# Deficiency in B7-H1 (PD-L1)/PD-1 Coinhibition Triggers Pancreatic β-Cell Destruction by Insulin-Specific, Murine CD8 T-Cells

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**OBJECTIVE—**RIP-B7.1 mice expressing the costimulator molecule B7.1 (CD80) on pancreatic β-cells are a well established model to characterize preproinsulin-specific CD8 T-cell responses and experimental autoimmune diabetes (EAD). Different immunization strategies could prime preproinsulin-specific CD8 T-cells in wild-type C57BL/6 (B6) mice, but did not induce diabetes. We tested whether altering the B7-H1 (PD-L1) coinhibition on pancreatic β-cells can reveal a diabetogenic potential of preproinsulin-specific CD8 T-cells.

**RESEARCH DESIGN AND METHODS—**DNA-based immunization and adoptive T-cell transfers were used to characterize the induction of preproinsulin-specific CD8 T-cell responses and EAD in RIP-B7.1, B6, B7-H1$^{-/-}$, PD-1$^{-/-}$ or bone marrow chimeric mice.

**RESULTS—**Preproinsulin-specific CD8 T-cells primed in B6 mice revealed their diabetogenic potential after adoptive transfer into congenic RIP-B7.1 hosts. Furthermore, preproinsulin-specific CD8 T-cells primed in anti-B7-H1 antibody-treated B6 mice, or primed in B7-H1$^{-/-}$ or PD-1$^{-/-}$ mice induced EAD. Immunization of bone marrow chimeric mice showed that deficiency of either B7-H.1 in pancreatic β-cells or of PD-1 in autoreactive CD8 T-cells induced EAD.

**CONCLUSIONS—**An imbalance between costimulator (B7.1) and coinhibitor (B7-H1) signals on pancreatic β-cells can trigger pancreatic β-cell-destruction by preproinsulin-specific CD8 T-cells. Hence, regulation of the susceptibility of the β-cells for a preproinsulin-specific CD8 T-cell attack can allow or suppress EAD. ***Diabetes*** **59:1966–1973, 2010**

Insulin-producing β-cells in the pancreatic islets are destroyed by an immune attack in autoimmune type 1 diabetes. Type 1 diabetes is triggered by a poorly defined breakdown in central or peripheral tolerance that allows activation of diabetogenic T-cells (1,2). Preclinical animal models have elucidated some aspects of the priming and effector phase of a diabetogenic immune response (3,4). Mice develop diabetes either spontaneously in the NOD model (5), or in response to transgene-encoded "neo-self" antigens selectively expressed in pancreatic β-cells (6–8). These studies indicated that priming of self-reactive T-cells and β-cell susceptibility to an autoaggressive T-cell attack are distinct steps in the pathogenesis of the disease.

Costimulating B7/CD28 family molecules provide critical signals for T-cell activation (9,10). RIP-B7.1 mice express the B7.1 costimulator in pancreatic β-cells under rat insulin promoter (RIP) control (11). We have shown that RIP-B7.1 mice develop CD8 T-cell–dependent experimental autoimmune diabetes (EAD) after immunization with preproinsulin-encoding vectors (12–14). Transgene-driven B7.1 expression in pancreatic β-cells thus makes them susceptible to T-cell–mediated immune attack.

Coinhibitory signals generated by "programmed death-1" (PD-1)/"programmed death-ligand-1" (B7-H1 or PD-L1) interaction downmodulated T-cell responses and maintain self-tolerance in autoimmune diabetes (15,16). Inducible or constitutive expression of B7-H1 is found in many peripheral tissues, including the β-cells of the pancreatic islets (17,18). Ligation of PD-1 (expressed by activated T-cells) to B7-H1 (expressed by epitope-presenting cells) downmodulates T-cell proliferation and IFNγ production (19). Furthermore, B7-H1 interacts specifically with the costimulatory B7.1 (CD80) molecule upregulated by activated T-cells and inhibits their responses (20). PD-1/B7-H1 interaction facilitates establishment of self-tolerance, thereby partially controlling diabetes development in NOD mice (15,21–23). Selective, transgene-driven overexpression of B7-H1 by pancreatic β-cells can, however, result in EAD, suggesting operation of a costimulatory B7-H1 pathway (24).

We investigated the impact of inhibitory (B7-H1, PD-1) molecules on the pathogenicity of preproinsulin-specific CD8 T-cells. We used RIP-B7.1 mice to characterize the specificity and diabetogenic potential of preproinsulin-specific CD8 T-cell responses. RIP-B7.1 mice were immunized with preproinsulin-encoding vectors, or used as hosts for adoptive T-cell transfers. We further analyzed preproinsulin-specific CD8 T-cell responses and EAD development in C57BL/6 (B6), B7-H1$^{-/-}$ (25), or PD-1$^{-/-}$ knockout mice (26), as well as bone marrow chimeras (using different donor T-cell and host β-cell phenotypes of B7-H1/PD-1).

## RESEARCH DESIGN AND METHODS

H-2$^b$ C57BL/6 (B6) mice were obtained from Janvier (Le Genets-St-Isle; France). B6.SJL-Ptprc$^a$Pep3$^b$/BoyJ (CD45.1$^+$ B6) mice (Jackson #002014), B7-H1$^{-/-}$ mice (25), PD-1$^{-/-}$ mice (26), and RIP-B7.1 mice (11) were bred and

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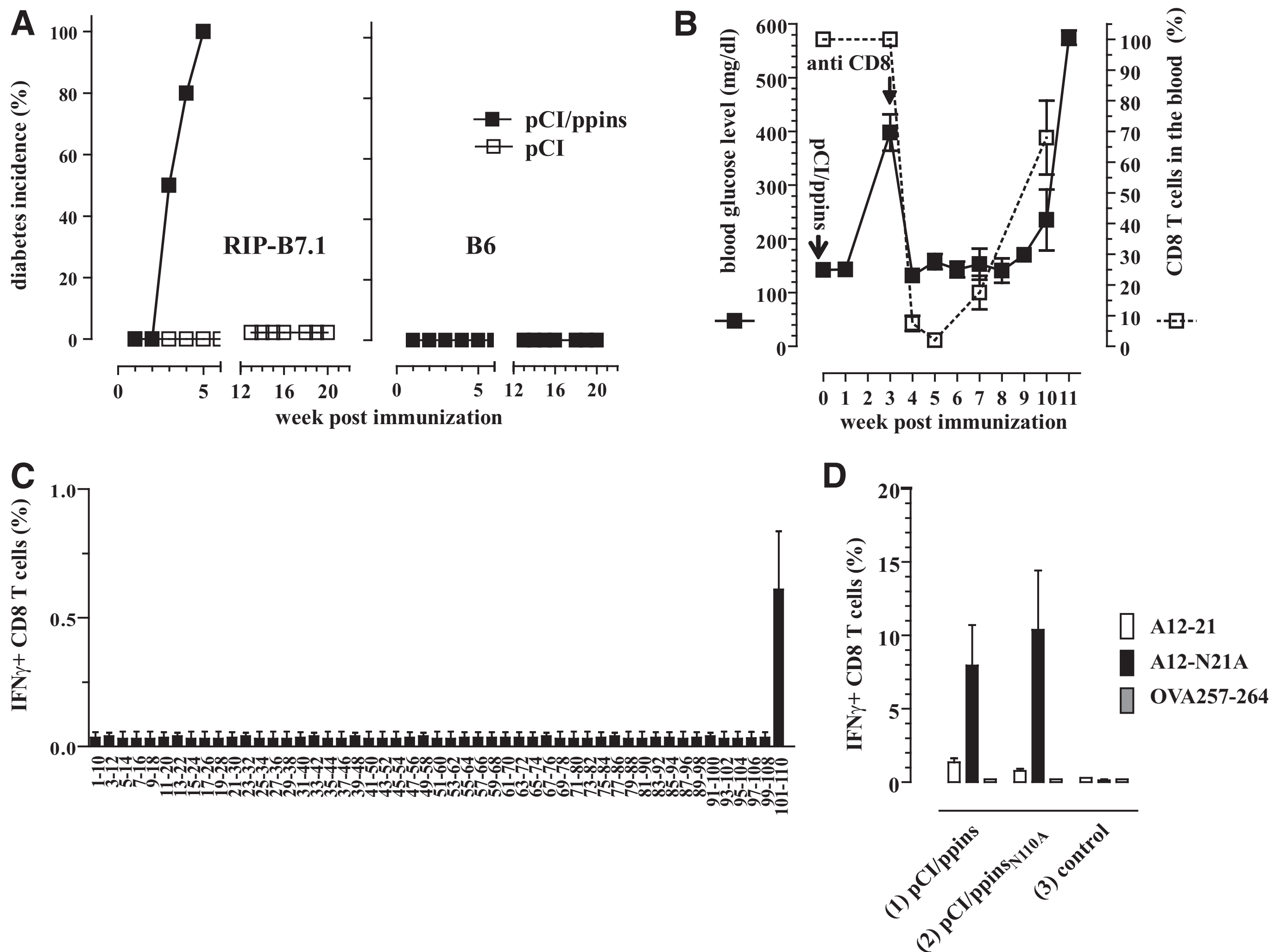


**FIG. 1. The RIP-B7.1 diabetes model.** ***A*****: RIP-B7.1 or C57BL/6 (B6) mice were immunized with pCI/preproinsulin (■, *n* = 8) or the noncoding pCI (□, *n* = 8). At indicated times after immunization cumulative diabetes incidences (%) were determined.** ***B*****: RIP-B7.1 mice were immunized with pCI/preproinsulin DNA. At 3 weeks after immunization, we selected three mice that had developed an early stage of EAD (with blood glucose levels between 300–440 mg/dl). These mice were injected twice (at days 21 and 23) with 200 μg mAb YTS-169 (anti-CD8). The glucose level (■; mg/dl) and CD8 T-cell numbers in the blood (□, CD8 T-cells number of nontreated mice were set for 100%) were determined at indicated time points after immunization. The injections of pCI/preproinsulin DNA and anti-CD8 mAb are indicated (*arrows*).** ***C*****: RIP-B7.1 mice (*n* = 10) were immunized with pCI/preproinsulin DNA. Pancreatic CD8 T-cells derived from diabetic mice (blood glucose level >400 mg/dl) were pooled and restimulated ex vivo with a preproinsulin-specific peptide library (i.e., 10mers with two amino acids offset), and frequencies of IFNγ$^+$ CD8 T-cells were determined by flow cytometry. The mean percentage of IFNγ$^+$ CD8 T-cells in the pancreatic CD8 T-cell population (obtained from two independent experiments) are shown.** ***D*****: Pancreatic cells were prepared from pCI/preproinsulin (group 1) or pCI/preproinsulin$_{N110A}$ (group 2) immunized, diabetic RIP-B7.1 mice, or control pCI-immunized, healthy (group 3) RIP-B7.1 mice and restimulated ex vivo with the K$^b$/A$_{12–21}$, K$^b$/A$_{12\text{-}N21A}$ or K$^b$/OVA$_{257–264}$ peptides, and specific IFNγ$^+$ CD8 T-cell levels were determined by flow cytometry. The mean percentage of IFNγ$^+$ CD8 T-cells in the pancreatic CD8 T-cell population (+ SD) of a representative experiment (*n* = three mice per group) are shown. pCI/ppins, pCI/preproinsulin.**

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kept under standard pathogen-free conditions in the animal colony of Ulm University (Ulm, Germany). All studies were conducted after Institutional Board approval in accordance with the Federal German Animal Protection Law.

**Immunization of mice.** Mice were immunized intramuscularly into the tibialis anterior muscle or injected intravenously with 3 × 10$^6$ magnetic-activated cell sorting (MACS)-purified splenic CD8 T-cells (cat. no. 130-090-859, Miltenyi Biotech). When indicated, mice were treated with blocking B7-H1 antibody (clone MIH5; cat. no. 16-5,982-85, eBioscience). Diabetes was diagnosed if two consecutive blood glucose values exceeded 250 mg/dl, i.e., 13.8 mmol/l (Disetronic Freestyle, Sulzbach, Germany).

**Histology.** Histology was performed as described previously (12,14).

**Isolation of CD8 T-cells from pancreatic tissue.** Pancreata were perfused in situ with collagenase P (cat. no. 11213865001, Roche) dissolved in 1 mg/ml Hank's 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, Germany) by centrifugation for 15 min at 2,400 rpm.

**Determination of specific CD8 T-cell frequencies.** Pancreatic cells (10$^5$/100 μl) were incubated for 14–16 h in UltraCULTURE medium with 5–20 μg/ml of the indicated peptides in the presence of brefeldin A (0.5 μg/ml) (cat. no. 15870; Sigma, 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 fluorescein isothiocyanate-conjugated anti-IFNγ antibody (cat. no.554411; BD Biosciences, Heidelberg, Germany). Frequencies of IFNγ$^+$ CD8 T-cells were determined by flow cytometry (FCM).

## RESULTS

**EAD induced in the RIP-B7.1 model.** A single injection of the pCI/preproinsulin DNA encoding murine preproinsulin efficiently induced hyperglycemia in RIP-B7.1 mice, but not in wild-type B6 mice (Fig. 1*A*) (13,14). PCI/

preproinsulin-immunized RIP-B7.1 mice with early EAD (blood glucose levels of 300–440 mg/dl) were treated 3 weeks after immunization with anti-CD8 antibody (Fig. 1*B*). This antibody treatment efficiently depleted CD8 T-cells within 2–3 days and transiently cured EAD (Fig. 1*B*). CD8 T-cell levels were restored 3–4 weeks after the anti-CD8 antibody treatment was discontinued, and EAD reappeared concomitant with the re-emerging CD8 T-cells (Fig. 1*B*). Anti-CD4 antibody treatment did not inhibit diabetes progression (data not shown). The effector phase of EAD thus depends on diabetogenic CD8 T-cells.

**Characterization of diabetogenic CD8 T-cells.** EAD development in preproinsulin-immunized RIP-B7.1 mice was accompanied by increasing infiltrations of CD8 T-cells into the pancreatic target tissue (13,14). CD8 T-cells isolated from immunized, diabetic RIP-B7.1 mice specifically recognized the immunodominant $K^b$-restricted $A_{12–21}$ (i.e., preproinsulin$_{101–110}$) epitope of preproinsulin (Fig. 1*C*) (13,27). Ex vivo stimulation of preproinsulin-primed, pancreas-infiltrating CD8 T-cells with the antigenic $A_{12–21}$ peptide, but not with all other peptides of a preproinsulin-specific library, revealed a CD8 T-cell population with specifically inducible IFNγ expression (Fig. 1*C*). In the course of EAD, we also found a significant influx of other lymphoid cells (e.g., CD4 T-cells and B cells) of unknown specificities into the pancreata (data not shown) (14). It is under investigation whether these bystander cells contribute to the diabetogenic immune response (5,28).

We usually detected $4–8 \times 10^3$ CD8 T-cells per pancreas in preproinsulin-immunized, diabetic RIP-B7.1 mice (with blood glucose levels of >400 mg/dl). The frequency of $A_{12–21}$-specific IFNγ$^+$ CD8 T-cells in these pancreatic CD8 T-cell populations was low (0.5–1.5%) (Fig. 1*C*). We identified an epitope variant ($A_{12\text{-}N21A}$) with an alanine (A) exchange for the COOH-terminal asparagine (N) at position $A_{21}$. This variant facilitated in vitro detection of primed CD8 T-cells (14). RIP-B7.1 mice immunized with either pCI/preproinsulin or the pCI/preproinsulin$_{N110A}$ variant (encoding preproinsulin with the mutant $A_{12\text{-}N21A}$ epitope) developed a similar EAD (data not shown). Pancreas-infiltrating IFNγ$^+$ CD8 T-cells expanded in vitro more efficiently after stimulation with the mutant $A_{12\text{-}N21A}$ than the $A_{12–21}$ peptide (Fig. 1*D*). Similarly, a monospecific $A_{12–21}$-encoding vector induced EAD in RIP-B7.1 mice (14), but pancreas-infiltrating CD8 T-cells preferentially expanded in vitro with the mutant $A_{12\text{-}N21A}$ peptide (data not shown). Hence, the alanine at position $A_{21}$ of the mutant epitope apparently modulates the specific steric structure of the peptide (29) and improves its major histocompatibility complex class I-presentation properties in vitro. We used the variant epitope to detect preproinsulin-specific CD8 T-cell responses.

**The effector phase of diabetogenic, preproinsulin-specific CD8 T-cell responses is blocked in C57BL/6 (B6) mice.** Preproinsulin-specific immunization efficiently induced EAD in RIP-B7.1 mice, but not in B6 mice (Fig. 1*A*). However, low numbers of CD8 T-cells were reproducibly found in the periphery of some (<2%) islets in immunized B6 mice (Fig. 2*A*). These CD8 T-cell populations were not found either in nonimmunized B6 (Fig. 2*A*) or nonimmunized RIP-B7.1 mice (13,14), suggesting that immunization had induced preproinsulin-specific CD8 T-cells in B6 mice.

We used adoptive transfer experiments to test whether functional preproinsulin-specific CD8 T-cells are primed in B6 mice. B6 mice were immunized with pCI/preproinsulin$_{N110A}$. Their splenic CD8 T-cells isolated 14 days after immunization were adoptively transferred into RIP-B7.1 mice (30). RIP-B7.1 hosts developed EAD after transfer with primed (Fig. 2*B*, group 2), but not with nonprimed (Fig. 2*B*, group 1), CD8 T-cells. Preproinsulin-specific CD8 T-cells accumulated in the pancreata of transplanted and diabetic hosts (Fig. 2*C*, group 2). IFNγ is critical for inducing EAD in RIP-B7.1 hosts because adoptively transferred CD8 T-cells from immunized IFNγ$^{-/-}$ mice did not induce EAD (Fig. 2*B*, group 3). Similarly, immunization of RIP-B7.1$^+$/IFNγ$^{-/-}$ mice with preproinsulin also did not induce EAD (13).

**B7-H1 expression by pancreatic β-cells controls the diabetogenic CD8 T-cell response.** We tested whether EAD can be triggered in preproinsulin-immune B6 mice by blocking B7-H1-mediated coinhibition. B6 mice injected with either pCI (Fig. 3*A*) or pCI/preproinsulin$_{N110A}$ (Fig. 3*B* and *C*) were treated at days 12 and 15 after immunization, either with blocking B7-H1 antibody (31) (Fig. 3*A* and *B*) or an isotype control antibody (Fig. 3*C*). PCI/preproinsulin$_{N110A}$-immunized B6 mice rapidly developed hyperglycemia after injection of the anti-B7-H1, but not control antibody (Fig. 3*B* and *C*). Control mice (injected with pCI and treated with anti-B7-H1 antibody) did not develop EAD (Fig. 3*A*).

Within 6–9 days after anti-B7-H1 antibody injection, mice developed either severe hyperglycemia (Fig. 3*B*, group 1) or moderate and transient hyperglycemia (Fig. 3*B*, groups 2a/b). Severe disease correlated with an influx of high levels of $A_{12\text{-}N21A}$-specific IFNγ$^+$ CD8 T-cells into the islets and an almost complete loss of insulin-producing islet β-cells (Fig. 3*D* and *E*; group 1). In mice with transient diabetes, only low numbers of CD8 T-cells were detectable in the islets during the hyperglycemic stage, and insulin expression was reduced, but still intact (Fig. 3*D* and *E*; group 2a). After recovery to normoglycemia, CD8 T-cells were no longer detectable in the islets (Fig. 3*D* and *E*; group 2b). Pancreatic β-cells that lose B7-H1 coinhibition are hence susceptible (at least transiently) to attack by preproinsulin-specific CD8 T-cells.

We used B7-H1$^{-/-}$ mice (25) to confirm that EAD induction by preproinsulin depends on B7-H1. A single injection of pCI/preproinsulin$_{N110A}$-induced hyperglycemia in B7-H1$^{-/-}$ mice. EAD developed in both male and female B7-H1$^{-/-}$ mice with a median onset of 3–5 weeks after immunization and a cumulative diabetes incidence of 80% by week 5 (Fig. 4*A*, data not shown). B7-H1$^{-/-}$ mice did not develop spontaneous EAD after injection with the noncoding pCI vector (Fig. 4*A*). Using the preproinsulin-specific peptide library described above (see Fig. 1*C*), we detected only $A_{12–21}$/$A_{12\text{-}N21A}$-specific IFNγ$^+$ CD8 T-cells in the pancreata of immunized and diabetic B7-H1$^{-/-}$ mice (data not shown). Thus, CD8 T-cells with this specificity play a prominent role in the destructive autoimmune response in these mice.

We used adoptive cell transfers to exclude that B7-H1-deficiency in CD8 T-cells is critical for EAD development in B7-H1$^{-/-}$ mice. RIP-B7.1 and B7-H1$^{-/-}$ mice were immunized with pCI/preproinsulin$_{N110A}$. We adoptively transferred $3 \times 10^6$ CD8 T-cells (derived from spleens of early diabetic mice) into RIP-B7.1 hosts (Fig. 4*B*). RIP-B7.1 hosts reconstituted with primed, RIP-B7.1- or B7-H1$^{-/-}$-derived CD8 T-cells induced EAD with a similar efficacy (Fig. 4*B*). Transfer of nonprimed CD8 T-cells from healthy RIP-B7.1 or B7-H1$^{-/-}$ donors did not induce EAD (data not

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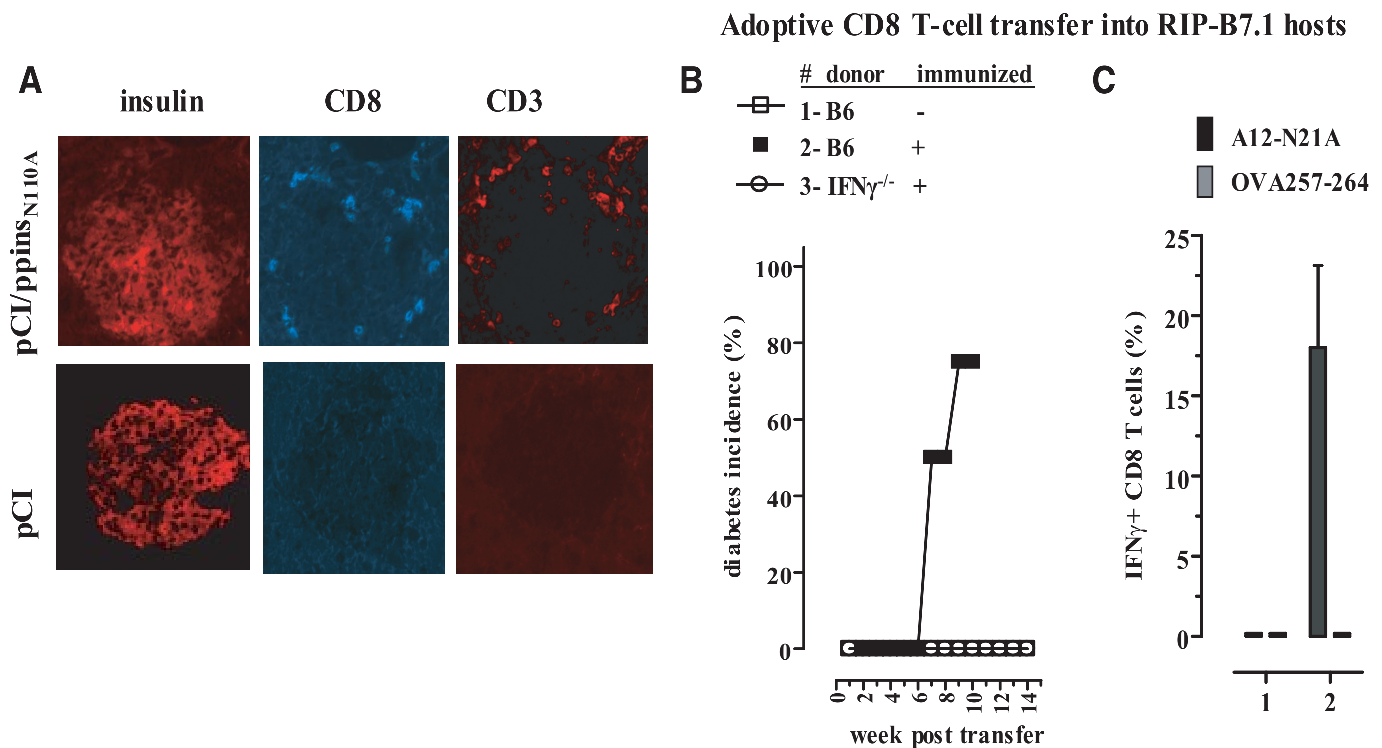


FIG. 2. Priming of preproinsulin-specific CD8 T-cells in B6 mice. *A*: C57BL/6 (B6) mice were immunized with pCI/preproinsulin$_{N110A}$ or control pCI. Twenty-four days after immunization, histology was performed by analyzing pancreatic sections for insulin, CD8, and CD3 T-cells. *B* and *C*: B6 mice remained either untreated (□, group 1) or were immunized with pCI/preproinsulin$_{N110A}$ (■, group 2). IFNγ-deficient mice were immunized with pCI/preproinsulin$_{N110A}$ (○, group 3). *B*: Fourteen days after injection, CD8 T-cells were isolated from spleens. Next, $3 \times 10^6$ splenic CD8 T-cells were transferred intravenously into sublethally (650rad) irradiated RIP-B7.1 hosts, and cumulative diabetes incidences were determined. *C*: Pancreatic CD8 T-cells were prepared from healthy hosts (transferred with nonimmune CD8 T-cells, group 1) or diabetic (transferred with immune CD8 T-cells, group 2) RIP-B7.1 hosts, and restimulated ex vivo with the $K^b/A_{12\text{-}N21A}$ or control $K^b/OVA_{257\text{-}264}$ peptide. Specific $IFN\gamma^+$ CD8 T-cell frequencies were determined by flow cytometry. The mean percentage of $IFN\gamma^+$ CD8 T-cells in the pancreatic CD8 T-cell population (± SD) of a representative experiment ($n = 3$ mice per group) is shown. pCI/ppins$_{N110A}$, pCI/preproinsulin$_{N110A}$. (A high-quality digital representation of this figure is available in the online issue.)

shown). Thus, B7-H1-deficiency in preproinsulin-specific CD8 T-cells did not alter their diabetogenic potential. In contrast, transfer of identical RIP-B7.1-derived CD8 T-cell preparations into B7-H1$^{-/-}$ hosts inefficiently induced late EAD (Fig. 4*C*). Only 1 of 8 B7-H1$^{-/-}$ hosts developed EAD at 18 weeks after transfer (Fig. 4*C*). The efficacy of EAD could be increased in adoptively transferred B7-H1-deficient hosts if the stimulatory B7.1 molecule is coexpressed in pancreatic β-cells of RIP-B7.1$^+$/B7-H1$^{-/-}$ mice (Fig. 4*C*).

**B7-H1/PD-1 coinhibition controls diabetogenic CD8 T-cells.** We used PD-1$^{-/-}$ knockout mice (26) to test whether induction of preproinsulin-specific EAD depends on the coinhibitory PD-1/B7-H1 interaction (19). A single injection of the pCI/preproinsulin$_{N110A}$ (but not of pCI vector) into PD-1$^{-/-}$ mice induced $A_{12\text{-}N21A}$-specific $IFN\gamma^+$ CD8 T-cell responses and EAD (Fig. 4*D*; data not shown), suggesting that B7-H1/PD-1 coinhibition is critical to induce EAD in preproinsulin-immune mice. To confirm this, we generated bone marrow chimeras. To distinguish between donor- and host-derived T-cells in bone marrow (BM) chimeras, we used wild-type CD45.1$^+$ B6 mice in this set of experiments. BM cells from CD45.1$^+$ donor mice (B7-H1$^+$ PD-1$^+$) were transferred into lethally irradiated, congenic B7-H1$^{-/-}$ or PD-1$^{-/-}$ (CD45.2$^+$), and BM cells from B7-H1$^{-/-}$ or PD-1$^{-/-}$ donor mice were transferred into wild-type CD45.1$^+$ hosts (Table 1). At 6 to 7 weeks after transplantation, chimeric mice contained >90% of donor T-cells (data not shown). Chimeric mice were immunized 7 weeks after transplantation with pCI/preproinsulin$_{N110A}$ (or control pCI). As is evident from Table 1, wild-type/B7-H1$^{-/-}$ (group 1) and PD-1$^{-/-}$/wild-type chimeras (group 7), but not wild-type /PD-1$^{-/-}$ (group 3) and B7-H1$^{-/-}$/wild-type chimeras (group 5), developed EAD after immunization with pCI/preproinsulin$_{N110A}$. EAD manifestation in groups 1 and 7 correlated with an influx of $A_{12\text{-}N21A}$-specific $IFN\gamma^+$ CD8 T-cells into the target tissue (data not shown). Hence, either the selective deficiency of B7-H1 on target cells (group 1) or the deficiency of PD-1 on T-cells (group 7) triggered CD8 T-cell–mediated EAD. Binding of PD-1 expressed by activated CD8 T-cells to B7-H1 expressed by pancreatic β-cells thus seems to control the diabetogenic immune response.

## DISCUSSION

There is increasing evidence from patients with type 1 diabetes that autoreactive CD8 T-cells specific for preproinsulin are involved in β-cell destruction (32–38). We established mouse models to study the pathogenic crosstalk between preproinsulin-specific CD8 T-cells and preproinsulin-expressing β-cells. RIP-B7.1 tg and two well defined (B7-H.1$^{-/-}$, PD-1$^{-/-}$) mouse lines allowed us to identify critical checkpoints for the control of diabetogenic, preproinsulin-specific CD8 T-cells: *1*) the costimulator molecule B7.1 (CD80) expressed on β-cells (RIP-B7.1 mice); *2*) the coinhibitor B7-H1 (PD-L1) expressed by

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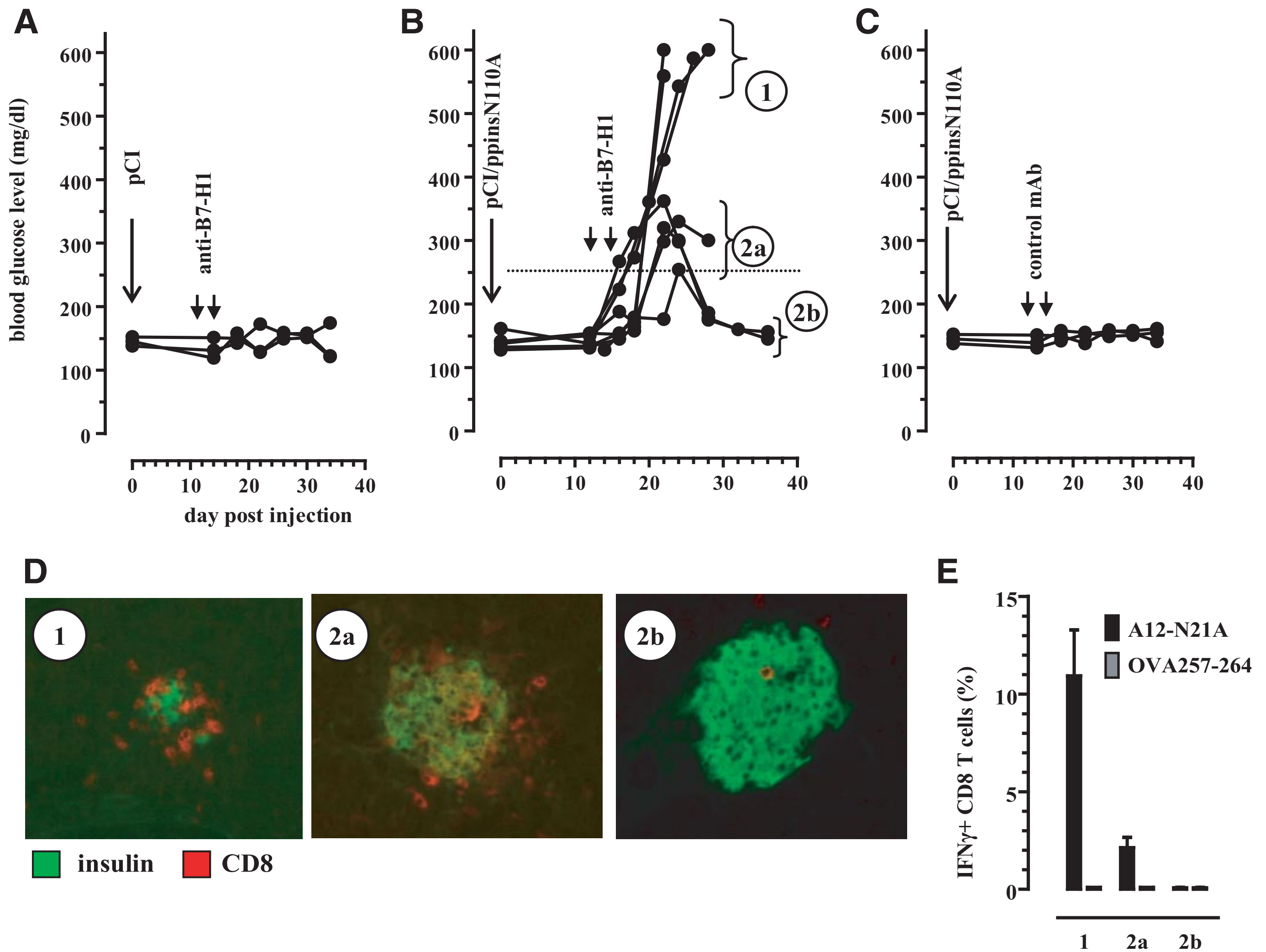


FIG. 3. Blocking B7–H1 coinhibition induced EAD in pCI/preproinsulin$_{N110A}$-immunized B6 mice. B6 mice were immunized with pCI (*A*) or pCI/preproinsulin$_{N110A}$ (*B* and *C*). At days 12 and 15 after immunization, mice were injected with blocking B7–H1 antibody (*A* and *B*) or control antibody (*C*), and blood glucose levels were followed up during the experiment. *B*,*D*, and *E*: Preproinsulin-immune and B7–H1 antibody-treated B6 mice developed either a severe hyperglycemia (with blood glucose levels >550 mg/dl, group 1) or a moderate hyperglycemia (with blood glucose levels of 250–360 mg/dl, group 2a) which subsequently returned to normoglycemia (with blood glucose levels of <200 mg/dl, group 2b). Representative mice in groups 1, 2a, and 2b were analyzed histologically for insulin expression and CD8 T-cell influx (*D*), or were analyzed for preproinsulin-specific CD8 T-cells (*E*). Pancreatic CD8 T-cells were restimulated ex vivo with the $K^b/A_{12\text{-}N21A}$ or control $K^b/OVA_{257–264}$ peptide, and $IFN\gamma^+$ CD8 T-cell levels were determined by flow cytometry (*E*). pCI/ppins$_{N110A}$, pCI/preproinsulin$_{N110A}$. (A high-quality digital representation of this figure is available in the online issue.)

β-cells (B7-H1$^{-/-}$ mice); or *3*) the coinhibitor PD-1 molecule expressed by CD8 T effector cells (PD-1$^{-/-}$ mice).

Transgene-driven B7.1 (CD80) expression in the pancreatic β-cells of RIP-B7.1 mice makes them susceptible to autoreactive CD8 T-cell attack. The nonphysiologic, costimulatory B7.1/CD28 interaction in RIP-B7.1 mice (9,10) may allow efficient effector function delivery by autoreactive CD8 T-cells. CD28-deficient RIP-B7.1$^+$/CD28$^{-/-}$ mice did not develop EAD after immunization with preproinsulin (data not shown). There is thus strong evidence that the interaction of B7.1 on the surface of β-cells with the costimulator molecule CD28 on T-cells is an essential component of T-cell–mediated EAD in RIP-B7.1 mice. 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 (Fig. 4*C*) (30). 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. For example, we previously showed that expression and processing of preproinsulin antigens in the endoplasmic reticulum favor priming of autoreactive CD8 T-cells (14). Thus, direct loading of the $A_{12–21}$ epitope on newly synthesized $K^b$-molecules in the endoplasmic reticulum may be an essential step for enabling presentation of this epitope (14). We currently establish a HLA-A*0201$^+$/RIP-B7.1 mouse model to define the diabetogenic potential of HLA-A*0201-restricted preproinsulin epitopes associated with human type 1 diabetes (35–37). Furthermore, RIP-B7.1 mice are a useful tool to identify novel β-cell antigens that are targets for CD8 T-cell–triggered diabetes (4).

We consider the key observation of this report to be the de novo induction of preproinsulin-specific CD8 T-cells (and EAD) in mice and their control by B7-H1/PD-1 interaction. It has been shown that PD-1 and its ligands, B7-H1 (PD-L1) and PD-L2, deliver inhibitory signals that



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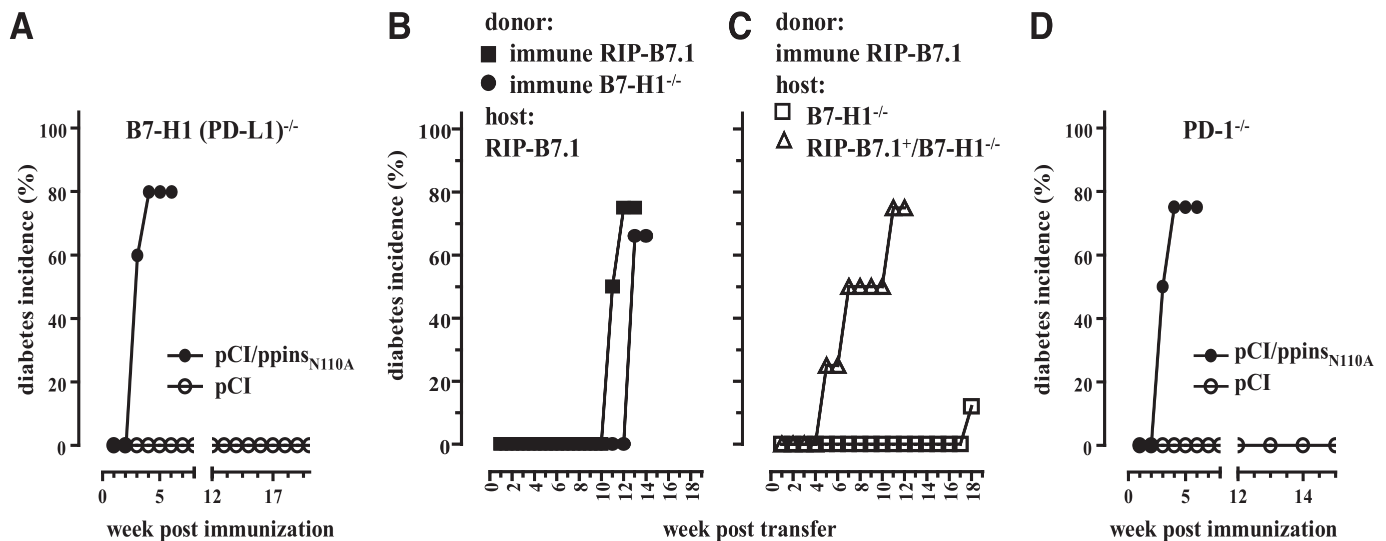


**FIG. 4. Preproinsulin-specific EAD development in B7–H1$^{-/-}$ and PD-1$^{-/-}$ mice. *A*: B7–H1$^{-/-}$ mice were immunized with pCI/preproinsulin$_{N110A}$ (●, $n$ = 5), or the noncoding pCI (○, $n$ = 8), and cumulative diabetes incidences (%) were determined. *B*: RIP-B7.1 (■) and B7–H1$^{-/-}$ (●) mice were immunized with pCI/preproinsulin$_{N110A}$. Spleens were removed from early diabetic mice and 3 × 10$^6$ CD8 T-cells were transferred intravenously into sublethally irradiated RIP-B7.1 hosts, and cumulative diabetes incidences (%) were determined. *C*: In addition, spleens were removed from early diabetic RIP-B7.1 mice, and 3 × 10$^6$ CD8 T-cells were transferred intravenously into sublethally irradiated B7–H1$^{-/-}$ (□, $n$ = 8) or RIP-B7.1$^+$/B7–H1$^{-/-}$ (△, $n$ = 4) hosts. *D*: PD-1$^{-/-}$ mice were immunized with pCI/preproinsulin$_{N110A}$ (●, $n$ = 12), or the noncoding pCI (○, $n$ = 12), and cumulative diabetes incidences (%) were determined. pCI/ppins$_{N110A}$, pCI/preproinsulin$_{N110A}$.**

regulate the balance between T-cell activation, tolerance, and immunopathology (19,39). B7-H1 (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, we confirmed that the deficiency of B7-H1 on target cells or PD-1 on T-cells was essential to trigger preproinsulin-specific, CD8 T-cell–mediated EAD by DNA-based immunization (Table 1). Binding of PD-1 on activated T-cells to B7-H1 expressed by pancreatic β-cells may hence downmodulate the diabetogenic potential of preproinsulin-specific CD8 T-cells. We further showed that B7-H1-deficiency in preproinsulin-specific CD8 T-cells did not alter their diabetogenic potential (Fig. 4*B*). B7-H1$^{-/-}$ mice inefficiently developed EAD after adoptive transfer of preproinsulin-specific CD8 T-cells, but coexpression of the stimulatory B7.1 molecule in B7-H1-deficient pancreatic β-cells (RIP-B7.1$^+$/B7-H1$^{-/-}$ mice) significantly accelerated disease induction (Fig. 4*C*). Transgene-driven B7.1 costimulation in pancreatic β-cells was thus more potent than the loss of B7-H1 coinhibition in promoting the pathogenic immune response of adoptively transferred preproinsulin-specific CD8 T-cells. In contrast, immunization of B7-H1$^{-/-}$ and RIP-B7.1 mice with preproinsulin-encoding DNA induced EAD with similar efficacies and kinetics (see Figs. 1*A*, 4*A*). We assume that additional factors (e.g., professional antigen-presenting dendritic cells or cells from the innate immune system) (40) are triggered by DNA-based immunization that facilitate expansion of preproinsulin-specific CD8 T-cells and/or maintain their diabetogenic potential in B7-H1$^{-/-}$ mice.

The B6 diabetes model described in this study is attractive to characterize distinct events in the regulation of β-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 it was unexpectedly easy to prime preproinsulin-specific CD8 T-cells in male and female B6 mice. CD8 T-cells revealed their diabetogenic potential after adoptive transfer into congenic RIP-B7.1 hosts or after conditioning the pancreatic target tissue by antibody-mediated blockage of B7-H1 coinhibiton (Figs. 2 and 3). Preproinsulin-specific CD8 T-cells in immunized B6 mice thus have a diabetogenic potential, but pancreatic β-cells are protected from immune attack by these cells. Similarly, in the lymphocytic choriomenin-

TABLE 1
Preproinsulin-specific EAD induction in bone marrow chimeras

| Group | Bone marrow chimera | Donor | Host | Immunization | EAD |
|---|---|---|---|---|---|
| 1 | wt/B7-H1$^{-/-}$ | CD45.1$^+$ wt | B7-H1$^{-/-}$ | pCI/ppins$_{N110A}$ | 4/4 |
| 2 | wt/B7-H1$^{-/-}$ | CD45.1$^+$ wt | B7-H1$^{-/-}$ | pCI | 0/3 |
| 3 | wt/PD-1$^{-/-}$ | CD45.1$^+$ wt | PD-1$^{-/-}$ | pCI/ppins$_{N110A}$ | 0/4 |
| 4 | wt/PD-1$^{-/-}$ | CD45.1$^+$ wt | PD-1$^{-/-}$ | pCI | 0/3 |
| 5 | B7-H1$^{-/-}$/wt | B7-H1$^{-/-}$ | CD45.1$^+$ wt | pCI/ppins$_{N110A}$ | 0/4 |
| 6 | B7-H1$^{-/-}$/wt | B7-H1$^{-/-}$ | CD45.1$^+$ wt | pCI | 0/2 |
| 7 | PD-1$^{-/-}$/wt | PD1$^{-/-}$ | CD45.1$^+$ wt | pCI/ppins$_{N110A}$ | 2/4 |
| 8 | PD-1$^{-/-}$/wt | PD1$^{-/-}$ | CD45.1$^+$ wt | pCI | 0/4 |

Bone marrow chimeras were generated by injecting wild-type CD45.1$^+$ bone marrow cells intravenously into lethally irradiated (950 rad) B7-H1$^{-/-}$ (CD45.2$^+$) or PD-1$^{-/-}$ (CD45.2$^+$) hosts, or by injecting B7-H1$^{-/-}$ or PD-1$^{-/-}$ bone marrow cells into wild-type CD45.1$^+$ hosts. Mice were injected 7 weeks after transplantation with either the pCI/ppins$_{N110A}$, or control pCI plasmid DNA. Blood glucose levels were determined, and the number of diabetic mice per group is indicated. wt, wild-type; ppins$_{N110A}$, preproinsulin$_{N110A}$.



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gitis 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 (8). The B6 model is a good example for translational medicine since our data illustrated the central role of the β-cell as a gatekeeper in the preproinsulin-specific insulitic process. Beta cells per se prevent the deleterious cross-talk with preproinsulin-specific CD8 T-cells. Changes in the β-cell milieu—e.g., by antibody treatment (Fig. 3), by interferon (8), or by viral infections (41)—can favor the susceptibiliy of β-cells for the CD8 T-cell–mediated immune attack. Further manipulations of the pancreatic β-cells or distinct arms of the immune system by specific drugs (42) 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 (3,41–43) could be helpful to design prophylactic vaccines.

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T.R., H.B., C.S., and A.S. researched data and contributed to discussion. B.O.B. contributed to discussion and reviewed/edited the manuscript. L.C. reviewed/edited the manuscript. J.R. contributed to discussion and wrote the manuscript. R.S. researched data and wrote the manuscript.

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## REFERENCES

1. Bach JF, Chatenoud L. Tolerance to islet autoantigens in type 1 diabetes. Annu Rev Immunol 2001;19:131–161
2. Ohashi PS, DeFranco AL. Making and breaking tolerance. Curr Opin Immunol 2002;14:744–759
3. von Herrath M, Nepom GT. Animal models of human type 1 diabetes. Nat Immunol 2009;10:129–132
4. Stadinski B, Kappler J, Eisenbarth GS. Molecular targeting of islet autoantigens. Immunity 2010;32:446–456
5. Anderson MS, Bluestone JA. The NOD mouse: a model of immune dysregulation. Annu Rev Immunol 2005;23:447–485
6. von Herrath MG, Guerder S, Lewicki H, Flavell RA, Oldstone MB. Coexpression of B7–1 and viral ("self") transgenes in pancreatic beta cells can break peripheral ignorance and lead to spontaneous autoimmune diabetes. Immunity 1995;3:727–738
7. Kurts C, Sutherland RM, Davey G, Li M, Lew AM, Blanas E, Carbone FR, Miller JF, Heath WR. CD8 T-cell ignorance or tolerance to islet antigens depends on antigen dose. Proc Natl Acad Sci U S A 1999;96:12703–12707
8. Lang KS, Recher M, Junt T, Navarini AA, Harris NL, Freigang S, Odermatt B, Conrad C, Ittner LM, Bauer S, Luther SA, Uematsu S, Akira S, Hengartner H, Zinkernagel RM. Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. Nat Med 2005;11:138–145
9. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol 2004;4:336–347
10. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol 2005;23:515–548
11. Harlan DM, Hengartner H, Huang ML, Kang YH, Abe R, Moreadith RW, Pircher H, Gray GS, Ohashi PS, Freeman GJ. 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 U S A 1994;91:3137–3141
12. Karges W, Pechhold K, Al Dahouk S, Riegger I, Rief M, Wissmann A, Schirmbeck R, Barth C, Boehm BO. Induction of autoimmune diabetes through insulin (but not GAD65) DNA vaccination in nonobese diabetic and in RIP-B7.1 mice. Diabetes 2002;51:3237–3244
13. Karges W, Rajasalu T, Spyrantis A, Wieland A, Boehm B, Schirmbeck R. The diabetogenic, insulin-specific CD8 T cell response primed in the experimental autoimmune diabetes model in RIP-B7.1 mice. Eur J Immunol 2007;37:2097–2103
14. Brosi H, Reiser M, Rajasalu T, Spyrantis A, Oswald F, Boehm BO, Schirmbeck R. Processing in the endoplasmic reticulum generates an epitope on the insulin A chain that stimulates diabetogenic CD8 T-cell responses. J Immunol 2009;183:7187–7195
15. Guleria I, Gubbels BM, Dada S, Fife B, Tang Q, Ansari MJ, Trikudanathan S, Vadivel N, Fiorina P, Yagita H, Azuma M, Atkinson M, Bluestone JA, Sayegh MH. Mechanisms of PDL1-mediated regulation of autoimmune diabetes. Clin Immunol 2007;125:16–25
16. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat Immunol 2007;8:239–245
17. Martin-Orozco N, Wang YH, Yagita H, Dong C. Cutting edge: programmed death (PD) ligand-1/PD-1 interaction is required for CD8+ T-cell tolerance to tissue antigens. J Immunol 2006;177:8291–8295
18. Okazaki T, Honjo T. The PD-1-PD-L pathway in immunological tolerance. Trends Immunol 2006;27:195–201
19. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008;26:677–704
20. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7–1 costimulatory molecule to inhibit T-cell responses. Immunity 2007;27:111–122
21. Ansari MJ. Salama AD, Chitnis T, Smith RN, Yagita H, Akiba H, Yamazaki T, Azuma M, Iwai H, Khoury SJ, Auchincloss H Jr, Sayegh MH. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. J Exp Med 2003;198:63–69
22. Fife BT, Guleria I, Gubbels BM, Eagar TN, Tang Q, Bour-Jordan H, Yagita H, Azuma M, Sayegh MH, Bluestone JA. Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway. J Exp Med 2006;203:2737–2747
23. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, Koulmanda M, Freeman GJ, Sayegh MH, Sharpe AH. Tissue expression of PD-L1 mediates peripheral T-cell tolerance. J Exp Med 2006;203:883–895
24. Subudhi SK, Zhou P, Yerian LM, Chin RK, Lo JC, Anders RA, Sun Y, Chen L, Wang Y, Alegre ML, Fu YX. Local expression of B7–H1 promotes organ-specific autoimmunity and transplant rejection. J Clin Invest 2004;113:694–700
25. Dong H, Zhu G, Tamada K, Flies DB, van Deursen JM, Chen L. B7–H1 determines accumulation and deletion of intrahepatic CD8(+) T lymphocytes. Immunity 2004;20:327–336
26. Nishimura H, Minato N, Nakano T, Honjo T. Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. Int Immunol 1998;10:1563–1572
27. Ma H, Ke Y, Li Q, Kapp JA. Bovine and human insulin activate CD8+-autoreactive CTL expressing both type 1 and type 2 cytokines in C57BL/6 mice. J Immunol 2000;164:86–92
28. Lennon GP, Bettini M, Burton AR, Vincent E, Arnold PY, Santamaria P, Vignali DA. T-cell islet accumulation in type 1 diabetes is a tightly regulated, cell-autonomous event. Immunity 2009;31:643–653
29. Sawaya MR, Sambashivan S, Nelson R, Ivanova MI, Sievers SA, Apostol MI, Thompson MJ, Balbirnie M, Wiltzius JJ, McFarlane HT, Madsen AO, Riekel C, Eisenberg D. Atomic structures of amyloid cross-beta spines reveal varied steric zippers. Nature 2007;447:453–457
30. Rajasalu T, Barth C, Spyrantis A, Durinovic-Bello I, Uibo R, Schirmbeck R, Boehm BO, Karges W. Experimental autoimmune diabetes: a new tool to study mechanisms and consequences of insulin-specific autoimmunity. Ann N Y Acad Sci 2004;1037:208–215
31. Lukens JR, Cruise MW, Lassen MG, Hahn YS. Blockade of PD-1/B7–H1 interaction restores effector CD8+ T-cell responses in a hepatitis C virus core murine model. J Immunol 2008;180:4875–4884
32. Eisenbarth GS, Moriyama H, Robles DT, Liu E, Yu L, Babu S, Redondo MJ, Gottlieb P, Wegmann D, Rewers M. Insulin autoimmunity: prediction/precipitation/prevention type 1A diabetes. Autoimmun Rev 2002;1:139–145
33. Pinkse GG, Tysma OH, Bergen CA, Kester MG, Ossendorp F, van Veelen PA, Keymeulen B, Pipeleers D, Drijfhout JW, Roep BO. Autoreactive CD8 T-cells associated with β-cell destruction in type 1 diabetes. Proc Natl Acad Sci U S A 2005;102:18425–18430

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34. Toma A, Haddouk S, Briand JP, Camoin L, Gahery H, Connan F, Dubois-Laforgue D, Caillat-Zucman S, Guillet JG, Carel JC, Muller S, Choppin J, Boitard C. Recognition of a subregion of human proinsulin by class I-restricted T-cells in type 1 diabetic patients. Proc Natl Acad Sci U S A 2005;102:10581–10586
35. Mallone R, Martinuzzi E, Blancou P, Novelli G, Afonso G, Dolz M, Bruno G, Chaillous L, Chatenoud L, Bach JM, van Endert P. CD8+ T-cell responses identify beta-cell autoimmunity in human type 1 diabetes. Diabetes 2007;56:613–621
36. Baker C, Petrich de Marquesini LG, Bishop AJ, Hedges AJ, Dayan CM, Wong FS. Human CD8 responses to a complete epitope set from preproinsulin: implications for approaches to epitope discovery. J Clin Immunol 2008;28:350–360
37. Skowera A, Ellis RJ, Varela-Calvino R, Arif S, Huang GC, Van Krinks C, Zaremba A, Rackham C, Allen JS, Tree TI, Zhao M, Dayan CM, Sewell AK, Unger W, Drijfhout JW, Ossendorp F, Roep BO, Peakman M. CTLs are targeted to kill β-cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J Clin Invest 2008;118:3390–3402
38. Zhang L, Nakayama M, Eisenbarth GS. Insulin as an autoantigen in NOD/human diabetes. Curr Opin Immunol 2008;20:111–118
39. Okazaki T, Honjo T. PD-1 and PD-1 ligands: from discovery to clinical application. Int Immunol 2007;19:813–824
40. Gurunathan S, Wu CY, Freidag BL, Seder RA. DNA vaccines: a key for inducing long-term cellular immunity. Curr Opin Immunol 2000;12:442–447
41. von Herrath M. Can we learn from viruses how to prevent type 1 diabetes?: the role of viral infections in the pathogenesis of type 1 diabetes and the development of novel combination therapies. Diabetes 2009;58:2–11
42. Luo X, Herold KC, Miller SD. Immunotherapy of type 1 diabetes: where are we and where should we be going? Immunity 2010;32:488–499
43. Santamaria P. The long and winding road to understanding and conquering type 1 diabetes. Immunity 2010;32:437–445

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