells were left untreated (−) or treated with epoxomicin (E) or lactacystin (L) for 6 h. Cell lysates were processed for 16% Tris-Tricine-urea-SDS-PAGE followed by western blot analysis using the H-86-α-insulin antibody. The position of pins isomer forms is indicated.

This revealed that pins but not pinsΔA12-21 was detectable 24 h after transfection (Fig. 7B, lane 1 and 2). 40 h post transfection, also pinsΔA12-21 expression levels
were detectable (Fig. 7B, lane 3 and 4). The same samples were analyzed on another SDS-PAGE system, especially developed for the separation and detection of small proteins (i.e., 16% Tris-Tricine-urea-SDS-PAGE; Fig. 7B, lane 5-8). Interestingly, conformational defined proinsulin isomers were detectable in ppins but not in ppins\(\Delta A_{12-21}\) transfectants (Fig. 7B, lane 5-8).

Since ppins\(\Delta A_{12-21}\) was barely detectable in transiently transfected cells I next asked, whether ppins\(\Delta A_{12-21}\) is more susceptible for proteasomal degradation than wt pins. Hek293 cells were transfected with pCl/ppins- or pCl/ppins\(\Delta A_{12-21}\)-DNA and treated with proteasome inhibitors for 6 h. Analyzing the samples on a 16% Tris-Tricine-urea-SDS-PAGE and western blotting revealed that ppins\(\Delta A_{12-21}\) isomers accumulated efficiently due to inhibition of proteasomal degradation by the proteasome inhibitors expoxomicin and lactacystin (Fig. 7D, lanes 5-7). In contrast, these inhibitors did not affect expression of pins in pCl/ppins transfected cells (Fig. 7D, lanes 2-4). Thus, the expression of ppins\(\Delta A_{12-21}\) (but not ppins) critically depends on proteasomal degradation.

### 3.1.3. Characterization of K\(^b\)/B\(^{22-29}\)-specific CD8 T cell responses

In the following, I characterized the development of ppins-B\(^{22-29}\) -specific diabetes in the RIP-B7.1 model. Mice were immunized with pCl/ppins (as a control for B\(^{22-29}\)-independent EAD, since K\(^b\)/B\(^{22-29}\)-epitope-specific CD8 T cell responses were not co-primed in RIP-B7.1 mice by ppins DNA) and pCl/ppins\(\Delta A_{12-21}\) (Fig. 8A). Splenocytes and pancreas infiltrating lymphocytes were prepared and CD8\(^+\) ppins-B\(^{22-29}\)-specific T cells were detected at d 12, d 21 and d 46 after immunization using K\(^b\)/B\(^{22-29}\)-tetramer\(^+\) CD8\(^+\) T cell numbers were detectable at d 46 in the spleens of pCl/ppins\(\Delta A_{12-21}\)-immunized mice (Fig. 8B). At d 12 CD8 T cells specific for the ppins-B\(^{22-29}\) epitope started to invade the pancreata of RIP-B7.1 mice, reaching the maximum cell number until d 46. This epitope specificity was not detectable in pCl/ppins-immunized mice (Fig. 8C).
Results

**Figure 8: Kinetic analyses of ppins-K\textsuperscript{b}/B\textsubscript{22-29}-specific EAD in RIP-B7.1 mice**

RIP-B7.1 mice were immunized with pCI/ppins (n=5) or pCI/ppins\textDelta\textsubscript{A\textsubscript{12-21}} (n=5) DNA. A) At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined. B) At the indicated times after immunization splenocytes were prepared and directly stained with K\textsuperscript{b}/B\textsubscript{22-29}-tetramers. Specific CD8 T cells were identified by flow cytometry. The percentages ± SD of K\textsuperscript{b}/B\textsubscript{22-29}-tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells within splenocytes at indicated times after immunization are shown. C) At the indicated times after immunization pancreatic lymphocytes were prepared and directly stained with K\textsuperscript{b}/B\textsubscript{22-29}-tetramers. Specific CD8 T cells were identified by flow cytometry. The total numbers ± SD of K\textsuperscript{b}/B\textsubscript{22-29}-tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells within pancreas-infiltrating CD8 T cells at indicated times after immunization are shown (left panel). Primary FACS data of pCI/ppins\textDelta\textsubscript{A\textsubscript{12-21}}-immunized mice are shown for representative mice (right panel).

In order to characterize pancreata of RIP-B7.1 mice immunized with pCI/ppins\textDelta\textsubscript{A\textsubscript{12-21}} morphological, histological methods were applied. Pancreatic sections were initially inquired for islet infiltrates using H+E staining. This revealed still healthy islets at d 12 (Fig. 9 group 1), islet surrounding cells at d 21 (Fig. 9 group 2) and massive cell infiltration at d 46 (Fig. 9 group 3) after immunization. Further immunohistochemical analysis of these sections manifested that insulitis in RIP-B7.1 mice immunized with...
pCl/ppinsΔA_{12-21} is characterized by the invasion of CD8 T lymphocytes (Fig. 9). Hence, flow cytometric and immunohistological analyses revealed that ppins-B_{22-29} specific diabetes development correlated with an influx of B_{22-29}-specific CD8 T cells and bystander lymphocytes into the pancreata of RIP-B7.1 mice.

**RIP-B7.1 mice immunized with pCl/ppinsΔA_{12-21}**

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<td>3 (d 46) diabetic [450 mg/dl]</td>
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**Figure 9: Histological characterization of ppins-K^b/B_{22-29}-specific diabetes**

RIP-B7.1 mice were immunized with pCl/ppinsΔA_{12-21} DNA and analyzed at day 12 (group 1 healthy), d 21 (group 2 pre-diabetic) and d 46 (group 3 diabetic) after immunization. Blood glucose levels were determined [mg/dl]. At the indicated time points (representative mice are shown), pancreatic sections were analyzed for general insulitis using H+E staining and in detail using immunohistochemical staining. Insulin expression is stained in green and CD8 T cell infiltration is stained in red. An overlay is shown.

### 3.1.4. Antigen requirements for K^b/B_{22-29}-specific CD8 T cell priming

To analyze the prerequisites for priming diabetogenic, B_{22-29}-specific CD8 T cells in RIP-B7.1 mice, I initially applied a peptide-based immunization approach. Immunostimulatory oligodeoxynucleotides (ODN) were incubated with short,
Results

synthetic peptides in which the cationic HIV-tat\textsubscript{49-57} RKKRRQRRRR domain (tat) was fused COOH-terminally to the A- or B-chain of insulin. This generated the cationic A/tat- or B/tat-peptides (Fig. 10A).

Figure 10: Antigen requirements for K\textsuperscript{β}/B\textsubscript{22-29}-specific EAD

A) The amino acid sequences of cationic peptides comprising the insulin A-chain (A-tat) and the insulin B-chain (B-tat) are shown. Increasing peptide concentrations were incubated with ODN and analyzed on an agarose gel. The positions of peptide/ODN-complexes and “free” ODN are indicated.

B) RIP-B7.1 mice were immunized with A-tat/ODN (n=5) or B-tat/ODN (n=5). At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

C) Schematic depiction of ppins-encoding DNA constructs containing different deletions is shown: pCI/ppins\_Δ\textsubscript{A12-21} lacks the ppins-A\textsubscript{12-21} sequence, pCI/SP-B-C lacks the complete A-chain and pCI/SP-B comprises only the signal peptide and the B-chain. Hek293 cells were transiently transfected with pCI (lane 1), pCI/SP-B-C (lane 2) or pCI/SP-B (lane 3). Cells were labeled with \textsuperscript{35}S-methionine/cysteine, lysed and cell lysates immunoprecipitated with the H-86-α-insulin antibody and protein A sepharose. Immunoprecipitates were processed for SDS-PAGE and analyzed by autoradiography. The slight band for pins is marked.

D) RIP-B7.1 mice were immunized with pCI/ppins\_Δ\textsubscript{A12-21} (n=7), pCI/SP-B-C (n=7) or pCI/SP-B (n=7) DNA. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.
RIP-B7.1 mice were immunized with A-tat/ODN or B-tat/ODN, and diabetes development was followed by measuring the blood glucose levels. Interestingly, while immunization with A-tat induced K\(^{b}\)/A\(_{12-21}\)-specific EAD in 60% of the mice, B-tat completely failed to induce diabetes in RIP-B7.1 mice (Fig. 10B). Thus, exogenous delivery of the 30-residue B-chain did not trigger EAD in RIP-B7.1 mice.

In the next step I shortened the ppins\(_{\Delta A_{12-21}}\) antigen by PCR-based cloning approaches: deletion of the residual A\(_{1-11}\) sequence generated the pCI/SP-B-C vector (containing the SP, the B- and the C-chain), and deletion of the residual A\(_{1-11}\) and the C-peptide encoding sequence created the pCI/SP-B vector (Fig. 10C). Expression analyses of these vectors in \[^{35}\text{S}\] methionine/cysteine-labeled Hek293 cells by a H-86-\(\alpha\)-insulin-specific IP revealed only very low levels of the SP-B-C protein. SP-B was not detectable (Fig. 10C). Neither pCI/SP-B-C nor pCI/SP-B were able to efficiently induce diabetes in RIP-B7.1 mice. Only one out of seven RIP-B7.1 mice immunized with pCI/SP-B developed diabetes within 16 weeks post injection, indicating that diabetes induction is very inefficient using DNA constructs lacking the A\(_{1-11}\) sequence (Fig. 10D). Thus, only pCI/ppins\(_{\Delta A_{12-21}}\) delivered the specific antigen for priming B\(_{22-29}\)-specific CD8 T cells.

3.1.5. Specific processing requirements within the B/C junction of ppins\(_{\Delta A_{12-21}}\) affects the efficacy of priming B\(_{22-29}\)-specific CD8 T cells and EAD

Pancreatic \(\beta\) cells are able to process ppins into bioactive insulin as they contain specific recognition sites for pancreas-specific proteolytic enzymes. These pairs of dibasic peptides (RR and KR) at the B/C and C/A junction of proinsulin display structural similarity to furin-specific recognition sequences. Since non-pancreatic cells process ppins only to proinsulin by cutting the signal peptide, I analyzed whether specific processing at the B/C junction of proinsulin has an impact on the generation of the B\(_{22-29}\) epitope (and of B\(_{22-29}\)-specific CD8 T cells). I introduced specifically engineered furin-specific recognition sites at the B/C junction (and also the C/A junction) of ppins\(_{\Delta A_{12-21}}\) (pCI/ppins-furin\(_{\Delta A_{12-21}}\)).
Results

In a second construct, these specific recognition sites were eliminated by alanine substitutions (pCI/ppins-minus-furinΔA₁₂-2₁) (Fig. 11A). Transient transfection of Hek293 cells revealed comparable expression levels of the different pinsΔA₁₂-2₁ constructs. Notably, I could not detect further processing products (Fig. 11A).

Figure 11: The antigenicity of Kᵇ/B₂₂-₂₉ depends on specific processing sites
A) The amino acid sequences at the B/C junction of wt ppins (wt), ppins with an engineered furin-specific recognition site (furin-plus) and ppins with alanine substitutions at the natural pancreas-specific cleavage site (furin-minus) are shown. Hek293 cells were transiently transfected with pCI/ppins-furinΔA₁₂-2₁ (lane 1) or pCI/ppins-minus-furinΔA₁₂-2₁ (lane 2). Samples were analyzed on SDS-PAGE. The position of proinsulin is indicated.
B) RIP-B7.1 mice were immunized with pCI/ppinsΔA₁₂-2₁ (n=5), pCI/ppins-furinΔA₁₂-2₁ (n=5) or pCI/ppins-minus-furinΔA₁₂-2₁ (n=5) DNA. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.
C) Pancreatic lymphocytes of pCI/ppinsΔA₁₂-2₁ - (group 1), pCI/ppins-furinΔA₁₂-2₁ - (group 2) or pCI/ppins-minus-furinΔA₁₂-2₁ - (group 3) immunized mice were prepared and directly stained with Kᵇ/B₂₂-₂₉-tetramers. Specific CD8 T cells were identified by flow cytometry. The total numbers ± SD of Kᵇ/B₂₂-₂₉-tetramer⁺ CD8⁺ T cells within pancreas-infiltrating CD8 T cells at indicated times after immunization are shown (left panel). Primary FACS data are shown for representative mice (right panel).
RIP-B7.1 mice were immunized with pCI/ppinsΔA_{12-21}, pCI/ppins-furinΔA_{12-21} and pCI/ppins-minus-furinΔA_{12-21}. Diabetes development was monitored by measuring the blood glucose levels and diabetes incidences were determined. While pCI/ppins-furinΔA_{12-21} enabled a faster induction of diabetes compared to pCI/ppinsΔA_{12-21}, diabetes development was significantly delayed in pCI/ppins-minus-furinΔA_{12-21}-immunized mice (Fig. 11B). Moreover, analysis of the pancreas-infiltrating lymphocytes using K^{b}/B_{22-29}-tetramers and FACS analysis demonstrated that pCI/ppins-furinΔA_{12-21} induced significant higher frequencies of K^{b}/B_{22-29}-tetramer^{+} CD8^{+} T cells compared to pCI/ppinsΔA_{12-21}. In contrast, K^{b}/B_{22-29}-tetramer^{+} CD8^{+} T cell numbers were decreased in pCI/ppins-minus-furinΔA_{12-21}-immunized mice (Fig. 11C). Hence, altering the specific recognition site for pancreas-specific proteolytic enzymes at the B/C junction of ppinsΔA_{12-21} had a significant effect on the efficacy of priming K^{b}/B_{22-29}-specific CD8 T cells and on the course of EAD development in RIP-B7.1 mice.

3.1.6. A ppins-mutant with specific furin sites enables co-priming of A_{12-21}-and B_{22-29}-specific CD8 T cells

Immunization of RIP-B7.1 mice with the full-length ppins DNA construct could not prime K^{b}/B_{22-29}-specific CD8 T cells (see Fig. 8). In order to identify the processing requirements of ppins for the induction of K^{b}/B_{22-29}-specific diabetes, I addressed the question whether introduction of furin-specific recognition sites at the B/C and C/A junctions of full-length ppins might improve the accessibility of the K^{b}/B_{22-29}-epitope for antigen presentation. To this end, I cloned a ppins-encoding DNA construct that contained specific furin protease recognition sequences at the B/C and C/A junctions of ppins (pCI/ppins-furin) (Fig. 12A). RIP-B7.1 mice were immunized with pCI/ppins or pCI/ppins-furin. Both DNA constructs efficiently induced diabetes in RIP-B7.1 mice between 2 and 4 weeks after immunization (Fig. 12B). FACS analysis of pancreas infiltrating cells in these diabetic mice revealed K^{b}/B_{22-29}-tetramer^{+} CD8^{+} T cells in RIP-B7.1 mice immunized with pCI/ppins-furin. This specificity was not detectable in pCI/ppins-immunized mice (Fig. 12C). Furthermore, ex vivo restimulation of islet infiltrates followed by FACS analysis displayed both, A_{12-21}- and B_{22-29}-specific
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IFNγ+ CD8+ T cells in mice immunized with pCI/ppins-furin, while in pCI/ppins-immunized mice only A12-21-specific IFNγ+ CD8+ T cells were detectable (Fig. 12D). Thus, immunization with pCI/ppins-furin, but not pCI/ppins, induced B22-29-specific CD8 T cells.

A) The amino acid sequences at the B/C and C/A junctions of wt ppins (wt) and ppins with engineered furin-specific recognition sites (furin-plus) are shown.

B) RIP-B7.1 mice were immunized with pCI/ppins (n=5) or pCI/ppins-furin (n=5) DNA. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

C) Pancreatic lymphocytes of pCI/ppins- (group 1) and pCI/ppins-furin-immunized mice (group 2) were prepared and directly stained with Kβ/B22-29-tetramers. Specific CD8 T cells were identified by flow cytometry. The total numbers ± SD of Kβ/B22-29-tetramer+ CD8 T cells within pancreas-infiltrating CD8 T cells are shown (left panel). Primary FACS data are shown for representative mice (right panel).

D) Pancreatic lymphocytes of pCI/ppins- (group 1) and pCI/ppins-furin-immunized mice (group 2) were prepared and ex vivo restimulated with Kα/A12-21- or Kβ/B22-29-peptides. IFNγ-producing CD8+ T cells were determined by flow cytometry. The total numbers ± SD of IFNγ+ CD8+ T cells within pancreas-infiltrating CD8 T cells are shown (right panel). Primary FACS data for pCI/ppins-furin-immunized mice are shown for representative mice (left panel).

Figure 12: Specific processing of full-length ppins enables priming of A12-21- and B22-29-specific CD8 T cells

A) The amino acid sequences at the B/C and C/A junctions of wt ppins (wt) and ppins with engineered furin-specific recognition sites (furin-plus) are shown.

B) RIP-B7.1 mice were immunized with pCI/ppins (n=5) or pCI/ppins-furin (n=5) DNA. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

C) Pancreatic lymphocytes of pCI/ppins- (group 1) and pCI/ppins-furin-immunized mice (group 2) were prepared and directly stained with Kβ/B22-29-tetramers. Specific CD8 T cells were identified by flow cytometry. The total numbers ± SD of Kβ/B22-29-tetramer+ CD8 T cells within pancreas-infiltrating CD8 T cells are shown (left panel). Primary FACS data are shown for representative mice (right panel).

D) Pancreatic lymphocytes of pCI/ppins- (group 1) and pCI/ppins-furin-immunized mice (group 2) were prepared and ex vivo restimulated with Kα/A12-21- or Kβ/B22-29-peptides. IFNγ-producing CD8+ T cells were determined by flow cytometry. The total numbers ± SD of IFNγ+ CD8+ T cells within pancreas-infiltrating CD8 T cells are shown (right panel). Primary FACS data for pCI/ppins-furin-immunized mice are shown for representative mice (left panel).
3.2. Co-inhibitory PD-L1 and co-stimulatory B7.1 signals regulate diabetes induction by ppins-specific CD8 T cells in an epitope-specific manner

3.2.1. EAD induction by K\textsuperscript{b}/A\textsubscript{12-21} and K\textsuperscript{b}/B\textsubscript{22-29}-specific CD8 T cells either depends or depends not on PD-1/PD-L1 interactions

Since the RIP-B7.1 mouse is an artificial system, I also used alternative mouse models without transgenically expressed co-stimulatory molecules for studying EAD. To analyze the influence of the PD-1/PD-L1 co-inhibition pathway on diabetes development, B6 mice were immunized with pCI/ppins or pCI/ppins\textsubscript{ΔA\textsubscript{12-21}} DNA. Afterwards one group was left untreated (pCI/ppins-immunized mice) and two other groups (pCI/ppins- and pCI/ppins\textsubscript{ΔA\textsubscript{12-21}}-immunized mice) were injected with α-PD-L1 antibodies on d 12 and d 15 after immunization. The blood glucose values were monitored during the experiment.

**Figure 13: EAD induction in B6 mice**

B6 mice were immunized with pCI/ppins (n=10) or pCI/ppins\textsubscript{ΔA\textsubscript{12-21}} (n=5) DNA. At d 12 and d 15 after immunization mice were left untreated (left panel; n=5) or injected with blocking PD-L1 antibody (middle and right panel (each n=5)) and blood glucose levels [mg/dl] were monitored during the experiment.
Results

B6 mice that received a pCl/ppins but no α-PD-L1 injection did not develop diabetes (Fig. 13 left panel). However, α-PD-L1-treatment triggered diabetes development in pCl/ppins-immunized, but not in pCl/ppinsΔA12-21-immunized B6 mice (Fig. 13 middle and right panel).

I further used PD-L1Δ−/− or PD1Δ−/− mice to confirm that EAD induction by ppins, but not by pCl/ppinsΔA12-21, depends on PD1/PD-L1 interactions. PD-1Δ−/− and PD-L1Δ−/− mice were immunized with pCl/ppins or pCl/ppinsΔA12-21. Diabetes development was monitored by measuring the blood glucose levels and diabetes incidences were determined. Interestingly, only PD-1Δ−/− and PD-L1Δ−/− mice immunized with the ppins DNA construct encoding full-length ppins developed diabetes. Disease incidences were 80% in PD-L1Δ−/− and 65% in PD-1Δ−/− mice. Neither PD-1Δ−/− nor PD-L1Δ−/− mice immunized with pCl/ppinsΔA12-21-DNA developed diabetes (Fig. 14A).

Figure 14: Characterization of EAD in PD-1Δ−/− and PD-L1Δ−/− mice
A) B6 (n=10), PD-1Δ−/− (n=10) and PD-L1Δ−/− mice (n=10) were immunized with pCl/ppins or pCl/ppinsΔA12-21 DNA. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.
B) At the indicated times after immunization pancreatic lymphocytes of B6 mice immunized with pCl/ppins (group 1), PD-1Δ−/− mice immunized with pCl/ppins (group 2), PD-L1Δ−/− mice immunized with pCl/ppins (group 3), B6 mice immunized with pCl/ppinsΔA12-21 (group 4), PD-1Δ−/− mice immunized with pCl/ppinsΔA12-21 (group 5) or PD-L1Δ−/− mice immunized with pCl/ppinsΔA12-21 (group 6) were prepared and ex vivo restimulated with Kα/βΔA12-21- or Kα/B22-29-peptides. IFNγ-producing CD8+ T cells were determined by flow cytometry. Total numbers ± SD of IFNγ+ CD8+ T cells within pancreas-infiltrating CD8 T cells are shown.
Moreover, the pancreas infiltrating cells of control B6, PD-1\(^{-/-}\) and PD-L1\(^{-/-}\) mice immunized with pCl/ppins or pCl/ppins\(\Delta A_{12-21}\) were isolated. *Ex vivo* restimulation of these cells with K\(^b/A_{12-21}\) and K\(^b/B_{22-29}\)-peptides revealed only IFN\(\gamma\)-producing CD8 T cells specific for the K\(^b/A_{12-21}\)-epitope in pCl/ppins-immunized PD-1\(^{-/-}\) and PD-L1\(^{-/-}\) mice (Fig. 14B). Thus, K\(^b/A_{12-21}\) (but not K\(^b/B_{22-29}\)) specific CD8 T cells induced EAD in immunized PD-1\(^{-/-}\) or PD-L1\(^{-/-}\) mice. For histological characterization, pancreata of PD-1\(^{-/-}\) and PD-L1\(^{-/-}\) mice immunized with pCl/ppins (Fig. 15 groups 1 and 2) or pCl/ppins\(\Delta A_{12-21}\) (Fig. 15 groups 3 and 4) were stained for insulitis.

**Figure 15: Histological characterization of EAD in PD-1\(^{-/-}\) and PD-L1\(^{-/-}\) mice**

PD-1\(^{-/-}\) and PD-L1\(^{-/-}\) mice were immunized with pCl/ppins or pCl/ppins\(\Delta A_{12-21}\) DNA. Blood glucose levels [mg/dl] were determined. Representative mice are shown. Pancreatic sections of diabetic PD-1\(^{-/-}\) mice immunized with pCl/ppins (group 1), diabetic PD-L1\(^{-/-}\) mice immunized with pCl/ppins (group 2), non-diabetic PD-1\(^{-/-}\) mice immunized with pCl/ppins\(\Delta A_{12-21}\) (group 3) and non-diabetic PD-L1\(^{-/-}\) mice immunized with pCl/ppins\(\Delta A_{12-21}\) (group 4) were analyzed for general insulitis using H+E staining and in detail using immunohistochemical staining. Insulin expression is stained in green and CD8 T cell infiltration is stained in red. An overlay is shown.
This revealed massive cell infiltrates in both diabetic PD-1\(^{−/−}\) and PD-L1\(^{−/−}\) mice, while in non-diabetic mice there was no insulitis detectable at all (Fig. 15). Furthermore, the cell types responsible for pancreas infiltration were analyzed in more detail using immunohistochemical methods. To this end, pancreatic sections were stained for insulin and CD8 T cells. CD8 T lymphocytes were present in pancreata of pCI/ppins-immunized PD-1\(^{−/−}\) and PD-L1\(^{−/−}\) mice (Fig. 15 groups 1 and 2). Sections of non-diabetic mice immunized with pCI/ppins\(_{ΔA_{12-21}}\) in contrast showed complete healthy islets and no CD8 T cells (Fig. 15 groups 3 and 4). Hence, immunization with pCI/ppins\(_{ΔA_{12-21}}\) could not induce or maintain diabetogenic CD8 T cells in PD-1\(^{−/−}\) and PD-L1\(^{−/−}\) mice.

To further characterize EAD development in PD-L1\(^{−/−}\) mice, I applied different prime/boost approaches. PD-L1\(^{−/−}\) mice were primed with pCI/ppins\(_{ΔA_{12-21}}\) and boosted with pCI/ppins DNA 3 weeks post injection. As a control, PD-L1\(^{−/−}\) mice received prime and boost injections with pCI/ppins DNA (Fig. 16A). Diabetes development was monitored by measuring the blood glucose levels and diabetes incidences were determined. Interestingly, prime immunization with pCI/ppins\(_{ΔA_{12-21}}\) reduced the diabetogenic potential of pCI/ppins in PD-L1\(^{−/−}\) mice. In contrast, prime/boost immunization using the pCI/ppins vector had no influence on diabetes development in PD-L1\(^{−/−}\) mice (Fig. 16A).

In a second approach, the effect of repeated boost immunizations on EAD development was analyzed. Therefore, mice were injected with pCI/ppins once per week for a period of four weeks. As a control, PD-L1\(^{−/−}\) mice were immunized only once with pCI/ppins. Diabetes development was followed by measuring the blood glucose levels and diabetes incidences were determined. Mice that received repeated boost immunizations with pCI/ppins did not develop diabetes, whereas mice immunized once with pCI/ppins showed a diabetes incidence of 80% (Fig. 16B). Thus, repeated booster injections prevented diabetes development in PD-L1\(^{−/−}\) mice.
Results

Figure 16: Boost immunizations affect EAD development in PD-L1⁻/⁻ mice
A) PD-L1⁻/⁻ mice received either prime and boost injections with pCI/ppins DNA or received pCI/ppinsΔA₁₂₋₂¹ for prime and pCI/ppins DNA for boost immunization. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.
B) PD-L1⁻/⁻ mice received either a single injection with pCI/ppins DNA or were repeated immunized with pCI/ppins DNA once per week for a period of four weeks. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

H+E staining was performed with pancreata of PD-L1⁻/⁻ mice immunized once with pCI/ppins or boosted with pCI/ppins to determine insulitis. Diabetic PD-L1⁻/⁻ mice immunized once with pCI/ppins (Fig. 17 group 1) had highly infiltrated islets. In contrast non-diabetic PD-L1⁻/⁻ mice four times boosted with pCI/ppins (Fig. 17 group 2) had complete healthy islets with no infiltrates detectable at all (Fig. 17). This was demonstrated by H+E staining. To specify these observations, pancreatic sections were also treated for immunohistochemical analysis. Therefore, they were stained for insulin and CD8 T lymphocytes. As expected, islets of diabetic PD-L1⁻/⁻
mice immunized once with pCI/ppins showed a massive CD8 T cell infiltration and almost no insulin was detectable any more (Fig. 17 group 1). Boost immunization with pCI/ppins instead, could protect islets in PD-L1°/− mice completely from CD8 T cell mediated autoimmune attack (Fig. 17 group 2). This showed that repeated injections with pCI/ppins DNA down-regulated priming of autoreactive CD8 T cells in PD-L1°/− mice.

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**Figure 17: Histological characterization of EAD in PD-L1°/− mice after boost immunization**

PD-L1°/− mice received either a single injection with pCI/ppins DNA (group 1) or were repeatedly immunized with pCI/ppins DNA once per week for a period of four weeks (group 2). Blood glucose levels [mg/dl] were determined. Pancreatic cryosections (representative mice are shown) were analyzed for general insulitis using H+E staining and in detail using immunohistochemical staining. Insulin expression is stained in green and CD8 T cell infiltration is stained in red. An overlay is shown.

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**3.2.2. Delivery of ppins by a recombinant adeno-associated virus prevents diabetes development in PD-L1°/− mice**

Type 1 diabetes development and the concomitant autoimmune mediated attack of pancreatic β cells results from failures in central and peripheral tolerance. I addressed the question whether experimentally altered insulin expression in non pancreatic cells and/or presentation of insulin-specific epitopes by liver-specific APCs could induce CD8 T cell tolerance. An adeno-associated virus encoding ppins
(AAV-INS wt) or ppins with specific furin recognition sites between the B/C and C/A junctions (AAV-INS-furin) was used (Fig. 18A). PD-L1⁻/⁻ mice were injected i.v. once with AAV-INS wt or AAV-INS-furin. Immunized mice did not develop EAD (Fig. 18B and C).

Moreover, groups of immunized mice were boosted 6 weeks after AAV-injection with pCI/ppins DNA. Interestingly, pre-immunization with AAVs efficiently inhibited diabetes development in mice boosted with pCI/ppins DNA (Fig. 19B and C).
Results

Diabetes incidences could be diminished from 80% (in PDL1\(^{-/-}\) control mice immunized once with pCI/ppins; Fig. 19A) to 33% (in PD-L1\(^{-/-}\) mice immunized with AAV-INS wt and pCI/ppins) (Fig. 19B), or completely deleted (in PD-L1 mice immunized with AAV-INS-furin x pCI/ppins) (Fig. 19C).

Figure 19: A ppins-encoding AAV prevents EAD in PD-L1\(^{-/-}\) mice
A) PD-L1\(^{-/-}\) mice were immunized with pCI/ppins DNA (n=5). At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.
B) PD-L1\(^{-/-}\) mice were injected with AAV-INSwt (n=5). 6 weeks after prime injection, mice were immunized with pCI/ppins DNA. At indicated time points after injection, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.
C) PD-L1\(^{-/-}\) mice were injected with AAV-INS-furin (n=5). 6 weeks after prime injection, mice were immunized with pCI/ppins DNA. At indicated time points after injection, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

Histological analyses performing H+E and immunohistochemical staining revealed that diabetic PD-L1\(^{-/-}\) mice immunized with pCI/ppins (Fig. 20 group 1), that served as a control, had massive CD8 T cell infiltrates in their pancreata. There was almost no
Results

insulin production detectable (Fig. 20 group 1). In non-diabetic PD-L1<sup>-/-</sup> mice immunized with pCI/ppins that had been pre-treated with AAV-INS wt or AAV-INS-furin, also distinct numbers of islet-infiltrating CD8 T cells could be detected. However, insulin production was almost completely preserved (Fig. 20 groups 2 and 3). Thus, pre-treatment of PD-L1<sup>-/-</sup> mice with an adeno-associated virus encoding wt ppins or ppins-furin could efficiently suppress EAD in PD-L1<sup>-/-</sup> mice immunized with pCI/ppins. This suggests that the experimentally altered insulin expression (provided by the AAV-INS) and antigen presentation by APCs might induce tolerance to ppins.

PD-L1<sup>-/-</sup> mice

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<td>(PD-L1&lt;sup&gt;-/-&lt;/sup&gt; x AAV-INS-furin + ppins) non-diabetic</td>
<td>165 mg/dl</td>
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Figure 20: Histological characterization of EAD in PD-L1<sup>-/-</sup> mice pre-treated with AAV-INS

PD-L1<sup>-/-</sup> mice were immunized with pCl/ppins DNA (group 1), PD-L1<sup>-/-</sup> mice were injected with AAV-INS<sup>wt</sup> and immunized with pCl/ppins DNA 6 weeks after prime injection (group 2) or were injected with AAV-INS-furin and immunized with pCl/ppins DNA 6 weeks after prime injection (group 3). The blood glucose values [%] were determined. Pancreatic cryosections of PD-L1<sup>-/-</sup> mice (representative mice are shown) were analyzed for general insulitis using H+E staining and in detail using immunohistochemical staining. Insulin expression is stained in green and CD8 T cell infiltration is stained in red. An overlay is shown.
3.3. The RIP-OVA diabetes model

3.3.1. Induction of Ova-specific, CD8 T cell-mediated EAD

In the following part of my work, I used Ovalbumin (Ova) as a model antigen. The main advantages for using Ova-specific EAD induction in mice, are the stable expression of Ova, the availability of Ova-encoding vectors and K\(^b\)/Ova\(_{257-264}\) (SIINFEKL)-specific tetramers, and the disposability of RIP-Ova-transgenic (expressing Ova in pancreatic \(\beta\) cells) and OT-I mice (expressing the K\(^b\)/Ova\(_{257-264}\)-TCR).

First I addressed the question whether the high affinity Ova-epitope inhibits either priming of ppins-specific CD8 T cells and EAD, and/or inhibits presentation of the ppins A\(_{12-21}\)-epitope by pancreatic \(\beta\) cells. For these purposes, a ppins-Ova vector expressing ppins and the K\(^b\)/Ova\(_{257-264}\)-epitope COOH-terminally attached was used (Fig. 21A). Expression analysis in transiently transfected Hek293 cells displayed comparable expression levels for pCl/ppins-Ova and pCl/ppins (Fig. 21A). For \textit{in vivo} characterization of pCl/ppins-Ova, RIP-Ova\(^{low}\) mice were bred with RIP-B7.1 mice resulting in mice expressing both, B7.1 and Ova in pancreatic \(\beta\) cells (RIP-B7.1/RIP-Ova\(^{low}\) mice). RIP-B7.1 or RIP-B7.1/RIP-Ova\(^{low}\) mice were immunized with pCl as a control, pCl/ppins or pCl/ppins-Ova. Diabetes development was monitored by measuring the blood glucose levels and diabetes incidences were determined. This experiment showed that pCl/ppins efficiently induced EAD in RIP-B7.1 and RIP-B7.1/RIP-Ova\(^{low}\) mice (Fig. 21B and C). However, the pCl/ppins-OVA vector efficiently induced EAD in RIP-B7.1/RIP-Ova\(^{low}\) but not in RIP-B7.1 mice (Figure 21B and C). Thus, pancreatic \(\beta\) cells were able to present both, ppins-specific and Ova-specific epitopes in RIP-B7.1/RIP-Ova\(^{low}\) mice, but Ova-specific immunodominance mechanisms may inhibit co-priming of ppins-specific CD8 T cells in pCl/ppins-Ova-immunized RIP-B7.1 mice.
Results

A) The map of pCI/ppins-Ova is shown. It contains the signal peptide (SP), B-, C- and A-chain of ppins containing the K\(^{b}/B_{22-29}\) and the K\(^{b}/A_{12-21}\) epitopes and the COOH-terminally-fused K\(^{b}/Ova_{257-264}\)-epitope sequence.

Hek293 cells were transiently transfected with pCI/ppins (lane 1) or pCI/ppins-Ova (lane 2). Cell lysates were processed for SDS-PAGE followed by western blot analysis using an H-86-\(\alpha\)-insulin antibody. The position of the fusion protein encoded by pCI/ppins-Ova is indicated.

B) RIP-B7.1 mice were either immunized with pCI (n=5), pCI/ppins (n=5) or pCI/ppins-Ova (n=5) DNA. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

C) RIP-B7.1/RIP-Ova\(^{low}\) mice were either immunized with pCI (n=5), pCI/ppins (n=5) or pCI/ppins-Ova (n=5) DNA. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

I further analyzed the kinetics of pCI/ppins-Ova-induced EAD. B6, RIP-Ova\(^{low}\) and RIP-B7.1/RIP-Ova\(^{low}\) mice were immunized with pCI/ppins-Ova, the blood glucose levels and diabetes incidences were determined. While 100% of RIP-B7.1/RIP-Ova\(^{low}\) mice developed diabetes until 8 weeks after immunization, RIP-Ova\(^{low}\) mice did not develop diabetes (Fig. 22A).
Results

Analyzing the splenocytes of pCI/ppins-Ova-immunized mice using the K\(^b\)/Ova\(_{257-264}\) tetramer revealed that maximal CD8 T cell numbers could be detected 2 weeks after immunization. B6 mice had higher Ova-specific CD8 T cell numbers in the spleen than both RIP-Ova-transgenic mice (Fig. 22B). In contrast, immunization of RIP-Ova\(_{low}\) and RIP-B7.1/RIP-Ova\(_{low}\) (but not B6) mice resulted in an influx of K\(^b\)/Ova\(_{257-264}\)-tetramer\(^+\) CD8\(^+\) T cells into the pancreatic target tissue (Fig. 22C).

Figure 22: Kinetic analyses of K\(^b\)/Ova\(_{257-264}\)-specific diabetes development in RIP-B7.1/RIP-Ova\(_{low}\) mice

A) B6 (n=5), RIP-Ova\(_{low}\) (n=5) or RIP-B7.1/RIP-Ova\(_{low}\) mice (n=5) were immunized with pCI/ppins-Ova DNA. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

B) At the indicated times after immunization splenocytes were prepared and directly stained with K\(^b\)/Ova\(_{257-264}\)-tetramers. Specific CD8 T cells were identified by flow cytometry. The percentages ± SD of K\(^b\)/Ova\(_{257-264}\)-tetramer\(^+\) CD8\(^+\) T cells within splenocytes at indicated times after immunization are shown.

C) At the indicated times after immunization pancreatic lymphocytes were prepared and directly stained with K\(^b\)/Ova\(_{257-264}\)-tetramers. Specific CD8 T cells were identified by flow cytometry. The total numbers ± SD of K\(^b\)/Ova\(_{257-264}\)-tetramer\(^+\) CD8\(^+\) T cells within pancreas-infiltrating CD8 T cells at indicated times after immunization are shown.
Ova-specific CD8 T cell frequencies were significantly higher in RIP-B7.1/RIP-Ova\textsuperscript{low} mice compared to RIP-Ova\textsuperscript{low} mice (Fig. 22C). K\textsuperscript{b}/Ova\textsubscript{257-264}tetramer\textsuperscript{+} CD8\textsuperscript{+} T cell numbers peaked 2 weeks post immunization and then declined (Fig. 22C). Hence, immunization with pCI/ppins-Ova could prime Ova-specific CD8 T cells in B6, RIP-Ova\textsuperscript{low} and RIP-B7.1/RIP-Ova\textsuperscript{low} mice. High K\textsuperscript{b}/Ova\textsubscript{257-264}tetramer\textsuperscript{+} CD8\textsuperscript{+} T cell numbers accumulated in the pancreata of RIP-B7.1/RIP-Ova\textsuperscript{low} mice and efficiently induced EAD (Fig. 22A).

To further characterize Ova-specific CD8 T cell-mediated EAD, I next performed immunization experiments using the pCI/Ova vector (encoding the wt Ova antigen) (Fig. 23A). The pCI/Ova vector failed to induce EAD in RIP-Ova\textsuperscript{low} mice (Fig. 23B and C).

**Figure 23:** Vaccination approaches with an Ova-encoding DNA construct in RIP-Ova\textsuperscript{low} mice

A) The map of pCI/Ova is shown. It encodes Ovalbumin containing the K\textsuperscript{b}/Ova\textsubscript{257-264} (SIINFEKL) epitope sequence.

B) B6 (n=5) and RIP-Ova\textsuperscript{low} (n=5) mice were immunized with pCI/Ova DNA. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

C) The blood glucose values [mg/dl] were followed during the experiment. The progress of the blood glucose levels of RIP-Ova\textsuperscript{low} mice (n=5) immunized with pCI/Ova are shown.
Immunization of B6 and RIP-Ova\textsuperscript{low} mice with pCI/Ova DNA efficiently primed K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells in the spleen (Fig. 24A), but Ova-specific CD8 T cells were not detectable in the pancreata of B6 mice. Very low levels of K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells were detectable in the pancreata of healthy RIP-Ova\textsuperscript{low} mice (Fig. 24B). This finding was confirmed by immunohistology as only few T cell infiltrates were found in the pancreata of pCI/Ova-immunized RIP-Ova\textsuperscript{low} mice (Fig. 25). Thus, selective Ova-specific CD8 T cells primed in RIP-Ova\textsuperscript{low} mice were not able to attack Ova-expressing β cells and therefore did not induce EAD.

**Figure 24: Kinetic analyses of K\textsuperscript{b}/Ova\textsubscript{257-264}-specific CD8 T cells primed in RIP-Ova\textsuperscript{low} mice**

B6 (n=5) and RIP-Ova\textsuperscript{low} (n=5) mice were immunized with pCI/Ova DNA.

A) At the indicated times after immunization splenocytes were prepared and directly stained with K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramers. Specific CD8 T cells were identified by flow cytometry. The percentages ± SD of K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells within splenocytes at indicated times after immunization are shown.

B) At the indicated times after immunization pancreatic lymphocytes were prepared and directly stained with K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramers. Specific CD8 T cells were identified by flow cytometry. The total numbers ± SD of K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells within pancreas-infiltrating CD8 T cells at indicated times after immunization are shown.
Results

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**Figure 25: Histological analyses of RIP-Ova\textsuperscript{low} mice immunized with pCI/Ova**

RIP-Ova\textsuperscript{low} mice were immunized with pCI/Ova DNA and analyzed at d 14 (group 1 healthy), d 21 (group 2 pre-diabetic) and d 42 (group 3 diabetic) after immunization. Blood glucose levels were determined [mg/dl]. At the indicated time points (representative mice are shown), pancreatic sections were analyzed for general insulitis using H+E staining and in detail using immunohistochemical staining. Insulin expression is stained in green and CD8 T cell infiltration is stained in red. An overlay is shown.

However, co-expression of B7.1 and Ova in pancreatic β cells (RIP-B7.1/RIP-Ova\textsuperscript{low} mice) facilitated induction of EAD using pCI/Ova-based immunization. A severe EAD (blood glucose levels ≥500 mg/dl) developed in these mice within 7 weeks after immunization (Fig. 26A and B). K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells were found in the spleens and pancreata of RIP-B7.1/RIP-Ova\textsuperscript{low} mice (Fig. 26C and 27). When mice developed severe diabetes (i.e. about 6 weeks after immunization), pancreas infiltrating K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells were hardly detectable (Fig. 27).
Results

Figure 26: $K^b$/Ova257-264- specific CD8 T cell priming and EAD in RIP-B7.1/RIP-Ova\textsuperscript{low} mice

A) RIP-B7.1/RIP-Ova\textsuperscript{low} (n=5) mice were immunized with pCI/Ova DNA. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

B) The blood glucose values [mg/dl] were followed during the experiment. The progress of the blood glucose levels of RIP-B7.1/RIP-Ova\textsuperscript{low} mice (n=5) immunized with pCI/Ova are shown.

C) At the indicated times after immunization splenocytes were prepared and directly stained with $K^b$/Ova257-264- tetramers. Specific CD8 T cells were identified by flow cytometry. The percentages ± SD of $K^b$/Ova257-264- tetramer$^+$ CD8$^+$ T cells within splenocytes at indicated times after immunization are shown.
Results

Pancreatic lymphocytes of RIP-B7.1/RIP-Ova\textsuperscript{low} mice immunized with pCI/Ova

![Graph showing kinetics of K\textsuperscript{b}/Ova\textsuperscript{257-264} specific EAD in RIP-B7.1/RIP-Ova\textsuperscript{low} mice.](image)

**Figure 27: Kinetics of K\textsuperscript{b}/Ova\textsuperscript{257-264} specific EAD in RIP-B7.1/RIP-Ova\textsuperscript{low} mice**

At the indicated times after immunization pancreatic lymphocytes were prepared and directly stained with K\textsuperscript{b}/Ova\textsuperscript{257-264}-tetramers. Specific CD8 T cells were identified by flow cytometry. The total numbers ± SD of K\textsuperscript{b}/Ova\textsuperscript{257-264}-tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells within pancreas-infiltrating CD8 T cells at indicated times after immunization are shown (left panel). Primary FACS data of pCI/Ova-immunized mice are shown for representative mice (right panel).

Histological analyses confirmed a massive influx of CD8 T cells into the islets of immunized RIP-B7.1/RIP-Ova\textsuperscript{low} mice that correlated with a significantly decreased insulin production and EAD development (Fig. 28).

Hence, the co-stimulatory molecule B7.1 enabled expansion of Ova-specific CD8 T cells in the pancreas, thereby mediating autoimmune destruction of the pancreatic β cells. This is in accordance with the results that were achieved for studying ppins-specific EAD, as B7.1 expression in pancreatic β cells can efficiently trigger EAD after ppins-DNA-based immunization.
Results

RIP-B7.1/RIP-Ovalo\textsuperscript{low} mice immunized with pCI/Ova

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Figure 28: Histological characterization of K\textsuperscript{b}/Ova\textsubscript{257-264}-specific EAD in RIP-B7.1/RIP-Ovalo\textsuperscript{low} mice

RIP-B7.1/RIP-Ovalo\textsuperscript{low} mice were immunized with pCI/Ova DNA and analyzed at d 14 (group 1 healthy), d 21 (group 2 pre-diabetic) and d 42 (group 3 diabetic) after immunization. Blood glucose levels were determined [mg/dl]. At the indicated time points (representative mice are shown), pancreatic sections were analyzed for general insulitis using H+E staining and in detail using immunohistochemical staining. Insulin expression is stained in green and CD8 T cell infiltration is stained in red. An overlay is shown.

To define the influence of the PD-1/PD-L1 signaling on CD8 T cell-mediated, OVA-specific EAD, PDL1\textsuperscript{-/-}/RIP-Ovalo\textsuperscript{low} mice were bred by crossing RIP-Ovalo\textsuperscript{low} mice with PDL1\textsuperscript{-/-} mice. PDL1\textsuperscript{-/-}/RIP-Ovalo\textsuperscript{low} mice were immunized with pCI/Ova and diabetes development was monitored by measuring the blood glucose levels. Immunization with pCI/Ova did not cause diabetes in PDL1\textsuperscript{-/-}/RIP-Ovalo\textsuperscript{low} mice (Fig. 29A), although some mice displayed fluctuating blood glucose values that sometimes exceeded the normoglycemic level (i.e., \( \leq 250 \text{ mg/dl} \)) (Fig. 29B), indicating that there is a limited cross-talk between diabetogenic, Ova-specific CD8 T cells and the PD-L1-deficient Ova-expressing \( \beta \) cells. Comparable with the studies in RIP-B7.1/RIP-Ovalo\textsuperscript{low} mice, I detected significant numbers of K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramer\textsuperscript{*} CD8\textsuperscript{*} T cells in the spleens and also the pancreata of pCI/Ova-immunized PDL1\textsuperscript{-/-}/RIP-Ovalo\textsuperscript{low} mice (Fig. 29C and D).
Results

Figure 29: The influence of PD-1/PD-L1 signaling on K^b/Ova_{257-264}-specific EAD

RIP-Ova_{low} (n=5) and PD-L1^{−/−}/RIP-Ova_{low} (n=5) mice were immunized with pCI/Ova. A) At indicated time points after immunization cumulative diabetes incidences [%] were determined. B) The blood glucose values [mg/dl] were followed during the experiment. The progress of the blood glucose levels of PD-L1^{−/−}/RIP-Ova_{low} mice (n=5) immunized with pCI/Ova are shown. C) At the indicated times after immunization splenocytes were prepared and directly stained with K^b/Ova_{257-264}-tetramers. Specific CD8 T cells were identified by flow cytometry. The mean percentages ± SD of K^b/Ova_{257-264}−tetramer^{+} CD8^{+} T cells in the spleen at indicated times after immunization are shown. D) At the indicated times after immunization pancreatic lymphocytes were prepared and directly stained with K^b/Ova_{257-264}−tetramers. Specific CD8 T cells were identified by flow cytometry. The total numbers ± SD of K^b/Ova_{257-264}−tetramer^{+} CD8^{+} T cells within pancreas-infiltrating CD8 T cells at indicated times after immunization are shown.

However, the influx of specific and bystander CD8 T cells in the pancreata was only moderate in pCI/Ova-immunized PDL1^{−/−}/RIP-Ova_{low} mice (Fig. 30). This indicates that, although significant K^b/Ova_{257-264}-specific CD8 T cells reached the pancreata of PD-L1^{−/−}/RIP-Ova_{low} mice, they were not able to destroy the pancreatic β cells and therefore mice did not develop autoimmune diabetes.
### Results

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#### Figure 30: Histological analyses of PD-L1\(^{-/}\)/RIP-Ova\(^{low}\) mice immunized with pCl/Ova

PD-L1\(^{-/}\)/RIP-Ova\(^{low}\) mice were immunized with pCl/Ova DNA and analyzed at d 14 (group 1 healthy), d 21 (group 2 pre-diabetic) and d 42 (group 3 diabetic) after immunization. Blood glucose levels [mg/dl] were determined. At the indicated time points (representative mice are shown), pancreatic sections were analyzed for general insulitis using H+E staining and in detail using immunohistochemical staining. Insulin expression is stained in green and CD8 T cell infiltration is stained in red. An overlay is shown.

#### 3.3.2. Characterization of Ova-specific CD8 T cell activation using the OT-I-based adoptive transfer system

I next studied the specific cross-talk between Ova-expressing \(\beta\) cells and K\(^{b}\)/Ova\(_{257-264}\)-specific CD8 T cells in vitro. The splenocytes of OT-I mice were isolated, the K\(^{b}\)/Ova\(_{257-264}\)-specific CD8 T cells purified by MACS and cells activated with the K\(^{b}\)/Ova\(_{257-264}\) peptide for 3 days. Pancreatic cells of B6 mice (expressing no Ova in pancreatic \(\beta\) cells) or RIP-Ova\(^{low}\) mice (expressing Ova in pancreatic \(\beta\) cells) were isolated, purified and either not treated or loaded with the K\(^{b}\)/Ova\(_{257-264}\) peptide for 2 h. Thereafter, cells were co-cultured with the activated OT-I cells for 24 h. The cell
culture supernatants were collected and IFNγ was detected by ELISA. As expected, B6-derived pancreatic cells presented the Kb/Ova257-264-epitope to OT-I cells only after pre-treatment with the Ova-peptide (Fig. 31 group 1 and 2). In contrast, RIP-Ova\textsuperscript{low}-derived pancreatic cells directly stimulated OT-I cells to produce IFNγ (Fig. 31 group 3). Stimulation of these pancreatic cells with the Ova-peptide significantly increased the IFNγ-production of OT-I cells (Fig. 31 group 4). Hence, the Kb/Ova257-264-epitope could be presented by Ova-expressing β cells of RIP-Ova\textsuperscript{low} mice.

**Coculture: OT-I cells with pancreatic cells**

![Coculture](image)

Pancreatic cells:
1: B6
2: B6 pulsed with Kb/Ova257-264
3: RIP-Ova\textsuperscript{low}
4: RIP-Ova\textsuperscript{low} pulsed with Kb/Ova257-264

**Figure 31: In vitro stimulation capability of Ova-expressing pancreatic cells**

Splenocytes of OT-I mice were isolated and pulsed with Kb/Ova257-264 peptide for 36 h. Pancreatic cells of B6 and RIP-Ova\textsuperscript{low} mice were isolated and either left untreated or pulsed with Kb/Ova257-264 peptide. OT-I blast cells were cocultured with either not pre-stimulated B6 (group 1), not pre-stimulated RIP-Ova\textsuperscript{low} (group 3), pre-stimulated B6 (group 2) or pre-stimulated RIP-Ova\textsuperscript{low} (group 4) pancreatic cells. After 1 d IFNγ production [ng/ml] was measured by ELISA.

To analyze whether the pathogenic cross-talk between autoreactive CD8 T cells and pancreatic β cells depends on PD-1/PD-L1 or B7.1-signaling in vivo, I employed different OT-I mouse strains as donor effector T cells for adoptive transfer experiments: PD-1-deficient (PD-1\textsuperscript{-/-}/OT-I mice), PD-L1-deficient (PD-L1\textsuperscript{-/-}/OT-I mice) or B7.1-deficient OT-I mice (B7.1\textsuperscript{-/-}/OT-I mice). Splenocytes of these mice were
prepared, CD8 T cells isolated by MACS purification and $3 \times 10^6$ CD8 T cells were transferred into RIP-Ova$^{\text{low}}$ hosts by i.v. injection. None of the different OT-I-specific CD8 T cell types was able to induce diabetes in RIP-Ova$^{\text{low}}$ mice (Fig. 32A and B), and no CD8 T cells were detectable in pancreatic sections 7 weeks after adoptive transfer (Fig. 32C). Hence, Ova-specific CD8 T cells can not directly interact with the Ova-expressing β cells to trigger EAD.

Figure 32: Adoptive transfer of OT-I cells into RIP-Ova$^{\text{low}}$-transgenic hosts
A) Splenocytes of OT-I, PD-1$^{-/-}$/OT-I, PD-L1$^{-/-}$/OT-I or B7.1$^{-/-}$/OT-I mice were isolated and purified by MACS. $3 \times 10^6$ OT-I cells (CD8$^+$ T cells) were adoptively transferred i.v. into RIP-Ova$^{\text{low}}$ (n=5 for each OT-I cell type) mice and cumulative diabetes incidences were determined.
B) The blood glucose values [mg/dl] were followed during the experiment. The progress of the blood glucose levels of RIP-Ova$^{\text{low}}$ mice (n=5) after adoptive transfer of OT-I cells is shown.
C) Histological characterization of RIP-Ova$^{\text{low}}$ mice 7 w after adoptive transfer of OT-I cells. Blood glucose levels [mg/dl] were determined. Pancreatic sections were analyzed for general insulitis using H+E staining and in detail using immunohistochemical staining. Insulin expression is stained in green and CD8 T cell infiltration is stained in red. An overlay is shown.
In contrast, adoptive transfer of OT-I-, PD-1⁻/⁻/OT-I-, PD-L1⁻/⁻/OT-I- or B7.1⁻/⁻/OT-I-derived CD8 T cells into RIP-B7.1/RIP-Ova<sub>low</sub> hosts (expressing the co-stimulatory molecule B7.1 and Ova in pancreatic β cells) efficiently triggered EAD (Fig. 33A). Following the blood glucose levels during the experiment showed that RIP-B7.1/RIP-Ova<sub>low</sub> mice reached high values around 7 weeks after adoptively transferred CD8 T cells isolated from OT-I mice (Fig. 33B). Histological analyses validated the correlation of a massive CD8 T cell infiltration with insulin deficiency (Fig. 33C).

**Figure 33: Adoptive transfer of OT-I cells into RIP-B7.1/RIP-Ova<sub>low</sub> hosts**

A) Splenocytes of OT-I, PD-1⁻/⁻/OT-I, PD-L1⁻/⁻/OT-I or B7.1⁻/⁻/OT-I mice were isolated and purified by MACS. 3 x 10⁶ OT-I cells (CD8⁺ T cells) were adoptively transferred i.v. into RIP-B7.1/RIP-Ova<sub>low</sub> (n=5 for each OT-I cell type) mice and cumulative diabetes incidences were determined.

B) The blood glucose values [mg/dl] were followed during the experiment. The progress of the blood glucose levels of RIP-B7.1/RIP-Ova<sub>low</sub> mice (n=5) after adoptive transfer of OT-I cells is shown.

C) Histological characterization of RIP-B7.1/RIP-Ova<sub>low</sub> mice 7 w after adoptive transfer of OT-I cells. Blood glucose levels [mg/dl] were determined. Pancreatic sections were analyzed for general insulitis using H+E staining and in detail using immunohistochemical staining. Insulin expression is stained in green and CD8 T cell infiltration is stained in red. An overlay is shown.
Moreover, pancreatic lymphocytes of RIP-B7.1/RIP-Ova\textsuperscript{low} mice were analyzed for Ova-specific CD8 T cell infiltrates at 4, 5 and 7 weeks after transfer of OT-I cells. The number of adoptively transferred K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells increased until 5 weeks post transfer and then declined (Fig. 34). Thus, co-expression of the co-stimulatory molecule B7.1 and Ova in pancreatic \(\beta\) cells is sufficient to expand and/or maintain diabetogenic OT-I T cell responses. However, it takes a long time (around 7 weeks) to induce EAD.

![Figure 34: Kinetic analyses of K\textsuperscript{b}/Ova\textsubscript{257-264}-specific EAD using adoptive transfer of OT-I cells into RIP-B7.1/RIP-Ova\textsuperscript{low} mice

Splenocytes of OT-I mice were isolated and purified by MACS. 3 x 10\textsuperscript{6} OT-I cells (CD8\textsuperscript{+} T cells) were adoptively transferred i.v. into RIP-Ova\textsuperscript{low} (n=5) or RIP-B7.1/RIP-Ova\textsuperscript{low} (n=5) mice. At the indicated times after transfer pancreatic were prepared and directly stained with K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramers. Specific CD8 T cells were identified by flow cytometry. The total numbers ± SD of K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells within pancreas-infiltrating CD8 T cells at indicated times after transfer are shown (left panel). Primary FACS data of RIP-B7.1/RIP-Ova\textsuperscript{low} mice after adoptive transfer of OT-I cells are shown for representative mice (right panel).

Furthermore, I analyzed the influence of the PD-1/PD-L1 signaling on the activation of Ova-specific CD8 T cells in the RIP-Ova diabetes model. For this purpose, OT-I-, PD-1\textsuperscript{-/-}/OT-I- or B7.1\textsuperscript{-/-}/OT-I- derived CD8 T cells were adoptively transferred into PDL1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low} hosts. Although none of these CD8 T cells could induce diabetes (Fig. 35A), PDL1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low} mice showed fluctuating blood glucose levels that sometimes exceeded the pre-diabetic state (i.e. \(\geq 250\) mg/dl) after adoptive transfer of OT-I cells (Fig. 35B). Islet-infiltrating CD8 T cells were not detectable in these
Results

PDL1⁻/⁻/RIP-Ova<sub>low</sub> hosts (Fig. 35C). Hence, altering the PD-1/PD-L1 signaling could not efficiently activate and expand antigen-specific CD8 T cells and did not induce diabetes in the RIP-Ova diabetes model.

Figure 35: The influence of PD-L1 signaling on OT-I based adoptive transfer into RIP-Ova<sub>low</sub>-transgenic hosts
A) Splenocytes of OT-I, PD-1⁻/⁻/OT-I or B7.1⁻/⁻/OT-I mice were isolated and purified by MACS. 3 x 10⁶ OT-I-specific CD8 T cells were adoptively transferred i.v. into PD-L1⁻/⁻/RIP-Ova<sub>low</sub> mice and cumulative diabetes incidences were determined.
B) The blood glucose values [mg/dl] were followed during the experiment. The progress of the blood glucose levels of PD-L1⁻/⁻/RIP-Ova<sub>low</sub> mice (n=5) after adoptive transfer of OT-I cells is shown.
C) Histological characterization of PD-L1⁻/⁻/RIP-Ova<sub>low</sub> mice 7 w after adoptive transfer of OT-I cells. Blood glucose levels [mg/dl] were determined. Pancreatic sections were analyzed for general insulitis using H+E staining and in detail using immunohistochemical staining. Insulin expression is stained in green and CD8 T cell infiltration is stained in red. An overlay is shown.

Table 10 shows an overview of the possible host-donor combinations that were tested and monitored for diabetes development.
Table 10: Host-donor combinations for adoptive transfer

<table>
<thead>
<tr>
<th>Donor</th>
<th>Host</th>
<th>Diabetes incidence</th>
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<tbody>
<tr>
<td>OT-I</td>
<td>B6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RIP-Ova\textsuperscript{low}</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RIP-B7.1/RIP-Ova\textsuperscript{low}</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PD-L1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low}</td>
<td>-</td>
</tr>
<tr>
<td>PD-1\textsuperscript{-/-}/OT-I</td>
<td>B6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RIP-Ova\textsuperscript{low}</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RIP-B7.1/RIP-Ova\textsuperscript{low}</td>
<td>+</td>
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<tr>
<td></td>
<td>PD-L1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low}</td>
<td>-</td>
</tr>
<tr>
<td>PD-L1\textsuperscript{-/-}/OT-I</td>
<td>B6</td>
<td>-</td>
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<td></td>
<td>RIP-Ova\textsuperscript{low}</td>
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<td></td>
<td>RIP-B7.1/RIP-Ova\textsuperscript{low}</td>
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<tr>
<td>B7.1\textsuperscript{-/-}/OT-I</td>
<td>B6</td>
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<td>RIP-Ova\textsuperscript{low}</td>
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<td></td>
<td>RIP-B7.1/RIP-Ova\textsuperscript{low}</td>
<td>+</td>
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<tr>
<td></td>
<td>PD-L1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low}</td>
<td>-</td>
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</table>

Summarized, only expression of the co-stimulatory molecule B7.1 in pancreatic β cells can efficiently stimulate Ova-specific CD8 T cells. Even the missing co-inhibitory signal on CD8 T cells (PD-1\textsuperscript{-/-}/OT-I) and/or on pancreatic β cells (PD-L1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low}) did not trigger diabetes development. These results indicate that the antigen presentation by Ova-expressing β cells in RIP-Ova\textsuperscript{low} mice is too weak for direct CD8 T cell activation. As a consequence, antigen-specific CD8 T cells do not expand and cannot display an autoreactive destructive character. I asked whether additionally Ova-epitope presentation by professional APCs could stimulate adoptively transferred CD8 T cells and trigger EAD. I used the well-established OT-I transfer model combined with a CD8 T cell stimulating Ova vaccine, composed of Ovalbumin and the ISCOM-derived adjuvant (AbISCO). CD8 T cells isolated from OT-I mice were adoptively transferred into RIP-Ova\textsuperscript{low} and PD-L1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low} mice. 1 day after adoptive transfer, mice were immunized i.m. with
Ova/AbISCO. All mice rapidly developed EAD within 5-10 days after adoptive transfer (Fig. 36A and B). Moreover, pancreatic infiltrates of diabetic RIP-Ova\textsuperscript{low} and diabetic PDL1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low} mice were analyzed at d 12 after transfer. Pancreas infiltrating cells were prepared, stained for CD8\textsuperscript{+} K\textsuperscript{b}/Ova\textsuperscript{257-264}+ tetramer\textsuperscript{+} cells and analyzed by flow cytometry. This revealed comparable high numbers of Ova-specific CD8 T cells in both, RIP-Ova\textsuperscript{low} and PDL1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low} mice (Fig. 36C).

Notably, several control experiments (i.e., immunization of mice with Ova/AbISCO without OT-I transfer or adoptive transfer followed by immunization with either Ova or AbISCO) did not induce EAD (Fig. 37). Thus, co-delivery of the Ova/AbISCO vaccine was critical for MHC class I presentation and subsequent CD8 T cell activation.
Results

Figure 36: Requirements for diabetes induction in RIP-Ova\textsubscript{low} and PD-L1\textsuperscript{-/-}/RIP-Ova\textsubscript{low} mice using adoptive transfer experiments
A) Splenocytes of OT-I mice were isolated and purified by MACS. 3 x 10\textsuperscript{6} OT-I-specific CD8 T cells were adoptively transferred i.v. into RIP-Ova\textsubscript{low} (n=5) or PD-L1\textsuperscript{-/-}/RIP-Ova\textsubscript{low} mice (n=5). 1 day after transfer mice were immunized with a combination of Ovalbumin protein and the adjuvant AbiSCO (Ova/AbiSCO). At the indicated time points after adoptive transfer cumulative diabetes incidences were determined.
B) The blood glucose values [mg/dl] were followed during the experiment. The progress of the blood glucose levels of RIP-Ova\textsubscript{low} and PD-L1\textsuperscript{-/-}/RIP-Ova\textsubscript{low} mice after adoptive transfer of OT-I cells combined with Ova/AbiSCO immunization is shown for representative mice.
C) On day 12 after transfer pancreatic lymphocytes of RIP-Ova\textsubscript{low} (group 1) or PD-L1\textsuperscript{-/-}/RIP-Ova\textsubscript{low} mice (group 2) were prepared and directly stained with K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramers. Specific CD8 T cells were identified by flow cytometry. The total numbers ± SD of K\textsuperscript{b}/Ova\textsubscript{257-264}-tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells within pancreatic lymphocytes are shown (left panel). Primary FACS data of RIP-Ova\textsubscript{low} or PD-L1\textsuperscript{-/-}/RIP-Ova\textsubscript{low} mice after adoptive transfer of OT-I cells plus immunization with Ova/AbiSCO are shown for representative mice (right panel).
Results

Figure 37: Diabetes induction in RIP-Ova\textsuperscript{low} and PD-L1\textsuperscript{−/−}/RIP-Ova\textsuperscript{low} mice after adoptive transfer of OT-I cells depends on the combined protein/adjuvant immunization

A) RIP-Ova\textsuperscript{low} (n=5) or PD-L1\textsuperscript{−/−}/RIP-Ova\textsuperscript{low} mice (n=5) were immunized with Ova/AbISCO. At indicated time points after immunization, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

B) Splenocytes of OT-I mice were isolated and purified by MACS. $3 \times 10^6$ OT-I-specific CD8 T cells were adoptively transferred i.v. into RIP-Ova\textsuperscript{low} (n=5) or PD-L1\textsuperscript{−/−}/RIP-Ova\textsuperscript{low} (n=5) mice. 1 day after transfer mice were immunized with Ova protein (20 µg). At indicated time points after adoptive transfer, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

C) Splenocytes of OT-I mice were isolated and purified by MACS. $3 \times 10^6$ OT-I-specific CD8 T cells were adoptively transferred i.v. into RIP-Ova\textsuperscript{low} (n=5) or PD-L1\textsuperscript{−/−}/RIP-Ova\textsuperscript{low} (n=5) mice. 1 day after transfer mice were immunized with AbISCO. At indicated time points after adoptive transfer, blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

These findings showed that additional K\textsuperscript{b}/Ova\textsuperscript{257-264}-presenting APCs are necessary for activation and/or maintenance of adoptively transferred Ova-specific CD8 T cells in RIP-Ova\textsuperscript{low} and PD-L1\textsuperscript{−/−}/RIP-Ova\textsuperscript{low} mice.
In order to perform proliferation studies of adoptively transferred OT-I cells *in vivo*, I bred double transgenic OT-I mice that express a red fluorescent protein under control of the actin promoter: B6.Cg-Tg(CAg) mice with OT-I mice (OT-I/red).

**Figure 38: Characterization of OT-I cell proliferation in RIP-Ova-transgenic mice after adoptive transfer**
Splenocytes of B6.Cg-Tg(CAg)/OT-I mice (expressing a red fluorescent protein under control of the actin promoter) were isolated and CD8 T cells purified by MACS. Cells were labeled with CFSE and 3 x 10^6 OT-I-specific CFSE-labeled CD8 T cells were adoptively transferred i.v. into RIP-Ova\textsuperscript{low} (n=4) or PD-L1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low} (n=4) mice. On d 2, d 4, d 6 and d 8 after adoptive transfer lymphocytes of the pancreatic lymph nodes were isolated and directly analyzed by flow cytometry. Cell proliferation is displayed as decreasing CFSE-specific fluorescence gated on red CD8 T cells. Primary FACS data of RIP-Ova\textsuperscript{low} and PD-L1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low} mice at the indicated time points after adoptive transfer are shown.

OT-I/red-derived CD8 T cells were additionally labeled with CFSE and adoptively transferred into control RIP-Ova\textsuperscript{low} or PDL1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low} mice. 1 day after adoptive transfer, one group was left untreated, while the other was immunized with Ova/AbISCO. Cell proliferation of Ova-specific CD8 T cells was monitored.
Pancreatic lymph nodes were prepared at d 2, d 4, d 6 and d 8 after adoptive transfer and CFSE\(^+\) OT-I/red\(^+\) CD8 T cells were directly analyzed using flow cytometry. RIP-Ova\(^{low}\) mice that were left untreated after adoptive transfer had only very few OT-I/red\(^+\) CD8 T cells in the pLNs and no cell proliferation was detectable (Fig. 38 upper panel). In contrast, OT-I/red CD8 T cell numbers significantly increased in PDL1\(^{-/-}\)/RIP-Ova\(^{low}\) mice and cells showed an increased proliferation in pLNs at 6-8 days after transfer (Fig. 38 lower panel). Remarkably, adoptively transferred OT-I/red CD8 T cells did not invade the pancreatic tissue in both, RIP-Ova\(^{low}\) and PDL1\(^{-/-}\)/RIP-Ova\(^{low}\) mice (Fig. 39).

**Figure 39: Characterization of the pancreas infiltration in RIP-Ova-transgenic mice after adoptive transfer**

Splenocytes of B6.Cg-Tg(CAg)/OT-I mice (expressing a red fluorescent protein under control of the actin promoter) were isolated and CD8 T cells purified by MACS. 3 x 10\(^6\) OT-I-specific red CD8 T cells were adoptively transferred i.v. into RIP-Ova\(^{low}\) (n=4) or PD-L1\(^{-/-}\)/RIP-Ova\(^{low}\) (n=4) mice. On d 2, d 4, d 6 and d 8 after adoptive transfer the pancreas infiltrating lymphocytes were isolated and directly analyzed by flow cytometry. Primary FACS data of RIP-Ova\(^{low}\) and PD-L1\(^{-/-}\)/RIP-Ova\(^{low}\) mice at the indicated time points after adoptive transfer are shown.
In contrast, mice that received Ova/AbISCO after adoptive transfer, showed a prominent accumulation of OT-I/red CD8 T cells in pLNs as well as in the pancreatic tissue (Figs. 40 and 41).

![Pancreatic lymph node](image)

**Figure 40: Characterization of OT-I-specific cell proliferation in RIP-Ova-transgenic mice after adoptive transfer and Ova/AbISCO immunization**

Splenocytes of B6.Cg-Tg(CAg)/OT-I mice (expressing a red fluorescent protein under control of the actin promoter) were isolated and CD8 T cells purified by MACS. Cells were labeled with CFSE and $3 \times 10^6$ OT-I-specific CFSE-labeled CD8 T cells were adoptively transferred i.v. into RIP-Ova$^{low}$ (n=4) or PD-L1$^{-/-}$/RIP-Ova$^{low}$ (n=4) mice. 1 day after adoptive transfer mice were immunized with Ova/AbISCO. On d 2, d 4, d 6 and d 8 after adoptive transfer lymphocytes of the pancreatic lymph nodes were isolated and directly analyzed by flow cytometry. Cell proliferation is displayed as decreasing CFSE-specific fluorescence gated on red CD8 T cells. Primary FACS data of RIP-Ova$^{low}$ and PD-L1$^{-/-}$/RIP-Ova$^{low}$ mice at the indicated time points after adoptive transfer are shown.

A very rapid cell proliferation was detectable at d 4 post transfer (Fig. 40). Pancreas infiltrating OT-I/red CD8 T cells declined between d 6 and d 8 after transfer (Fig. 41).
Thus, it could be demonstrated, that without additional treatment, OT-I/red CD8 T cells proliferated in pLNs of PDL1^{-/-}/RIP-Ova^{low}, but not of RIP-Ova^{low} mice (Fig. 38 lower panel). Proliferation and influx of OT-I cells into the pancreatic tissue is facilitated by Ova/AbISCO immunization (Figs. 40 and 41).

**Figure 41: Characterization of the pancreas infiltration in RIP-Ova-transgenic mice after adoptive transfer and Ova/AbISCO immunization**

Splenocytes of B6.Cg-Tg(CAg)/OT-I mice (expressing a red fluorescent protein under control of the actin promoter) were isolated and CD8 T cells purified by MACS. 3 x 10^{6} OT-I-specific red CD8 T cells were adoptively transferred i.v. into RIP-Ova^{low} (n=4) or PD-L1^{-/-}/RIP-Ova^{low} (n=4) mice. 1 day after adoptive transfer mice were immunized with Ova/AbISCO. On d 2, d 4, d 6 and d 8 after adoptive transfer the pancreas infiltrating lymphocytes were isolated and directly analyzed by flow cytometry. Primary FACS data of RIP-Ova^{low} and PD-L1^{-/-}/RIP-Ova^{low} mice at the indicated time points after adoptive transfer are shown.

I could demonstrate that Ova/AbISCO-mediated T cell activation and proliferation occurs between d 2 and d 6 after adoptive transfer. Similarly, histological analyses revealed high numbers of islet-infiltrating CD8 T cells on d 7 after adoptive transfer and Ova/AbISCO-immunization (Fig. 42).
Results

Transfer OT-I cells into RIP-Ova\textsuperscript{low} + Ova/AbISCO

<table>
<thead>
<tr>
<th>1 (d 3 after transfer)</th>
<th>2 (d 7 after transfer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (d 3 after transfer)</td>
<td>2 (d 7 after transfer)</td>
</tr>
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</table>

Figure 42: Histological characterization of RIP-Ova\textsuperscript{low} mice after adoptive transfer and Ova/AbISCO immunization

Splenocytes of OT-I mice were isolated and CD8 T cells purified by MACS. 3 x 10\textsuperscript{6} OT-I-specific CD8 T cells were adoptively transferred i.v. into RIP-Ova\textsuperscript{low} (n=5) mice. 1 day after transfer mice were immunized with Ova/AbISCO. Pancreata of non-diabetic RIP-Ova\textsuperscript{low} (group 1) on d 3 or diabetic RIP-Ova\textsuperscript{low} mice (group 2) on d 7 after adoptive transfer were analyzed. At the indicated time points (representative mice are shown), pancreatic sections were analyzed using H+E staining and in detail using immunohistochemical staining. Insulin expression is stained in green and CD8 T cell infiltration is stained in red. An overlay is shown.

3.3.3. Characterization of the RIP-Ova x OT-I model

In a third approach, I used double-transgenic mice characterized by the expression of an Ova-specific TCR and Ova under the rat insulin promoter in pancreatic β cells. I used this double-transgenic mouse system to analyze whether T cells expressing a TCR for a self-antigen (transgenically expressed in pancreatic β cells) are deleted during development or escape selection and display an autoreactive character. To this end, RIP-Ova\textsuperscript{low} mice were bred with OT-I mice (RIP-Ova\textsuperscript{low} x OT-I). The procreation of these mice is characterized on the one hand by the expression of Ova in pancreatic β cells and on the other hand by endogenously provided Ova-specific CD8 T cells. In a second approach, OT-II mice (containing the genetic information for a CD4 T cell receptor for the I-A\textsuperscript{b}/Ova\textsuperscript{323-339} epitope) were bred with RIP-Ova\textsuperscript{low} mice (RIP-Ova\textsuperscript{low} x OT-II). The procreation of these mice is characterized by the
expression of Ova in pancreatic β cells and by endogenously provided Ova-specific CD4 T cells. I determined diabetes incidences in RIP-Ova^{low} x OT-I and RIP-Ova^{low} x OT-II mice. Therefore, blood glucose levels were measured starting in 4 weeks old mice. 100% of RIP-Ova^{low} x OT-I mice were already diabetic at this age (Fig. 43). Blood glucose levels slightly increased until mice were 7 weeks old (Fig. 43). In contrast, RIP-Ova^{low} x OT-II mice were not diabetic and displayed normal blood glucose levels until 9 weeks of age (Fig. 43). Hence, Ova-specific CD8 T cells induced diabetes in double-transgenic RIP-Ova^{low} x OT-I mice.

Figure 43: Characterization of RIP-Ova x OT-I double transgenic mice
A) RIP-Ova^{low} mice were bred with OT-I or with OT-II mice. Diabetes incidences [%] of their procreation were determined by measuring the blood glucose levels.
B) The blood glucose levels [mg/dl] of RIP-Ova^{low} x OT-I and RIP-Ova^{low} x OT-II mice were followed until 9 weeks of age. The progress of the blood glucose levels of RIP-Ova^{low} x OT-I and RIP-Ova^{low} x OT-II mice is shown for representative mice.
C) Diabetic RIP-Ova^{low} x OT-I mice (a representative mouse is shown) were analyzed at the age of 8 weeks. Pancreatic sections were analyzed for general insulitis using H+E staining and in detail using immunohistochemical staining. Insulin expression is stained in green and CD8 T cell infiltration is stained in red. An overlay is shown.
Finally I addressed the question whether Ova-specific CD8 T cells of RIP-Ova\textsubscript{low} x OT-I mice display a pathogenic phenotype. This was first tested \textit{in vitro}. OT-I cells were prepared from pancreatic lymph nodes of diabetic RIP-Ova\textsubscript{low} x OT-I mice and \textit{ex vivo} restimulated with a control peptide or the K\textsuperscript{b}/Ova\textsubscript{257-264}-peptide. Flow cytometric analyses showed a specific IFN\textsubscript{γ} response of these OT-I cells after K\textsuperscript{b}/Ova\textsubscript{257-264}-peptide-stimulation (Fig. 44A).

**Figure 44: CD8 T cells of RIP-Ova\textsubscript{low} x OT-I mice display an active character \textit{in vitro} and \textit{in vivo}**

A) Lymphocytes of the pancreatic lymph nodes of diabetic RIP-Ova\textsubscript{low} x OT-I mice were prepared and \textit{ex vivo} restimulated with K\textsuperscript{b}/exo\textsubscript{208-215} (group 1) or K\textsuperscript{b}/Ova\textsubscript{257-264} (group 2) peptides. IFN\textsubscript{γ}-producing CD8 T cells were determined by flow cytometry. The total numbers ± SD of IFN\textsubscript{γ} CD8\textsuperscript{+} T cells in the pancreatic lymph nodes are shown.

B) Splenocytes of OT-I or RIP-Ova\textsubscript{low} x OT-I mice were isolated and purified by MACS. 3 x 10\textsuperscript{6} OT-I-specific CD8 T cells were adoptively transferred i.v. into RIP-Ova\textsubscript{low} (n=10) mice. 1 day after transfer mice were immunized with Ova/AbsICO. At the indicated time points after adoptive transfer blood glucose levels [mg/dl] and cumulative diabetes incidences [%] were determined.

I analyzed whether these Ova-specific CD8 T cells can be re-activated \textit{in vivo}. Splenocytes of control OT-I and RIP-Ova\textsubscript{low} x OT-I mice were prepared, CD8 T cells isolated and adoptively transferred into RIP-Ova\textsubscript{low} mice. 1 day after transfer, mice were immunized with Ova/AbsICO. Diabetes development was monitored by
measuring the blood glucose levels. This revealed that Ova-specific CD8$^+$ T cells isolated from RIP-Ova$^{low}$ x OT-I mice were able to induce diabetes in RIP-Ova$^{low}$ mice comparable with naïve OT-I cells (Fig. 44B). Summarized, crossing RIP-Ova$^{low}$ mice with OT-I mice provided a double transgenic mouse model for spontaneous diabetes development. It could be demonstrated that Ova-specific CD8 T cells display an active phenotype isolated from RIP-Ova$^{low}$ x OT-I double transgenic mice.
4. Discussion

4.1. The processing requirements for $K^b/B_{22-29}$-specific EAD

There is increasing evidence from patients with type 1 diabetes that autoreactive CD8 T cells specific for preproinsulin are involved in β cell destruction [119]. Mouse models have been established to study the pathogenic crosstalk between preproinsulin-specific CD8 T cells and preproinsulin-expressing β cells. The RIP-B7.1 mouse provides a well defined model system for studying ppins-specific EAD. Transgene-driven B7.1 expression in the pancreatic β cells of RIP-B7.1 mice makes them susceptible to autoreactive CD8 T cell attack. The non-physiologic, co-stimulatory B7.1/CD28 interaction in RIP-B7.1 mice [20, 38] may allow efficient effector function delivery by autoreactive CD8 T-cells. There is strong evidence that the interaction of B7.1 on the surface of β cells with the co-stimulator molecule CD28 on T cells is an essential component of T cell–mediated EAD in RIP-B7.1 mice [102]. Preproinsulin-specific immunization induced EAD in almost all RIP-B7.1 mice with a strikingly similar time course and histopathology. Expression of B7.1 in islet β cells facilitated diabetes development by adoptively transferred preproinsulin-specific CD8 T cells [101, 102]. The RIP-B7.1 diabetes model is thus well suited to study distinct events in the priming and effector phase of preproinsulin-specific CD8 T cells. Hence, RIP-B7.1 mice are a useful tool to identify novel β cell antigens that are targets for CD8 T cell-triggered diabetes [120].

During this work, I characterized the immunogenicity of two previously reported ppins-encoding DNA constructs [16, 17]: the pCI/ppins vector (encoding the full length ppins sequence) efficiently induced $K^b/A_{12-21}$-specific CD8 T cell-mediated EAD in RIP-B7.1 mice, whereas pCI/ppinsΔA_{12-21} (containing a mutant ppins with a deletion of the A_{12-21}-sequence) efficiently induced $K^b/B_{22-29}$-specific CD8 T cell-mediated EAD in RIP-B7.1 mice. Interestingly, $K^b/B_{22-29}$-specific CD8 T cells were not detectable in pCI/ppins immune RIP-B7.1 mice. The same pattern of EAD induction was obtained in immunized RIP-B7.1/B7.1−/− mice (expressing the co-stimulatory
molecule B7.1 only in β cells), indicating that expression of the co-stimulatory molecule B7.1 in β cells is sufficient to trigger K\(^b\)/A\(_{12-21}\)-or K\(^b\)/B\(_{22-29}\)-specific CD8 T cells and EAD, respectively (Fig. 6). This suggested that immunodominance mechanisms i.e., the K\(^b\)/A\(_{22-29}\)-specific CD8 T cell response suppressed priming of K\(^b\)/B\(_{22-29}\)-specific CD8 T cells, operate in pCI/ppins-immunized mice [16]. However, comparing the binding affinities of these two epitopes to K\(^b\)-restricted MHC class I molecules revealed that the K\(^b\)/B\(_{22-29}\)-epitope has a higher avidity for K\(^b\) than the K\(^b\)/A\(_{12-21}\)-epitope (Fig. 5). It is thus unlikely, that direct interactions of the weak K\(^b\)/A\(_{12-21}\)-epitope suppress presentation of the K\(^b\)/B\(_{22-29}\)-epitope. Moreover, it could not be excluded that the K\(^b\)/B\(_{22-29}\)-epitope is not generated by processing of the wt ppins. I analyzed whether antigen expression and processing of ppins and ppins\(_{ΔA_{12-21}}\) differed. Transient transfection studies in Hek293 cells revealed weaker expression levels of ppins\(_{ΔA_{12-21}}\) compared to ppins. The ppins\(_{ΔA_{12-21}}\)-antigen accumulated to barely detectable expression levels, as determined by specific western blot analyses (Fig. 7). The expression levels of ppins\(_{ΔA_{12-21}}\) (but not ppins) were significantly increased by inhibitors of proteasomal degradation (epoxomicin, lactacystin) (Fig. 7). This showed that both antigens are processed by different mechanisms.

Proinsulin folding in the ER is linked to the building of three disulfide-bridges (A6-A11, A7-B7 and A20-B19), which are necessary for the stability and bioactivity of insulin [24, 31, 43, 51, 53, 54, 56, 87, 140, 146]. Deletion of the K\(^b\)/A\(_{12-21}\)-epitope, thereby preventing the formation of the A20-B19 disulfide bridge, may have a strong impact on the folding of the ppins\(_{ΔA_{12-21}}\) protein. Thus, changes in the conformation of the ppins\(_{ΔA_{12-21}}\) protein might trigger its translocation from the ER to the cytosol and its accessibility for proteasomal degradation. Examples for such retro-translocated misfolded proteins or polypeptides from the ER back to the cytosol have already been reported [15, 130]. Similarly, it has been shown that proinsulin directly expressed in the cytosol is degraded by the proteasome [17].

Pancreatic β cells are able to process ppins into mature bioactive insulin [36, 123, 124]. In contrast, in non-pancreatic cells, ppins is expressed and translocated into the ER, where the signal peptide is removed to generate pins. ER-associated pins is expected to be a major substrate for generating diabetogenic epitopes in non-pancreatic APCs [60]. Since pCI/ppins-immunized RIP-B7.1 mice developed
K\textsuperscript{b}/A\textsubscript{12-21}-specific [17] and pCI/ppins\Delta A\textsubscript{12-21}-immunized mice developed K\textsuperscript{b}/B\textsubscript{22-29}-specific EAD (Fig. 8 and 9), it can be concluded that pancreatic β cells present both ppins-derived epitopes, to be targeted by autoreactive CD8 T cells. Thus, the limitation to present the K\textsuperscript{b}/B\textsubscript{22-29}-epitope is obviously linked to antigen-presenting cells targeted by ppins-specific DNA. In order to analyze the antigenic requirements for efficient K\textsuperscript{b}/B\textsubscript{22-29}-specific CD8 T cell priming, I shortened the ppins\Delta A\textsubscript{12-21} antigen (Fig. 10A). The resulting pCI/SP-B-C or pCI/SP-B vectors did not allow induction of K\textsuperscript{b}/B\textsubscript{22-29}-specific CD8 T cells and EAD in RIP-B7.1 mice (Fig. 10D). This was surprising, since even small antigens containing the A-chain or the A\textsubscript{12-21}-epitope with an ER-targeting signal efficiently elicited K\textsuperscript{b}/A\textsubscript{12-21}-specific CD8 T cells and EAD in RIP-B7.1 mice [17]. The shortened DNA constructs could not elicit K\textsuperscript{b}/B\textsubscript{22-29}-specific CD8 T cells and EAD in RIP-B7.1 mice. A possible explanation for this observation might be that pCI/SP-B-C and pCI/SP-B are even weaker expressed than pCI/ppins\Delta A\textsubscript{12-21} in Hek293 cells (Fig. 10C). Using cationic peptide-based immunization [108], A-tat/ODN (but not B-tat/ODN) induced diabetes in RIP-B7.1 mice (Fig. 10B). Thus, exogenous delivery of the 30-residue B-chain did not trigger EAD in RIP-B7.1 mice.

Furin is a ubiquitous type-I membrane-bound proteinase responsible for processing of various proproteins. It cleaves at the general recognition motif R-X-(R/K)R [110]. Furin acts in the trans-golgi network, on the cell surface or in recycling endosomes [129]. It has been shown that a construct encoding mutant human proinsulin, modified by introduction of specific furin cleavage sites at the B/C and C/A junctions, can be processed into mature human insulin in transiently transfected Hek293 cells [41].

I designed similar constructs based on the murin ppins-II sequence. To exclude any influence of bioactive insulin, I first focussed on the ppins\Delta A\textsubscript{12-21} construct. I introduced specifically engineered furin-specific recognition sites at the B/C junction (and also the C/A junction) of ppins\Delta A\textsubscript{12-21} (pCI/ppins-furin\Delta A\textsubscript{12-21}). In a second construct, these specific recognition sites were eliminated by alanine substitutions (pCI/ppins-minus-furin\Delta A\textsubscript{12-21}). RIP-B7.1 mice immunized with pCI/ppins-furin\Delta A\textsubscript{12-21} developed an early and severe EAD. Higher K\textsuperscript{b}/B\textsubscript{22-29}-specific CD8 T cell numbers were induced in these mice compared to pCI/ppins\Delta A\textsubscript{12-21}-immunized mice.
In contrast, pCI/ppins-minus-furinΔA₁₂-2₁ significantly delayed diabetes incidence, indicating that priming of diabetogenic Kᵇ/B₂₂–₂₉-specific CD8 T cells is inefficient (Fig. 11). These findings suggested that furin-specific processing at the B/C junction increases the efficacy of Kᵇ/B₂₂–₂₉-epitope processing and presentation. I further confirmed this observation with a mutant ppins-construct encoding full length ppins with engineered furin recognition sites at the B/C and C/A junction (pCI/ppins-furin). In comparison to the wt ppins, this construct induced Kᵇ/B₂₂–₂₉-specific CD8 T cells and EAD. Both, Kᵇ/A₁₂–₂₁- and Kᵇ/B₂₂–₂₉-epitope specificities were detectable in pCI/ppins-furin-immunized diabetic mice (Fig. 12).

4.2. The influence of PD-1/PD-L1 signaling on EAD

Recent studies analyzed the de novo induction of preproinsulin-specific CD8 T cells (and EAD) in mice and their control by PD-L1/PD-1 interaction [102]. It has been shown that PD-1 and its ligands, PD-L1 and PD-L2, deliver inhibitory signals regulating the balance between T cell activation, tolerance, and immunopathology [61, 94]. PD-L1 is expressed on antigen-presenting cells as well as effector CD8 T cells, whereas PD-1 is expressed on T cells. Using bone marrow chimeric mice, it was confirmed that the deficiency of PD-L1 on target cells or PD-1 on T cells was essential to trigger preproinsulin-specific CD8 T cell–mediated EAD by DNA based immunization [102]. Moreover, transgene-driven B7.1 co-stimulation in pancreatic β cells was more potent than the loss of PD-L1 co-inhibition in promoting the pathogenic immune response of adoptively transferred preproinsulin-specific CD8 T cells. In contrast, immunization of PD-L1⁻/⁻ and RIP-B7.1 mice with ppins-encoding DNA induced Kᵇ/A₁₂–₂₁-specific CD8 T cells and EAD with similar efficacies and kinetics [102]. It was assumed that additional factors (e.g., professional antigen-presenting dendritic cells or cells from the innate immune system) [44] are triggered by DNA-based immunization that facilitate expansion of preproinsulin-specific CD8 T cells and/or maintain their diabetogenic potential in PD-L1⁻/⁻ mice. Immunization of RIP-B7.1 mice with pCI/ppinsΔA₁₂-2₁ efficiently induced B-chain (Kᵇ/B₂₂–₂₉)-specific CD8 T cells and EAD (Figure 8 and 9). In contrast, immunization
with pCl/ppins\(\Delta A_{12-21}\) completely failed to induce K\(^b\)/B\(_{22-29}\)-specific CD8 T cells and EAD in PD-L1\(^-/-\) mice (Fig. 14, 15). Similarly, B6 mice immunized with pCl/ppins but not with pCl/ppins\(\Delta A_{12-21}\) developed diabetes upon \(\alpha\)-PD-L1 antibody treatment (Fig. 13). Therefore, I assumed that the diabetogenic K\(^b\)/A\(_{12-21}\), but not the K\(^b\)/B\(_{22-29}\)-specific CD8 T cell-mediated EAD is regulated by PD-L1/PD-1 signaling. The direct interaction of diabetogenic CD8 T cells with B7.1 expressing \(\beta\) cells may either facilitate expansion of K\(^b\)/B\(_{22-29}\)-specific CD8 T cells and/or regulate the susceptibility of the \(\beta\) cells for a K\(^b\)/B\(_{22-29}\)-specific CD8 T cell attack.

It is critical for autoimmune diseases that co-stimulatory and/or co-inhibitory signaling pathways mediate the balance of autoreactive and regulatory T cells [14]. In this context, it might be possible that, due to the missing PD-1/PD-L1 signaling, specific regulatory mechanisms prevent autoreactive T cell responses directed against the K\(^b\)/B\(_{22-29}\)-epitope. Moreover, it has been reported that T cell receptor ligands characterized by a high agonist activity are able to mediate the conversion of naïve T cells (that have not been activated) into FoxP3-expressing regulatory T cells [5, 67, 100]. A recent study revealed that immunization with a strong insulin mimetope prevented type 1 diabetes in NOD mice by conversion of naïve T cells into FoxP3 regulatory T cells [26]. Similar, the strong K\(^b\)/B\(_{22-29}\)-epitope of ppins characterized by high avidity for K\(^b\) molecules, might be able to induce regulatory mechanisms and therefore prevent diabetes development in PD-L1\(^-/-\) mice.

In this work, I established a new approach for inhibiting the immune response by repeated DNA injections. Interestingly, PD-L1\(^-/-\) mice that received five injections of pCl/ppins DNA once per week did not develop EAD (Fig. 16B). Hence, K\(^b\)/A\(_{12-21}\)-specific CD8 T cells were not (or inefficiently) primed, deregulated by yet unknown mechanisms or lost their diabetogenic potential. Thus, continuous “over-stimulation” of autoreactive CD8 T cells specifically down-regulates their numbers or attenuates their diabetogenic potential. Moreover, priming of PD-L1\(^-/-\) mice with pCl/ppins\(\Delta A_{12-21}\) and boosting them with pCl/ppins also diminished the ability of pCl/ppins for inducing K\(^b\)/A\(_{12-21}\)-specific diabetogenic CD8 T cells (Fig. 16A), indicating that ppins\(\Delta A_{12-21}\) provides regulatory signals.

Adeno-associated viruses (AAVs) are the most commonly used vectors for gene therapy [6, 50, 118]. Such vectors are based on a single-stranded, non-pathogenic
member of the parvovirus family characterized by replication deficiency and a 4.7 kb single-stranded DNA genome [34]. They can be used for efficient gene transfer to non-dividing cells like muscle fibers or hepatocytes [28]. The combination of AAV vectors with efficient, liver-specific promoters led to transgene expression at high-levels in the hepatocytes of small and large animals [112]. Several studies reported that hepatic gene transfer induces immune tolerance to various antigens by induction of regulatory T cells responsible for suppression of humoral and cellular immune responses [27, 28, 84]. Thus, liver-directed gene transfer using AAV vectors is suitable for induction of immune tolerance against the transgene product [28].

I used these findings to develop a model for induction of peripheral tolerance in PDL1\(^{-/-}\) mice. An adeno-associated vector encoding preproinsulin that was targeted to the liver by i.v. injection, could efficiently decrease diabetes incidence in PDL1\(^{-/-}\) mice immunized with pCI/ppins. Moreover, a vector encoding ppins with engineered furin sites completely protected PDL1\(^{-/-}\) mice immunized with pCI/ppins from diabetes development (Fig. 19). Histological analysis of these mice revealed that islets expressed insulin despite lymphocyte infiltrates (Fig. 20). I assume that providing alternative insulin expressing cells (by infection with an adeno-associated vector) thereby increasing the presentation of ppins-derived antigens can induce peripheral tolerance in PDL1\(^{-/-}\) mice.

The B6-derived diabetes model described in this study is attractive to characterize distinct events in the regulation of \(\beta\) cell susceptibility to manifest or control preproinsulin-specific, CD8 T cell–mediated EAD. B6 is not a privileged strain for type 1 diabetes studies, but unexpectedly priming of preproinsulin-specific CD8 T cells was possible in male and female B6 mice. Preproinsulin-specific CD8 T cells in immunized B6 mice have a diabetogenic potential, but pancreatic \(\beta\) cells are protected from immune attack by these cells. Similarly, in the lymphocytic choriomeningitis virus diabetes model, the pancreatic target tissue must be exposed to stimulatory signals from the innate immune system, to become susceptible to the destructive CD8 T cell attack [74]. The B6 model is a good example for translational medicine, since recent data illustrated the central role of the \(\beta\) cell as a gatekeeper in preproinsulin-specific diabetes [102]. \(\beta\) cells per se prevent the deleterious cross-talk with preproinsulin-specific CD8 T cells. Changes in the \(\beta\) cell milieu, e.g., by antibody

Discussion
treatment [102], by interferon [74], or by viral infections [136], can favor the susceptibility of \( \beta \) cells for the CD8 T cell-mediated immune attack. Further manipulations of the pancreatic \( \beta \) cells, or distinct arms of the immune system by specific drugs [76], or by using different mouse strains with defects in specific cell types or immune mediators, may define conditions that inactivate (tolerize/anergize) autoreactive CD8 T cells. “Translation” of these approaches to human type 1 diabetes [76, 105, 135, 136] could be helpful to design prophylactic vaccines.

4.3. Ova-specific EAD

Type 1 diabetes results from the autoimmune-mediated destruction of the pancreatic \( \beta \) cells. Molecular mechanisms and cell types that contribute to \( \beta \) cell destruction were mainly analyzed in animal models [81]. Beside the NOD mouse, which is the most common model system to study type 1 diabetes and the RIP-B7.1 mouse, that are both based on non-transgenically expressed self-antigens, several other mouse models exist, based on the transgenic expression of antigens (for example Ova [11, 68, 70]) under the insulin promoter in \( \beta \) cells. A well defined transfer model for studying type 1 diabetes is based on RIP-Ova-transgenic mice. Performing adoptive transfer experiments with OT-I cells as donors and RIP-Ova-transgenic mice as hosts, offers an attractive model for selectively studying the effector phase of EAD. It could be shown that adoptively transferred OT-I cells into RIP-mOva mice were activated in pancreas-draining lymph nodes, as exogenously-derived Ova was presented via MHC class I on bone-marrow-derived APCs [68]. Cross-presentation of antigens to naïve CD8 T cells requires relative high antigen levels expressed by peripheral tissues. However, the accessibility of low expressed exogenous antigens for cross-presentation can be enhanced by cellular destruction [69, 71]. Focussing on co-signaling pathways, it was shown that the PD-1/PD-L1 signaling is critical for maintaining peripheral tolerance by limiting the effector function and differentiation of autoreactive CD8 T cells [79]. In this context it was reported that both, PD-1 and PD-L1 are critical for peripheral tolerance [62]. A previous study revealed that CTL-mediated tissue damage releases self-antigens leading to deletion of autoreactive
CD8 T cells [98]. Moreover, it was demonstrated that providing Ova combined with a foreign T helper peptide sequence, as antigen can break B cell tolerance in all three RIP-Ova-transgenic strains, but is only sufficient to induce self-reactive CTLs in RIP-mOva and RIP-Ova\textsuperscript{low} mice [121]. Recent studies could show that a DNA immunization approach enhanced by GM-CSF and imiquimod (a TLR-7-stimulating agent) as adjuvant elicited high Ova-specific CD8 and CD4 T cell frequencies, and is thus sufficient for breaking tolerance in RIP-Ova\textsuperscript{low} mice [104].

The well-defined RIP-Ova\textsuperscript{low} mouse model provides an attractive system for characterization of the different phases during autoimmune diabetes, as it offers several advantages compared to ppins-specific diabetes models (i.e., the high avidity K\textsuperscript{b}/Ova\textsubscript{257-264}-epitope, available antibodies or tetramers and the possibility to use the OT-I-based adoptive transfer model).

Using a ppins-OVA vector expressing ppins plus the K\textsuperscript{b}/Ova\textsubscript{257-264}-epitope at the COOH-terminus, I could show that pancreatic β cells were able to present both, ppins-specific and Ova-specific epitopes in RIP-B7.1/RIP-Ova\textsuperscript{low} mice. However, Ova-specific immunodominance mechanisms inhibited co-priming of ppins-specific CD8 T cells in pCI/ppins-Ova-immunized RIP-B7.1 mice (Fig. 22). For selective induction of Ova-specific EAD, a DNA-based vector encoding the antigen Ovalbumin was used. I analyzed the kinetics of Ova-specific CD8 T cell responses. I could demonstrate that only RIP-Ova\textsuperscript{low} mice also co-expressing the co-stimulatory B7.1 molecule on pancreatic β cells (RIP-B7.1/RIP-Ova\textsuperscript{low} mice) were able to elicit Ova-specific CD8 T cell responses and EAD (Fig. 26-28). Ova-specific CD8 T cells were only detectable during the initial phase of diabetes development in RIP-B7.1/RIP-Ova\textsuperscript{low} mice (Fig. 27). At later stages, when diabetes onset had already been diagnosed, reduced numbers of Ova-specific CD8 T cells were detectable in the pancreata as shown by FACS analysis (Fig. 27). However, histological analysis of these pancreata revealed massive islet infiltrates (Fig. 28). I thus concluded, that at the time of diabetes onset mainly non-specific “bystander T cells” are present in the pancreas. This effect was already shown during type 1 diabetes development in NOD mice as insulin-specific T cells play a major role in the initiation of the disease but at diabetes onset islet-associated T cells are characterized by a different antigen specificity [85].
I studied the effector phase of Ova-specific EAD by focusing on the pathogenic cross-talk between Ova-expressing target cells (i.e. the pancreatic β cells) and autoreactive CD8 T cells. For studying the requirements for effective stimulation of antigen-specific CD8 T cells, I used the well-defined transfer model, in which OT-I mice, that contain the genomic information for an Ova-specific TCR, served as donors while RIP-Ova-transgenic mice were used as hosts. *In vitro* data showed that pancreatic β cells of RIP-Ova\textsuperscript{low} mice were able to stimulate Ova-specific CD8 T cells (Fig. 31). In contrast, this presentation was not sufficient to stimulate and/or expand adoptively transferred OT-I cells and cause EAD (Fig. 32). Similarly, transfer of naïve OT-I or PD-1\textsuperscript{−/−}/OT-I cells into RIP-Ova\textsuperscript{low} mice was not efficient to trigger diabetes development [62, 71] (Fig. 32). I could also demonstrate that PD-L1\textsuperscript{−/−}/OT-I or B7.1\textsuperscript{−/−}/OT-I cells did not induce diabetes in RIP-Ova\textsuperscript{low} mice (Fig. 32). Even the use of a RIP-Ova\textsuperscript{low} knockout strain (PD-L1\textsuperscript{−/−}/RIP-Ova\textsuperscript{low}) as acceptors in combination with various OT-I knockout strains could not elicit Ova-specific CD8 T cells and EAD (Tab. 10). In contrast, cells of all tested OT-I knockout strains were efficiently activated and caused diabetes in RIP-B7.1/RIP-Ova\textsuperscript{low} mice until 7 weeks after transfer (Fig. 33 and 34). Thus, only the transgenic expression of the co-stimulatory molecule B7.1 on pancreatic β cells could provide signals sufficient for activation of antigen-specific CD8 T cells in the RIP-Ova\textsuperscript{low} mouse model. Since Ova-specific CD8 T cells could not be activated leading to β cell attack in RIP-Ova\textsuperscript{low} mice, I tested whether additional stimulation of CD8 T cells by professional APCs presenting the K\textsuperscript{b}/Ova\textsuperscript{257-264}-epitope could trigger EAD. Similar, a recent study could demonstrate that an immunization approach using Ova-encoding DNA combined with the adjuvants GM-CSF and iquimod induced high CD8 T cell frequencies in RIP-Ova\textsuperscript{low} mice [104].

I combined the adoptive transfer of OT-I cells into RIP-Ova\textsuperscript{low}-transgenic mice with a protein/adjuvant immunization (24 h post transfer) approach. Using this model, I could demonstrate that transfer of OT-I cells into RIP-Ova\textsuperscript{low} or PD-L1\textsuperscript{−/−}/RIP-Ova\textsuperscript{low} mice followed by immunization with Ova/AbISCO could efficiently elicit Ova-specific CD8 T cells and EAD (Fig. 36). These observations were further defined by analyzing the cell proliferation of Ova-specific CD8 T cells in different RIP-Ova\textsuperscript{low}-transgenic mice after adoptive transfer of OT-I cells, either without additional treatment or
combined with an Ova/AbISCO immunization approach. I could show that Ova-specific CD8 T cells were not able to proliferate in the pLNs and did not invade the pancreas without Ova/AbISCO immunization in RIP-Ova\textsuperscript{low} mice. In contrast, OT-I cells showed a distinct proliferation in PD-L1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low} mice, but these cells were not able to invade the pancreas until d 8 after adoptive transfer (Fig. 38 and 39). Injection of Ova/AbISCO led to massive cell proliferation and pancreas infiltration in all RIP-Ova\textsuperscript{low}-transgenic mice (Fig. 40 and 41). Thus, immunization with Ova/AbISCO provided a sufficient signaling by APCs presenting Ova, to activate OT-T cell proliferation and diabetes development in RIP-Ova\textsuperscript{low} and PD-L1\textsuperscript{-/-}/RIP-Ova\textsuperscript{low} mice.

In a third approach, I used double-transgenic mice characterized by the expression of an Ova-specific TCR and Ova under the rat insulin promoter in pancreatic \(\beta\) cells. Such double-transgenic mice are well suitable for monitoring disease initiation and progression and can be manipulated easily [141]. Previous research mainly focused on CD4 T cells using DO11.10 x RIP-mOva mice (expressing a membrane-bound form of Ova in pancreatic \(\beta\) cells and the DO11.10 CD4-TCR). Studies revealed that \(T_{reg}\) cells display the ability for proliferation and accumulation in response to their local antigen environment \textit{in vivo} [138]. Recently it was suggested that during diabetes onset, the balance between regulatory and pathogenic islet-reactive T cells is disturbed [21].

During this work, I used this double-transgenic mouse system for studying the development of diabetes focusing on CD8 T cells that express the Ova-specific TCR (OT-I), to analyze whether CD8 T cells expressing a TCR for a self-antigen (transgenically expressed in pancreatic \(\beta\) cells) are deleted during development or escape selection and display an autoreactive character. To this end, RIP-Ova\textsuperscript{low} mice were bred with OT-I mice. RIP-Ova\textsuperscript{low} x OT-I mice are characterized by developing spontaneous diabetes about 3 weeks after birth (Fig. 43). However it is likely, that the onset of diabetes occurs even earlier. RIP-Ova\textsuperscript{low} mice that express the Ova-specific CD8 TCR (OT-I), but not the CD4 TCR (OT-II), developed EAD (Fig. 43). Thus, EAD in this model critically depends on CD8 T cells. Interestingly diabetic double-transgenic RIP-Ova\textsuperscript{low} x OT-I mice, that displayed already high blood glucose levels early after birth, were able to survive until 12 weeks of age. The main question in this
context was, how mice can survive with such high blood glucose levels over weeks. Histological analysis of pancreata from diabetic double-transgenic RIP-Ova\textsuperscript{low} x OT-I mice could explain this phenomenon at least partially. H+E staining and immunohistological analysis revealed that pancreata of these mice were characterized by massive insulitis due to CD8 T cells. However, it was still possible to detect some partially healthy islets that were still able to produce insulin (Fig. 43).

\textit{Ex vivo} restimulation experiments revealed that Ova-specific CD8 T cells isolated from RIP-Ova\textsuperscript{low} x OT-I double-transgenic mice displayed effector functions as measured by IFN\textgamma-production (Fig. 44A). Moreover, \textit{in vivo} transfer studies demonstrated that Ova-specific CD8 T cells isolated from RIP-Ova\textsuperscript{low} x OT-I double-transgenic mice were able to induce diabetes in RIP-Ova-transgenic mice (Fig. 44B).
5. Summary

RIP-B7.1 mice expressing the co-stimulatory molecule B7.1 (CD80) on pancreatic β cells are an attractive model to characterize the de novo induction of preproinsulin (ppins)-specific CD8 T cells and experimental autoimmune diabetes (EAD). The model is suitable for studying the pathogenic cross-talk between autoreactive CD8 T cells and their insulin-expressing pancreatic target cells. Different preproinsulin-encoding vectors selectively induce either insulin A-chain-(K^b/A_{12-21})- (immunization with wild type ppins) or insulin B-chain-(K^b/B_{22-29})-specific (immunization with a mutant ppinsΔA_{12-21}-antigen lacking the COOH-terminal A_{12-21} epitope) CD8 T cells and EAD. The induction of K^b/B_{22-29}-specific CD8 T cells is thus limited using wt ppins as DNA-based antigen, but this epitope is efficiently presented to CD8 T cells by pancreatic β cells.

In this study, RIP-B7.1 mice were used to characterize the priming conditions of both, K^b/A_{12-21}- and K^b/B_{22-29}-specific CD8 T cells. Proteasome-mediated degradation of mutant ppinsΔA_{12-21}-antigen resulted in a high turnover of it. In contrast, expression of wild type ppins in the ER was not modulated by proteasome inhibitors. Both antigens, ppins and ppinsΔA_{12-21} are thus processed by different mechanisms. The presence of the co-stimulatory molecule B7.1 on pancreatic β cells (but not on all other cell types) is necessary to trigger EAD by both CD8 T cell specificities. Induction of pCI/ppinsΔA_{12-21}-induced EAD correlated with an influx of K^b/B_{22-29}-specific CD8 T cells into the pancreatic islets. Interestingly, only the ppinsΔA_{12-21}-antigen efficiently elicited K^b/B_{22-29}-specific CD8 T cells and EAD in RIP-B7.1 mice. Further shortening of the ppinsΔA_{12-21}-antigen (e.g., Sp-B) resulted in an inefficient induction of diabetogenic K^b/B_{22-29}-specific CD8 T cells. Modulation of the processing sites at the B/C junction of ppinsΔA_{12-21} antigen by adding specific furin-recognition sites (ppins-furinΔA_{12-21}) enhanced priming of K^b/B_{22-29}-specific CD8 T cells and accelerated EAD progression in RIP-B7.1 mice. When wt ppins was engineered with furin recognition sequences K^b/B_{22-29} (and K^b/A_{12-21})-specific CD8 T cells and EAD were induced in RIP-B7.1 mice. Thus, specific processing of wt ppins at the B/C junction may be an
essential step in generating the K\textsuperscript{b}/B\textsubscript{22-29}-epitope in APCs and insulin-expressing \(\beta\) cells.

Both, distinct activation of diabetogenic CD8 T cells and \(\beta\) cell susceptibility to an autoimmune attack are required for diabetes development. It has been shown that PD-1/PD-L1 co-inhibitory signaling on CD8 T cells and \(\beta\) cells regulate EAD development by K\textsuperscript{b}/A\textsubscript{12-21}-specific CD8 T cells. I investigated whether inhibitory (PD-1/PD-L1) signaling has an impact on the priming and/or effector phase of autoreactive CD8 T cells specific for the high affine K\textsuperscript{b}/B\textsubscript{22-29}-epitope. Interestingly, immunization with full-length ppins- (triggering K\textsuperscript{b}/A\textsubscript{12-21}-specific CD8 T cells), but not with ppins\(\Delta\)A\textsubscript{12-21}-antigen (triggering K\textsuperscript{b}/B\textsubscript{22-29}-specific CD8 T cells) induced severe EAD in PD-1\textsuperscript{−/−} and PD-L1\textsuperscript{−/−} mice. Thus, EAD development by K\textsuperscript{b}/B\textsubscript{22-29}-specific CD8 T cells is triggered by co-stimulator (B7.1) but not by co-inhibitor (PD-L1) expression on \(\beta\) cells. I further established novel immunization strategies (repeated injections of pCI/ppins in short intervals; pre-immunization with a recombinant insulin-expressing AAV followed by pCI/ppins DNA immunization) that could suppress K\textsuperscript{b}/A\textsubscript{12-21}-specific diabetes development in PD-L1\textsuperscript{−/−} mice. Specific regulation of K\textsuperscript{b}/A\textsubscript{12-21}-specific CD8 T cells may thus operate in the priming phase of this immune response in PD-L1\textsuperscript{−/−} mice.

Similar to the ppins\(\Delta\)A\textsubscript{12-21}-(K\textsuperscript{b}/B\textsubscript{22-29}) specific EAD model, a high affine K\textsuperscript{b}/Ova\textsubscript{257-264}-specific CD8 T cell response efficiently induced severe EAD in pCI/Ova-immunized RIP-B7.1/RIP-Ova\textsubscript{low} (expressing the Ova-antigen and the co-stimulatory B7.1 molecule in \(\beta\) cells) but not in PDL1\textsuperscript{−/−}/RIP-Ova\textsubscript{low} mice (expressing the Ova-antigen in PD-L1-deficient \(\beta\) cells). Similarly, adoptive transfer of Ova-specific CD8 T cells into RIP-B7.1/RIP-Ova\textsubscript{low} but not PDL1\textsuperscript{−/−}/RIP-Ova\textsubscript{low} induced EAD. Additionally, professional Ova-specific antigen presentation (delivered by a single Ova/adjuvant injection) was necessary to activate the diabetogenic potential of adoptively transferred Ova-specific CD8 T cells in RIP-Ova\textsubscript{low} and PDL1\textsuperscript{−/−}/RIP-Ova\textsubscript{low} mice. Co-inhibitory (PD-L1) and co-stimulatory (B7.1) signals thus regulate diabetes induction by preproinsulin- and Ova-specific CD8 T cells in an epitope-specific manner.
6. Bibliography


# A. Appendix

## Chemicals and reagents

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Media and buffers

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Publication

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Der Lebenslauf wurde aus Gründen des Datenschutzes entfernt.