A Missing PD-L1/PD-1 Coinhibition Regulates Diabetes Induction by Preproinsulin-Specific CD8 T-Cells in an Epitope-Specific Manner

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Abstract

Coinhibitory PD-1/PD-L1 (B7-H1) interactions provide critical signals for the regulation of autoreactive T-cell responses. We established mouse models, expressing the costimulator molecule B7.1 (CD80) on pancreatic beta cells (RIP-B7.1 tg mice) or are deficient in co-inhibitory PD-L1 or PD-1 molecules (PD-L1−/− and PD-1−/− mice), to study induction of preproinsulin (ppins)-specific CD8 T-cell responses and experimental autoimmune diabetes (EAD) by DNA-based immunization. RIP-B7.1 tg mice allowed us to identify two CD8 T-cell specificities: pCIPpins DNA exclusively induced Kβ/A12–21-specific CD8 T-cells and EAD, whereas pCIPppinsΔA12–21 DNA (encoding ppins without the COOH-terminal A12–21 epitope) elicited Kβ/B22–29−specific CD8 T-cells and EAD. Specific expression/processing of mutant ppinsΔA12–21 (but not ppins) in non-beta cells, targeted by intramuscular DNA-injection, thus facilitated induction of Kβ/B22–29-specific CD8 T-cells. The A12–21 epitope binds Kβ molecules with a very low avidity as compared with B22–29. Interestingly, immunization of coinhibition-deficient PD-L1−/− or PD-1−/− mice with pCIPppins induced Kβ/A12–21-monompecific CD8 T-cells and EAD but injections with pCIP/ ppinsΔA12–21 did neither recruit Kβ/B22–29-specific CD8 T-cells into the pancreatic target tissue nor induce EAD. PpipsΔA12–21/(Kβ/B22–29)-mediated EAD was efficiently restored in RIP-B7.1/PD-1−/− mice, differing from PD-L1−/− mice only in the tg B7.1 expression in beta cells. Alternatively, an ongoing beta cell destruction and tissue inflammation, initiated by ppins/(Kβ/A12–21)-specific CD8 T-cells and EAD but injections with pCIP/ ppinsΔA12–21/(Kβ/B22–29)-specific CD8 T-cells in pCIPppins+pcIPppinsΔA12–21 co-immunized PD-L1−/− mice, facilitated the expansion of ppinsΔA12–21/(Kβ/B22–29)-specific CD8 T-cells. CD8 T-cells specific for the high-affinity Kβ/B22–29 (but not the low-affinity Kβ/A12–21)-epitope thus require stimulatory help from beta cells or inflamed islets to expand in PD-L1-deficient mice. The new PD-1/PD-L1 diabetes may be valuable tools to study under well controlled experimental conditions distinct hierarchies of autoreactive CD8 T-cell responses, which trigger the initial steps of beta cell destruction or emerge during the pathogenic progression of EAD.

Introduction

Type 1 diabetes (T1D) is an autoimmune disorder, in which insulin-producing beta cells are destroyed by the cellular immune system [1,2,3]. Diabetes development is characterized by progressive infiltration of T-cells into the pancreatic islets and beta cell destruction, resulting in severe hyperglycemia. Disease in man is triggered by poorly defined antigens and factors that finally result in the breakdown of central and/or peripheral tolerance and activation of autoreactive CD4+ and/or CD8+ T-cells [1,4]. There is increasing evidence from patients with T1D that autoreactive CD8+ T-cells are involved in the development of disease but it is difficult to detect these rare lymphocytes and to assign their individual effects during the progression of diabetes [5,6,7]. It is assumed that the nature of an autoantigen-derived peptide and its presentation by MHC class I molecules plays a central role in the development of T-cell-mediated autoimmunity [8]. In the NOD mouse model [9], the binding of insulin-derived self peptides to MHC class I or class II molecules is weak and caused by unfavoured binding registers [10,11,12]. This suggests that unconventional antigenic epitope processing and presentation may contribute to the induction of autoreactive immune responses [7,13].

Spontaneous diabetes development in the NOD mouse model elucidates many aspects of diabeticogenic immune responses [9]. Furthermore, different mouse models have been used to characterize de novo induction of well-defined T-cell responses and their pathogenic cross-talk with beta cells, which selectively express transgene-encoded ‘neo-self’ antigens under rat insulin promoter (RIP) control [14]. We used transgenic (tg) RIP-B7.1 mice, expressing the costimulatory molecule B7.1 (CD80) on pancreatic beta cells [15], to characterize induction of preproinsulin (ppins)-specific CD8 T-cells and experimental autoimmune diabetes (EAD) by DNA-based immunization [16,17,18,19]. A single injection of ppins-encoding DNA (pCIP/ppins) efficiently induced CD8 T-cell-mediated EAD in both, male and female RIP-B7.1 tg
mice with a median onset of 2–3 weeks post immunization and a cumulative diabetes incidence of >95% by week 4 [17]. In these mice, progressive invasion of insulin A-chain-derived K\(^{b}/\)A\(_{12-21}\)-specific CD8 T-cells into pancreatic islets precedes hyperglycemia and insulin deficiency. K\(^{b}/\)A\(_{12-21}\)-specific CD8 T-cells and EAD were efficiently induced by pCI/ppins in MHC class II-deficient (A\(_{2-21}\)/-/-) RIP-B7.1 mice (RIP-B7.1/MHC-II/-/-) with no conventional CD4 T-cells and in RIP-B7.1 tg mice acutely depleted of CD4 T-cells with anti CD4 antibody [17,18]. The RIP-B7.1 tg model hence provides an attractive experimental approach to study CD4 T-cell-independent induction of EAD by ppins-specific CD8 T-cells.

We further investigated the impact of coinhibitory ‘programmed death-1’ (PD-1)/‘programmed death-ligand-1’ (PD-L1) interactions on CD8 T-cell specificity and EAD. We further used immunization with pCI/ppins DNA efficiently primed K\(^{b}/\)A\(_{12-21}\)-specific CD8 T-cells and EAD in coinhibiton-deficient PD-L1/-/- and PD-1/-/- mice [19]. K\(^{b}/\)A\(_{12-21}\)-specific CD8 T-cells were also primed in wild type (wt) C57BL/6 (B6) mice but these cells revealed their diabetogenic potential only after treatment with anti PD-L1 antibody [19]. Furthermore, a deficiency of either PD-L1 in antigen-presenting cells or PD-1 in T-cells was required to induce K\(^{b}/\)A\(_{12-21}\)-mediated EAD in bone marrow chimeric mice [19]. This suggested that PD-1/PD-L1-mediated signals regulate beta cell-destruction by K\(^{b}/\)A\(_{12-21}\)-specific CD8 T-cells.

During the course of EAD in RIP-B7.1 tg mice ex vivo stimulation of ppins-primed CD8 T-cells with the K\(^{b}/\)A\(_{12-21}\) peptide, but not with all other peptides of a ppins-specific library, revealed a CD8 T-cell population with specifically inducible IFN\(\gamma\) expression [19]. This suggested that the K\(^{b}/\)A\(_{12-21}\) is the only diabetogenic epitope in ppins-immune RIP-B7.1 tg mice. However, a mutant ppins\(\Delta_{12-21}\) antigen (with a deletion of the COOH-terminal A\(_{12-21}\) sequence) also induced CD8 T-cell-mediated EAD in RIP-B7.1 tg mice, indicating that EAD can be induced by CD8 T-cell responses that have specificities other than K\(^{b}/\)A\(_{12-21}\) [18]. In this study, we mapped the alternative CD8 T-cell epitope in pCI/ppins\(\Delta_{12-21}\)-immune RIP-B7.1 tg mice and investigated the antigen expression and processing requirements to prime this CD8 T-cell specificity and EAD. We further used coinhibiton-deficient PD-1/-/-, PD-1/-/-/ and RIP-B7.1/PD-L1/-/- mice (differing from PD-L1/-/- mice only in the tg B7.1 expression in beta cells) to determine whether induction of ppinsand ppins\(\Delta_{12-21}\)-specific CD8 T-cell responses and diabetes development depends on specific costimulatory and coinhibitory signals from pancreatic beta cells.

Mice

RIP-B7.1 mice were backcrossed for >15 generations to the C57BL/6 (H-2\(^{b}\)) background as described [13]. C57BL/6 (B6) H-2\(^{b}\) mice were obtained from Janvier (Le Genets-St-Ise; France). PD-L1/-/- (B7-H1/-/- or CD274/-/-) mice [29], PD-1/-/- mice [30], CD28/-/- mice (Jackson Laboratory, USA), MHC class II-deficient A\(_{2-21}\)/-/- mice [31] and RIP-B7.1 mice [15] were bred and kept under standard pathogen-free conditions in the animal colony of Ulm University (Ulm, Germany). We further generated RIP-B7.1 mice that were deficient for PD-L1 (RIP-B7.1/PD-L1/-/-) or MHC class II molecules (RIP-B7.1/MHC-II/-/-). Male and female mice were used in the experiments at 6–8 weeks of age.

Construction of Expression Plasmids

The sequences of the different ppins antigens were codon-optimized and synthesized by GeneArt (Regensburg, Germany). All constructs were cloned into the pCI vector (cat.no. E1731, Promega, Mannheim, Germany) using the NheI and NotI restriction sites. Batches of plasmid DNA were produced in E. coli by PlasmidFactory GmbH (Bielefeld, Germany).

Characterization of Antigen Expression

Human embryonal kidney cells (HEK-293 cells) were transiently transfected with the indicated plasmid DNAs using the calcium phosphate method. Cells were labeled with 100 \(\mu\)Ci \(^{35}\)S-methionine/cysteine (cat. no. IS103, Hartmann Analytic GmbH) between 36 and 48 h post transfection and subsequently lysed with pH 8.0 lysis buffer (100 mM NaCl, 0.5% NP40 and 100 mM Tris-hydrochloride) supplemented with the protease inhibitors, leupeptin and aprotinin. Extracts were cleared by centrifugation and precipitated with polyclonal rabbit H-86 anti-insulin (cat. no. sc-9168, Santa Cruz Biotechnology) and protein G sepharose. Precipitates were processed for SDS-PAGE (15%) and subsequent fluorography of the gels. Alternatively, non-labeled cells were lysed in a SDS-containing buffer (3% SDS, 50 mM Tris-hydrochloride, 5% \(\beta\)-mercaptoethanol) and, for high resolution of protein bands, samples were directly loaded onto urea-containing SDS-polyacrylamide gels (16%) [32]. Gels were blotted onto a nitrocellulose membrane using the iBlot\textsuperscript{TM} Dry Blotting System (cat. no. IB3010-01; Invitrogen, Carlsbad, CA, USA). Membranes were blocked for 20 min at RT in a buffer supplemented with 0.1% Tween 20, 0.1% gelatine and 3% milk powder, followed by successive incubations with rabbit H-86 anti-insulin antibody and HRP-conjugated anti rabbit IgG (cat. no. NA9340; GE Healthcare, Chalfont St Giles, UK). Specific protein bands were detected using the Immobilon\textsuperscript{TM} Western Chemoluminescent HRP substrate (cat. no. WBKLS0100; Millipore, Bedford, MA, USA) followed by subsequent exposure of the membranes to an Amersham Hyperfilm ECL (cat. no. 92004; GE Healthcare).

Immunization of Mice

Plasmid DNA (75–100 \(\mu\)g/mouse) dissolved in PBS was injected into both tibialis anterior muscles. Diabetes was diagnosed if two consecutive blood glucose values (within 2 days) exceeded 250 mg/dl, i.e. 13.8 mmol/l (Dositronic Freestyle, Sulzbach, Germany).

Histology

H&E staining and immunohistochemistry of pancreatic sections was performed as described previously [16]. For the staining of insulin, CD8\(^{\text{a}}\) or CD4\(^{\text{a}}\) cells the following primary antibodies were used: polyclonal guinea pig anti insulin serum (cat. No. A0564; Dako, Carpinteria, CA, USA), rat \(\alpha\)-CD8 (cat. No. MCA2694;
AbD Serotec, Oxford, UK) and rat α-CD4 (cat. No. MCA1767GA; AbD Serotec). These primary antibodies were detected with the secondary antibodies anti guinea pig IgG-FITC (cat. No. F-6261; Sigma-Aldrich, St Louis, MO, USA) and anti rat IgG-TRITC (cat. No. T4280; Sigma-Aldrich). Furthermore, sections were directly stained with PE-conjugated antibodies α-F4/80 (cat. No. 12-4001-80; eBioscience, Frankfurt, Germany) and α-CD11c (cat. No. 553802; BD Biosciences, Heidelberg, Germany). Sections were covered and mounted with Cytoseal60 mounting medium (cat. no. 10006, EMD). Finally, the images were captured with an Olympus IX71 fluorescence microscope equipped with a digital camera (C4742, Hamamatsu). Edition of the pictures was performed using ImageJ software (http://rsbweb.nih.gov/ij/).

Isolation of CD8 T-cells from Pancreatic Tissues
Pancreata were perfused in situ with collagenase P (cat. no. 1213856001, Roche) dissolved at 1 mg/ml in Hanks Balanced Salt Solution (HBSS), removed, digested again with collagenase P for 8 min at 37 °C and washed twice with cold HBSS supplemented with 10% FCS. Pancreatic cells were purified with Histopaque-1077 (cat. no. 10771, Sigma-Aldrich, Taufkirchen, Germany). Cells were harvested, surface stained with APC-conjugated anti CD8 antibody (cat. no. 17-0081-83, BD Biosciences, Heidelberg, Germany), fixed with 2% paraformaldehyde, resuspended in permeabilization buffer (HBSS, 0.5% BSA, 0.5% saponin, 0.05% sodium azide), and stained with FITC-conjugated anti-IFN-γ antibody (cat. no. 554411; BD Biosciences, Heidelberg, Germany). Non-specific binding of antibodies to Fc-receptor was blocked by preincubating cells with mAb 2.4G2 (cat. no. 12-4041; BD Biosciences, Heidelberg, Germany) directed against the FcγRIII/II CD16/CD32 (0.5 μg mAb/10⁶ cells/100 μl). Frequencies of IFNγ⁺ CD8 T-cells were determined by flow cytometry (FCM) using a BD LSR-II Flow Cytometer.

Furthermore, specific CD8 T-cells were analyzed with Kb/B22-29 tetramers (Glycotope, Heidelberg, Germany). Freshly isolated cells were washed twice in PBS/0.3% w/v BSA/0.1% w/v sodium azide. Non-specific binding of antibodies to Fc-receptor was blocked by preincubating cells with mAb 2.4G2 as described above. Cells were incubated for 30 min at 4 °C with FITC-labeled anti-CD8 mAb (BD Biosciences, Heidelberg, Germany) and PE- or APC-conjugated tetramers. Cells were washed and analyzed by FCM.

Results

Induction of Distinct ppins-specific CD8 T-cells is Critically Dependent on the Antigen Used
A single injection of pCI/ppins plasmid DNA (Figure 1A) efficiently induced CD8 T-cell-mediated EAD in RIP-B7.1 tg mice (Figure 1B and C) [19]. CD8 T-cells isolated from pancreata of ppins-primer, diabetic RIP-B7.1 tg mice recognized the Kb⁻⁻⁻restricted A₁₂–21-i.e., ppins₁₀₁–₁₁₀-epitope of ppins and, with a better efficacy, an epitope variant (A₁₂–Ν₂₁Α) with an alanine (Α) exchange for the COOH-terminal asparagine (Ν) at position Α₂₁ (Figure 1A) [19]. Similarly, a pCI/ppinsΔ₁₂–21 DNA encoding a truncated ppins protein without the COOH-terminal Kβ/B₂₂–₂₉-epitope; Figure 1A) also induced severe hyperglycemia and diabetes in RIP-B7.1 tg mice (Figure 1B and C) [18]. The kinetics and diabetes incidences were comparable in pCI/ppins- and pCI/ppinsΔ₁₂–₂₁-immune RIP-B7.1 tg mice (Figure 1B and C).

CD8 T-cells isolated from pancreata of pCI/ppinsΔ₁₂–₂₁-primer and diabetic RIP-B7.1 tg mice specifically recognized the overlapping ppins₄₅–₅₄ and ppins₄₇–₅₆-peptides of a ppins library (i.e., 10mers with two amino acids offset; Figure 1D). These sequences contain an optimal Kβ-binding motif, i.e., Y at anchor position P5 and M at anchor position P8 [33]. Ex vivo restimulation of CD8 T-cells with this antigenic ppins₄₆–₅₃ (B₂₂–₂₉) peptide revealed a CD8 T-cell population with specifically inducible IFN-γ expression in pCI/ppinsΔ₁₂–₂₁- (but not in pCI/ppins+-) immune and diabetic RIP-B7.1 tg mice (Figure 1E, groups 2 and 3; Table S1). We could exclude that a simple immune competition between Kβ/Α₁₂–₂₁- and Kβ/B₂₂–₂₉-specific CD8 T-cells [34] limits the priming and expansion of Kβ/B₂₂–₂₉-specific CD8 T-cells in pCI/ppins-immune RIP-B7.1 tg mice. Kβ/Α₁₂–₂₁- and Kβ/B₂₂–₂₉-specific CD8 T-cells were efficiently co-primed when pCI/ppins and pCI/ppinsΔ₁₂–₂₁ plasmids were co-injected into different sites of the same mouse (Figure 1E, group 4). Comparable numbers of Kβ/Α₁₂–Ν₂₁Α- and Kβ/B₂₂–₂₉-specific IFNγ⁺ CD8 T-cells were detectable in the pancreata of diabetic RIP-B7.1 tg mice immunized with pCI/ppins, pCI/ppinsΔ₁₂–₂₁ or both, pCI/ppins+π/pπsΔ₁₂–₂₁ vectors, respectively (Figure 1E, groups 2-4; Table S1). Hence, after successful priming, the two CD8 T-cell populations do not interfere with each another. Furthermore, immunization of MHC class II-deficient (A⁻⁻⁻) mice with pCI/ppinsΔ₁₂–₂₁-immune RIP-B7.1 tg mice (RIP-B7.1) with pCI/ppinsΔ₁₂–₂₁ vectors (Figure 1E, group 4) efficiently induced EAD. This showed that diabetogenic ppins/(Kβ/Α₁₂–₂₁)- and ppinsΔ₁₂–₂₁/i.e., pCI/ppinsΔ₁₂–₂₁/(Kβ/B₂₂–₂₉)-specific CD8 T-cell responses do not require CD4 T-cell help. The novel insulin B-chain epitope B₂₂–₂₉ efficiently stabilized the class I molecules Kβ on the surface of TAP-deficient RMA-S cells (Figure 2A) [35]. The B₂₂–₂₉ epitope stabilized Kβ-molecules more efficiently than the A₁₂–₂₁ or mutant A₁₂–Ν₂₁Α epitopes (Figure 2A, data not shown) and we could generate Kβ/B₂₂–₂₉- (but neither Kβ/Α₁₂–₂₁- nor A₁₂–Ν₂₁Α) specific dimers or tetramers (Figure 2B; data not shown). Kβ/B₂₂–₂₉-tetramer⁺ CD8 T-cells were specifically detectable in pCI/ppinsΔ₁₂–₂₁- (but not in pCI/ppins+-) primed and diabetic RIP-B7.1 tg mice (Figure 2B and C). During the course of EAD, the development of hyperglycemia correlated with an increasing influx of lymphocytes and CD8 T-cells into the pancreatic islets (Figure 2C, groups 1–3). In diabetic mice (with blood glucose levels between 400 and 550 mg/dl) 0.8–2.0×10⁶ Kβ/B₂₂–₂₉-tetramer⁺ CD8 T-cells were detectable in the pancreata. This corresponds to 7–12% of all pancreas-infiltrating CD8 T-cells (Figure 2C, group 3). The influx of Kβ/B₂₂–₂₉-specific CD8 T-cells into the pancreata thus specifically correlated with the development of disease in ppinsΔ₁₂–₂₁-immune RIP-B7.1 tg mice.

Statistics
The statistical significance of differences in the mean CD8 T-cell frequencies between groups was determined by the unpaired student’s t-test. The statistical significance of diabetes induction in immunized mice was determined by the log-rank test. Data were analyzed using PRISM software (GraphPad, San Diego, CA). Values of P<0.05 were considered significant.
Characterization of Antigen Expression Requirements that Favour Priming of K\textsuperscript{b}/B22–29-Specific CD8 T-cells and EAD by DNA-based Immunization

The efficient induction of K\textsuperscript{b}/B22–29-specific CD8 T-cells and EAD by mutant ppins\textsubscript{D}A12–21 was unexpected because immunization with different insulin B-chain-encoding vectors did not (or very inefficiently) induce EAD in RIP-B7.1 tg mice. A pCI/SP-B construct (encoding the ER-targeting signal peptide and the insulin B-chain; Figure 3A), inefficiently induced late EAD in one out of eight RIP-B7.1 tg mice (Figure 3B, group 2). Similarly, a pCI/SP-B-C construct (encoding the ER-targeting signal peptide up to the C-peptide; Figure 3A) did not induce EAD in RIP-B7.1 tg mice within three months post immunization (Figure 3B, group 3). We thus conclude that efficient priming of K\textsuperscript{b}/B22–29-reactive CD8 T-cells by pCI/ppins\textsubscript{D}A12–21 critically depends on specific properties of the mutant antigen itself.

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We next characterized the expression of mutant ppins\textsubscript{D}A12–21 and ppins in transiently transfected HEK-293 cells (Figure 3C and D). In these non-beta cells, the ppins signal peptide (SP) targets the proteins into the ER, where the SP is removed to generate proinsulin (pins) or pins\textsubscript{D}A12–21 but further downstream processing of pins to insulin was not detectable [17;36]. The expression levels of \textsuperscript{35}S-methionine/cysteine labeled pins\textsubscript{D}A12–21 were lower than that of pins (Figure 3C, lanes 2 and 3) and significant steady-state levels of pins (but not of mutant pins\textsubscript{D}A12–21) were detectable by specific western blot analyses (Figure 3D, lanes 1 and 2).
CD8 T-Cell-Mediated Induction of Diabetes

A  
RMA-S cells pulsed with:
- /-
- K\(^{b}\)/A\(_{12-N21A}\)
- K\(^{b}\)/B\(_{22-29}\)

RIP-B7.1 tg mice immunized with:
- pCI
- pCI/pps
- pCI/pps\(_{A_{12-21}}\)

B

CD8+

C

RIP-B7.1 tg mice immunized with:
- pCI/pps
- pCI/pps\(_{A_{12-21}}\)

blood glucose levels:
1) <200 mg/dl (healthy)
2) 250-350 mg/dl
3) 400-560 mg/dl

H&E

CD8+
However, treatment of transfectants with the proteasome inhibitors epoxomicin or lactacystin efficiently restored pins\textsubscript{DA12–21} levels within 6 hours (Figure 3D, lanes 2 to 4). This showed that pins\textsubscript{DA12–21} is efficiently processed by proteasomal degradation. In contrast, the expression of ppins in transiently transfected HEK-293 cells was not changed by proteasome inhibitors [18]. This implies that proteasomes play an essential role in the pCI/ppins\textsubscript{DA12–21}-specific antigen processing/presentation and the induction of Kb/B22–29 specific CD8 T-cells.
Differential Regulation of Diabetogenic K<sup>b</sup>/A<sub>12–21</sub>- and K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cell Responses in Coinhibition-deficient PD-L1~/~/ Mice

Coinhibition interactions of PD-1 (expressed on T-cells) with PD-L1 (expressed on APCs) inhibit T-cell activation and promote induction of peripheral T-cell tolerance [23,25]. We used coinhibition-deficient PD-L1~/~/ [29] and PD-1~/~/ [30] mice to determine whether EAD is equally induced by ppins- and ppinsΔ<sub>12–21</sub>-specific CD8 T-cells. Immunization of PD-L1~/~/ mice with pCl/pipins efficiently induced CD8 T-cell-mediated EAD (Figure 4A, left panel, group 2) [19]. Comparable with RIP-B7.1 tg mice, a K<sup>b</sup>/A<sub>12–21</sub>-monospecific CD8 T-cell response was detectable in pCl/ppins-primed and diabetic PD-L1~/~/ mice (Figure 4A, middle panel), and K<sup>b</sup>/B<sub>22–29</sub>-specific tetramer<sup>+</sup> CD8 T-cells were not detectable (Figure 4A, right panel, group 2). Unexpectedly, PD-L1~/~/ mice did not develop EAD after single or repeated immunizations with pCl/ppinsΔ<sub>12–21</sub> (Figure 4A, left panel, group 3; data not shown). We could neither detect T-cell infiltrations into the pancreatic islets (Figure S2A) nor K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells in these healthy mice (Figure 4A, right panel, group 3; Table S1). We could not induce EAD in PD-L1~/~/ mice after immunization with pCl/ppinsΔ<sub>12–21</sub> and acutely depletion of regulatory CD25<sup>+</sup> T<sub>reg</sub>-cells by anti CD25 antibody treatment (data not shown) [37]. It is thus unlikely that T<sub>reg</sub>-cells inhibit the outcome of K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells in ppinsΔ<sub>12–21</sub>-immune PD-L1~/~/ mice. Similarly, coinhibition-deficient PD-1~/~/ mice efficiently developed EAD after immunization with pCl/ppins [19] but not pCl/ppinsΔ<sub>12–21</sub> (Figure S3). An imbalance between PD-1/PD-L1-interactions thus facilitated development of EAD by pCl/ppins/Δ<sub>12–21</sub> but not pCl/ppinsΔ<sub>12–21</sub>-coimmunized PD-L1~/~/ mice.

K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells were either inefficiently primed in PD-L1~/~/ and PD-1~/~/ mice by pCl/ppinsΔ<sub>12–21</sub> and/or inefficiently expanded and targeted to the pancreatic islets. We next generated PD-1-deficient mice, which selectively express the costimulatory B7.1 molecule on beta cells (RIP-B7.1~/~/PD-L1~/~/) by crossing PD-L1~/~/ with RIP-B7.1 tg mice. These mice differ from PD-L1~/~/ mice only in the tg B7.1 expression in beta cells. Interestingly, immunization of RIP-B7.1~/~/PD-L1~/~/ mice with pCl/ppinsΔ<sub>12–21</sub> efficiently induced EAD and high frequencies of K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells accumulated in the pancreata of diabetic mice (Figure 4B, groups 2). Notably, pCl/ppins/Δ<sub>12–21</sub>-specific CD8 T-cells efficiently induced EAD in both, PD-L1~/~/ and RIP-B7.1~/~/PD-L1~/~/ mice (Table S1) [19]. K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells thus require B7.1-mediated costimulatory signals from PD-L1-deficient beta cells to expand and/or develop their diabetogenic potential.

A Concomitant ppins/(K<sup>b</sup>/A<sub>12–21</sub>)-specific CD8 T-cell Response in PD-L1~/~/ Mice Facilitates Induction of ppinsΔ<sub>12–21</sub>-(K<sup>b</sup>/B<sub>22–29</sub>)-specific CD8 T-cells

We next asked whether alternative (B7.1 tg-independent) beta cell-mediated signals could trigger the diabetogenic ppinsΔ<sub>12–21</sub>-(K<sup>b</sup>/B<sub>22–29</sub>)-specific CD8 T-cell response in PD-L1~/~/ mice. It was well established that an initial damage or destruction of beta cells by autoreactive T-cells induces a complex inflammatory milieu in the islets, thereby attracting different non-specific “bystander” cells [38,39,40]. These events play a prominent role in the amplification of autoimmune immune responses and beta cell destruction [38,39,40]. We thought that an initial islet-destructive K<sup>b</sup>/A<sub>12–21</sub>-specific CD8 T-cell response (primed in PD-L1~/~/ mice by pCl/ppins) could facilitate the expansion of pCl/ppinsΔ<sub>12–21</sub>-coprimed K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells. To test this assumption, we co-immunized PD-L1~/~/ mice with both, pCl/ppins and pCl/ppinsΔ<sub>12–21</sub> vectors (pCl/ppins+pCl/ppinsΔ<sub>12–21</sub>) into the left and right tibialis anterior muscles, respectively. These mice efficiently developed an early and severe EAD (Figure 4C, left panel, group 2). Comparable with pCl/ppins-immune and diabetic PD-L1~/~/ mice, high numbers of K<sup>b</sup>/A<sub>12–21</sub>-specific CD8 T-cells were detectable in pCl/ppins+pCl/ppinsΔ<sub>12–21</sub>-coimmunized and diabetic PD-L1~/~/ mice (see Figure 4A; data not shown). In these mice, we found a significant influx of CD8 T-cells (Figure S2B) and other bystander cells (e.g., CD4+ T-cells, macrophages, DCs) into or closely attached to the pancreatic islets of early diabetic PD-L1~/~/ mice (Figure 5). The inflammatory islet invasion by these cell populations was comparable in pCl/ppins- and pCl/ppins+pCl/ppinsΔ<sub>12–21</sub>-immune and diabetic PD-L1~/~/ mice (data not shown). Most interestingly, high numbers of K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells accumulated in the pancreata of pCl/ppins+pCl/ppinsΔ<sub>12–21</sub>-coimmunized PD-L1~/~/ mice (Figure 4C, right panel, group 2). Because K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells were detectable in pCl/ppins+pCl/ppinsΔ<sub>12–21</sub>-coimmunized PD-L1~/~/ mice, but not in PD-L1~/~/ mice injected with the individual ppins- and ppinsΔ<sub>12–21</sub>-encoding vectors (Figure 4A and C; Table S1), their expansion was apparently induced by events triggered by the initial ppins/(K<sup>b</sup>/A<sub>12–21</sub>)-specific CD8 T-cell response.

Discussion

We continued work on the specific priming of ppins-specific CD8 T-cells and EAD in RIP-B7.1 tg mice [16,17,18,19]. DNA-based immunization of RIP-B7.1 tg mice revealed two monospecific CD8 T-cell responses that were exclusively induced by either pCl/ppins (primed K<sup>b</sup>/A<sub>12–21</sub>-specific CD8 T-cells) or pCl/ppinsΔ<sub>12–21</sub> (primed K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells). This indicated that the mutant ppinsΔ<sub>12–21</sub> antigen (but not the ppins) efficiently induced K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells in RIP-B7.1 tg mice. We further characterized the antigen expression requirements that favour in vivo priming of K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells and EAD by DNA-based immunization. Different insulin B-chain-encoding vectors (pCl/SP-B or pCl/SP-B-C) did not or very inefficiently induce K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells and EAD in RIP-B7.1 tg mice (Figure 3A and B). Deletion of the A<sub>12–21</sub> sequence may thus generate a specifically folded ppinsΔ<sub>12–21</sub> antigen, which is efficiently processed for K<sup>b</sup>/B<sub>22–29</sub>-specific epitope presentation. Expression analyses in transiently transfected HEK-293 cells showed that ppinsΔ<sub>12–21</sub> (but not ppins) is efficiently processed by proteasomes, resulting in a high turnover expression of this mutant antigen. Proteasomes could thus play an essential role in the generation/presentation of the K<sup>b</sup>/B<sub>22–29</sub>-specific epitope and the induction of K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells by pCl/ppinsΔ<sub>12–21</sub>.

We here showed that K<sup>b</sup>/B<sub>22–29</sub>-specific CD8 T-cells are efficiently primed by ppinsΔ<sub>12–21</sub>- but not ppins-expressing vectors. This indicates that ppins is inefficiently processed for K<sup>b</sup>/B<sub>22–29</sub> epitope presentation in non-beta cells targeted by intra-muscular DNA injection. However, the K<sup>b</sup>/B<sub>22–29</sub> epitope is efficiently processed and presented in vivo by ppins-insulin-expressing beta cells because ppinsΔ<sub>12–21</sub>-(K<sup>b</sup>/B<sub>22–29</sub>)-monospecific CD8 T-cells specifically recognize and destroy these cells and induce fulminant EAD in RIP-B7.1 tg mice (Figure 1). The efficient beta cell-specific presentation of the K<sup>b</sup>/B<sub>22–29</sub> epitope could be explained by different antigen expression and/or processing mechanisms, operating in ppins/insulin producing
beta cells and in ppins-expressing non-beta APCs [36]. However, further studies are needed to define the inefficient processing of ppins in non-beta cells and/or the inefficient induction of Kb/B22–29-specific CD8 T-cells by pCI/ppins.

We consider the differential regulation of Kb/A12–21- and Kb/B22–29-monospecific CD8 T-cell responses (and EAD) the key observation of this report. Kb/A12–21-monospecific CD8 T-cells and EAD were efficiently induced in RIP-B7.1 tg and coinhibition-deficient PD-L1/-/- mice by pCI/ppins, whereas Kb/B22–29-specific CD8 T-cells and EAD were efficiently induced in RIP-B7.1 tg (but not in PD-L1/-/- or PD-1/-/-) mice by pCI/ppinsΔA12-21. The missing coinhibition in PD-L1/-/- or PD-1/-/- mice is thus sufficient to induce and expand vector-primed Kb/B22–29-specific CD8 T-cells. PD-L1 expressed by antigen presenting beta cells can interact with PD-1 or B7.1 expressed by CD8 T-cells to inhibit immune responses [25,41]. Interestingly, expression of PD-L1 has no impact on the priming of Kb/A12–21-specific CD8 T-cells in PD-L1-competent wt B6 mice. However, ppins-immune B6 mice rapidly developed EAD after treatment with anti PD-L1 antibody [19]. This suggested that PD-L1-mediated signals delivered by pancreatic beta cells are sufficient to regulate their susceptibility for the destructive Kb/A12–21-specific CD8 T-cell attack [19].

We found no evidence for an autoreactive immune response in pCI/ppinsΔA12-21-immune and healthy PD-L1/-/- or PD-1/-/- mice. Interestingly, a single manipulation of the PD-L1 mouse model (i.e., the tg expression of the costimulatory B7.1 molecule in beta cells) restored the induction of Kb/B22–29-specific CD8 T-cells and EAD in these RIP-B7.1/-/PD-L1/-/- mice by pCI/ppinsΔA12-21. It is thus unlikely that the initial CD8 T-cell priming phase (i.e., intramuscular injection of pCI/ppinsΔA12-21 DNA;
local antigen expression/processing and K̂/B22−29-specific epitope presentation in PD-L1-deficient myocytes and professional APCs; priming of PD-L1-deficient K̂/B22−29-specific CD8 T-cells differs in RIP-B7.1+/PD-L1−/− and PD-L1−/− mice. Expression of the tg B7.1 costimulator molecule by PD-L1-deficient beta cells is thus a key event that decides whether a K̂/B22−29-specific T-cell response can progress and develop a functional pathogenic phenotype. This implies that primed K̂/B22−29-specific CD8 T-cells must directly interact with RIP-B7.1+ beta cells to expand and/or develop their diabetogenic potential. B7.1 on the surface of beta cells could bind in trans to CD28 costimulator molecule or CTLA-4/PD-L1 coinhibitor molecules [21]. CD28-deficient RIP-B7.1+/CD28−/− mice do not develop EAD after immunization with pCI/ppins [19] or pCI/ppinsDA12−21 (data not shown). The interaction of B7.1 on the surface of beta cells with CD28 costimulator molecules on CD8 T-cells may thus promote T-cell-driven EAD in RIP-B7.1+ mice by facilitating effector function delivery but a critical effect of CD28 in CD8 T-cell priming can not be excluded.

We here identified an alternative mechanism to promote the expansion and influx of diabetogenic K̂/B22−29-specific CD8 T-cells into the pancreas of pCI/ppinsΔA12−21-primed and diabetic PD-L1−/− mice. Co-immunization of PD-L1−/− mice with both, pCI/ppins or pCI/ppinsΔA12−21 vectors efficiently elicited both, K̂/A12−21− and K̂/B22−29−specific CD8 T-cells (Table S1). This suggested that the initial beta cell destruction, triggered by pCI/ppins/(K̂/A12−21)-specific CD8 T-cells in PD-L1−/− mice, facilitates expansion and invasion of K̂/B22−29-specific CD8 T-cells but also other bystander cells into the pancreatic target tissue. The specific molecular mechanisms and signals expanding and attracting K̂/B22−29−specific CD8 T-cells to the pancreas and the role of bystander cells are not well understood [38,39,40]. An initial beta cell death and antigen release could facilitate activation of circulating, pCI/ppinsDA12−21-preprimed K̂/B22−29-specific CD8 T-cells in the regional lymph nodes by professional APCs [42]. Furthermore, an altered local cytokine milieu and expression of cell surface receptors [40] or an enhanced antigen presentation by beta cells [43] in inflamed islets may favour the attraction and/or activation of K̂/B22−29−specific CD8 T-cells. Taken together, our findings suggested that the K̂/A12−21−specific CD8 T-cell response directly initiates beta cell destruction in PD-L1−/− mice, whereas a downstream K̂/B22−29−specific CD8 T-cell response requires additional activation signals in vivo and emerge during the pathogenic destruction of beta cells. Interestingly, distinct hierarchies of diabetogenic T-cell responses were also detectable in the NOD mouse model. The insulin B9–23 domain, containing both, a dominant CD4 and a K̂-restricted B15–23 CD8 T-cell epitope, plays a prominent role in the diabetes development in NOD mice.

Figure 5. Recruitment of different ‘bystander’ cell populations into the pancreatic target tissue. PD-L1−/− mice were immunized with both, pCI/ppins+pCI/ppinsΔA12−21 vectors into the right and the left tibialis anterior muscles, respectively. Pancreata of representative healthy (at 3 days post immunization) (A) or early diabetic mice (at 15–20 days post immunization) (B) were analyzed histologically for insulin expression (insulin) and influx of CD4+ T-cells (CD4+), macrophages (F4/80+) or DCs (CD11c+).

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Prasad et al. previously showed that an initial insulin B9–23-specific T-cell response is immunodominant and autoimmune responses to epitope(s) distinct from B9–23 emerge during the pathogenic progression of diabetes in NOD mice [46]. Similarly, CD8 T-cells specific for the id-type-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) were detected in NOD mice but not in tg NOD mice tolerant to proinsulin, indicating that an initial T-cell response against proinsulin is necessary for the development of IGRP-specific CD8 T-cells [47]. This suggests that distinct CD8 T-cell responses, triggering the initial steps of beta cell destruction, play a prime role in the induction of diabetes [44].

In conclusion, we showed in this study that inhibitory interactions between ppins/insulin-presenting beta cells and autoreactive CD8 T-cells either allow or prevent activation of pathogenic effector responses. K\textsuperscript{\textbeta}B\textsubscript{29–29}-reactive CD8 T-cells additionally require stimulatory signals from beta cells or inflamed islets to expand and develop their diabetogenic potential in PD-L1-deficient mice. In contrast, CD8 T-cells directed against the weak Kb/A12–21 epitope (binding Kb molecules with a relatively low avidity as compared with the K\textbeta/B\textsubscript{29–29} epitope; see Figure 2A) do not depend on the tg B7.1-mediated help to reveal their diabetogenic potential in ppins-immune PD-L1−/− or PD-L1−/− mice. Differences in the MHCH-binding avidity of ppins-derived epitopes may thus have a strong impact on the regulation of autoreactive CD8 T-cell responses in PD-1− or PD-L1-deficient mice. The novel PD-1/PD-L1 diabetes models are thus valuable tools to study under well controlled experimental conditions the induction and regulation of autoreactive CD8 T-cell responses.

Supporting Information

Figure S1 Induction of EAD in MHC II-deficient RIP-B7.1 tg mice. MHC class II-deficient RIP-B7.1 tg mice (RIP-B7.1/MHCII−/−) were immunized with pCI (group 1, n = 3) or pCI/ppins\textalpha\textbeta\textsubscript{12–21} (group 2, n = 3, n = 3). At indicated times after immunization, blood glucose levels and cumulative diabetes incidences were determined.

Figure S2 Ppins/(K\textbeta\textalpha\textbeta\textsubscript{12–21})-mediated recruitment of autoreactive T-cells into the pancreatic target tissue. PD-L1−/− mice were immunized with pCI/ppins\textalpha\textbeta\textsubscript{12–21} (A) or with both, pCI/ppins+pCI/ppins\textalpha\textbeta\textsubscript{12–21} vectors into the right and the left tibial anterior muscles, respectively (B). Pancreata of representative healthy (A) and early diabetic mice (B) were analyzed histologically for insulin expression (insulin) and influx of CD8 T-cells (CD8\textalpha), or stained with hematoxylin-eosin (H&E).

Table S1 Induction of autoreactive CD8 T-cell responses and EAD in RIP-B7.1+ mice. doi:10.1371/journal.pone.0071746.t001

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Author Contributions

Conceived and designed the experiments: CS HB KS RS. Performed the experiments: CS HB KS. Analyzed the data: CS HB KS. Wrote the paper: RS. Contributed to discussion and reviewed/edited manuscript: BOB.

References

1. Zhang L, Nakayama M, Eisenbarth GS (2008) Insulin as an autoantigen in NOD/human diabetes. Curr Opin Immunol 20: 111–118.
2. Luo X, Herold KC, Miller SD (2010) Immunotherapy of type 1 diabetes targeting the long and winding road to understanding and conquering type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J Clin Invest 118: 3390–3402.
3. Santamaria P (2010) The long and winding road to understanding and conquering type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J Clin Invest 118: 3390–3402.
4. Jeker LT, Bour-Jordan H, Bluestone JA (2012) Breakdown in peripheral T-cell unresponsiveness. Proc Natl Acad Sci USA 109: 18425–18430.
5. Pinkie GG, Tyma OH, Bergen CA, Kester MG, Ossendorp F, et al (2005) Autoimmune CD8 T-cells associated with beta cell destruction in type 1 diabetes. Proc Natl Acad Sci USA 102: 10425–10430.
6. Mallone R, Martinuzzi E, Blancou P, Novelli G, Alonso G, et al (2007) CD8 T-cell responses identify beta-cell autoimmunity in human type 1 diabetes. Diabetes 56: 615–621.
7. Skowera A, Ellis RJ, Varela-Calvino R, Arif S, Huang GC, et al (2008) CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J Clin Invest 118: 3390–3402.
8. Stadinski B, Kappler J, Eisenbarth GS (2010) Molecular targeting of islet autoantigens. Immunity 32: 446–456.
9. Skowera A, Ellis RJ, Varela-Calvino R, Arif S, Huang GC, et al (2008) CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J Clin Invest 118: 3390–3402.
10. Wong FS, Moustakas AK, Wen L, Papadopoulos GK, Janeway CA (2002) Deficiency in B7-H1 (PD-L1)/PD-1 coinhibition triggers pancreatic beta cell destruction by insulin-specific, murine CD8 T cells. Diabetes 59: 1966–1973.
11. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ (2007) The function of PD-1 and its ligands in infection. Nat Immunol 8: 239–245.
12. Brosi H, Reiser M, Rajasalu T, Spryantis A, Wieland A, Boehm B et al (2007) The diabetogenic, insulin-specific CD8 T cell response primed in the experimental autoimmune diabetes model in RIP-B7.1 mice. Eur J Immunol 37: 2097–2103.
13. Mohan JF, Petzold SJ, Unanue ER (2011) Register shifting of an insulin peptide-binding register. Proc Natl Acad Sci USA 107: 10978–10983.
14. von Herrath M, Nepom GT (2009) Animal models of human type 1 diabetes. Nat Immunol 10: 129–132.
15. Haslan IM, Hemenger H, Huang ML, Kang VH, Abe R et al (1994) Mice expressing both B7-1 and viral glycoprotein on pancreatic beta cells along with glycoprotein-specific transgenic T cells develop diabetes due to a breakdown of T-lymphocyte unresponsiveness. Proc Natl Acad Sci USA 91: 5309–5314.
16. Karges W, Pechhold K, Al Daboul S, Rieger I, Rief M et al (2008) Induction of autoimmune diabetes through insulin (but not GAD65) DNA vaccination in nonobese diabetic and in RIP-B7.1+ mice. Diabetes 57: 3237–3244.
17. Karges W, Rajasalu T, Spryantis A, Wieland A, Boehm B et al (2007) The diabetogenic, insulin-specific CD8 T cell response primed in the experimental autoimmune diabetes model in RIP-B7.1 mice. Eur J Immunol 37: 2097–2103.
18. Brosi H, Reiser M, Rajasalu T, Spryantis A, Oswald F et al (2009) Processing in the endoplasmic reticulum generates an epitope on the insulin A chain that stimulates diabetogenic CD8 T cell responses. J Immunol 183: 7187–7195.
19. Rajasalu T, Brosi H, Schuster C, Spryantis A, Boehm BO, et al (2010) Deficiency in B7-H1 (PD-1)/PD-1 coinhibition triggers pancreatic beta cell destruction by insulin-specific, murine CD8 T cells. Diabetes 59: 1966–1973.
20. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ (2007) The function of programmed cell death 1 and its ligands in regulating autoimmune and infectious. Nat Immunol 8: 239–245.
21. Keir ME, Butte MJ, Freeman GJ, Sharpe AH (2008) PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 26: 677–704.
22. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ et al (2009) PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. J Exp Med 206: 3015–3029.
23. Ansari MJ, Salama AD, Chitnis T, Smith RN, Yagita H et al (2003) The PD-1/PD-L1 pathway regulates the development and regulation of autoreactive T cells. J Exp Med 202: 1219–1229.
24. Fife BT, Guleria I, Gubbels BM, Eagar TN, Tang Q et al (2006) Insulin-induced dysregulation. Annu Rev Immunol 23: 447–485.
25. Karges W, Rajasalu T, Spryantis A, Wieland A, Boehm B et al (2007) The diabetogenic, insulin-specific CD8 T cell response primed in the experimental autoimmune diabetes model in RIP-B7.1 mice. Eur J Immunol 37: 2097–2103.
25. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A et al (2006) Tissue expression of PD-L1 mediates peripheral T cell tolerance. J Exp Med 203: 883–895.
26. Guleria I, Gubbels BM, Dada S, Fife B, Tang Q, et al (2007) Mechanisms of PD-L1-mediated regulation of autoimmune diabetes. Clin Immunol 125: 16-25.
27. Nielsen C, Hansen D, Husby S, Jacobsen BB, Lillevang ST (2003) Association of a putative regulatory polymorphism in the PD-1 gene with susceptibility to type 1 diabetes. Tissue Antigens 62: 492–497.
28. Ni R, Ibara K, Miyake K, Kurnatowski R, Inoue M et al (2007) PD-1 gene haplotype is associated with the development of type 1 diabetes mellitus in Japanese children. Hum Genet 121: 223–232.
29. Dong H, Zhu G, Tamada K, Fiers DB, van Deursen JM et al (2004) B7-H1 determines accumulation and deletion of intrahepatic CD8(+) T lymphocytes. Immunity 20: 327–336.
30. Nishimura H, Minato N, Nakano T, Honjo T (1998) Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. Int Immunol 10: 1563–1572.
31. Kourtzen F, Suss G, Stewart C, Steinmetz M, Bluethmann H (1993) Targeted disruption of the MHC class II Aa gene in C57BL/6 mice. Int Immunol 5: 957–964.
32. Schagger H (2006) Tricine-SDS-PAGE. Nat Protoc 1: 16–22.
33. Kammensee HG, Friede T, Stevanovic S (1995) MHC ligands and peptide motifs: first listing. Immunogenetics 41: 178–228.
34. Kedl RM, Kappler JW, Marrack P (2003) Epitope dominance, competition and T cell affinity maturation. Curr Opin Immunol 15: 120–127.
35. Ljunggren HG, Stam NJ, Ohlen C, Neefjes JJ, Hoglund F et al (1990) Empty MHC class I molecules come out in the cold. Nature 346: 476–480.
36. Sneathen SP, Montag AG, Thomas G, Alligres-Rao C, Carroll R et al (1992) Proinsulin processing by the subtilisin-related proprotein convertases furin, PC2, and PC3. Proc Natl Acad Sci USA 89: 8022–8026.
37. Sakaguchi S (2005) Naturally arising Foxp3-expressing CD25(+)CD4(+) regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 6: 345–352.
38. Wong FS, Siew LK, Wen L (2008) CD8(+) T-cells and their interaction with other cells in damage to islet beta-cells. Biochem Soc Trans 36: 316–320.
39. Lennon GP, Bettini M, Burton AR, Vincent E, Arnold PY et al (2009) T cell islet accumulation in type 1 diabetes is a tightly regulated, cell-autonomous event. Immunity 31: 643–653.
40. Calderon B, Uyarne ER (2012) Antigen presentation events in autoimmune diabetes. Curr Opin Immunol 24: 119–128.
41. Paterson AM, Brown KE, Keir ME, Yanguri VK, Riella LV et al (2011) The programmed death-1 ligand 1-B7-1 pathway restrains diabetogenic effector T cells in vivo. J Immunol 187: 1097–1105.
42. Turley S, Poirier L, Hattori M, Benoist C, Mathis D (2003) Physiological beta cell death triggers priming of self-reactive T cells by dendritic cells in a type-1 diabetes model. J Exp Med 198: 1527–1537.
43. Lang KS, Recher M, Junt T, Navarini AA, Harris NL (2005) Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. Nat Med 11: 138–145.
44. Nakayama M, Abaru N, Moriyama H, Bahaya N, Liu E et al (2005) Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. Nature 435: 220–223.
45. Wong FS, Siew LK, Scott G, Thomas IJ, Chapman S et al (2009) Activation of insulin-reactive CD8(+) T-cells for development of autoimmune diabetes. Diabetes 58: 1136–1144.
46. Prasad S, Kohm AP, McMahon JS, Luo X, Miller SD (2012) Pathogenesis of NOD diabetes is initiated by reactivity to the insulin B chain 9–23 epitope and involves functional epitope spreading. J Autoimmun 39: 347–353.
47. Krishnamurthy B, Duleek NL, McKenzie MD, Purcell AW, Brooks AG et al (2006) Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP. J Clin Invest 116: 3258–3265.