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Low Dose Streptozotocin-Induced Diabetes in Rat Insulin Promoter-mCD80-Transgenic Mice Is T Cell Autoantigen-Specific and CD28 Dependent

Klaus Pechhold, Noelle B. Patterson, Carmen Blum, Christine L. Fleischacker, Bernhard O. Boehm, and David M. Harlan

Although transgenic mice expressing murine B7-1 (mCD80) on their pancreatic β cells under the rat insulin-1 promoter (RIP-mCD80β mice) rarely develop spontaneous β cell destruction and diabetes, we have previously reported the transgene-dependent induction of profound insulitis and lethal diabetes following multiple low dose injections of the β cell toxin streptozotocin (MLDS) in RIP-mCD80β mice. Here, we have further characterized this MLDS-induced diabetes model using the RIP-mCD80β mice and now demonstrate that disease is critically dependent on T cell signaling via CD28. Thus, although naïve RIP-mCD80β and nontransgenic littermates have comparable gross β cell mass, and immediately following MLDS induction the mice display similar degrees of insulitis and decrements in the β cell mass, only transgenic mice continued to destroy their β cells and develop insulin-dependent diabetes mellitus. Strikingly, MLDS-induced diabetes was completely prevented in CD28-deficient mice (RIP-mCD80β CD28−/−) due to abrogation of leukocytes infiltrating their pancreatic islets. We further characterized MLDS-induced diabetes in the RIP-mCD80β mice by demonstrating that the MLDS-induced lymphocytic islet infiltrate contained a substantial frequency of autoantigen-specific, IFN-γ-secreting, CD8+ T cells. We conclude that MLDS-induced β cell destruction and subsequent insulin-dependent diabetes mellitus in RIP-mCD80β mice is T cell-mediated as it involves both Ag-specific recognition of self-target molecules in the inflamed pancreatic islet (signal 1) and is CD28 costimulation dependent (signal 2). The Journal of Immunology, 2001, 166: 2531–2539.

Activation of naïve T cells to proliferate and secrete lymphokines is believed to require two signals. The first signal is conferred by the TCR upon its cognate interaction with antigenic peptide presented in the context of appropriate MHC molecules. The important second signal(s) is provided by the interaction of nonpolymorphic receptor-ligand pairs, most notably those involving CD80 (B7-1) or CD86 (B7-2) on APC and CD28 expressed by T cells (1, 2). Although costimulatory CD28 ligands of the B7 family have been found predominantly on professional APCs like dendritic cells, activated monocytes/macrophages, and B cells (3–5), we have speculated that costimulatory molecules of the B7 family may be locally and/or temporarily elaborated by nonprofessional APC such as pancreatic β cells. Indeed, we and others have demonstrated the existence of B7 family costimulatory ligand expression on cells unrelated to hematopoietic stem cells (6–9).

In an attempt to study the possible consequences of β cell costimulatory ligand expression, we created and have partially characterized a transgenic mouse expressing murine B7-1 (mCD80) selectively on pancreatic β cells under the rat insulin-1 promoter (RIPβ) control (10, 11). Although these mice rarely develop diabetes spontaneously (<7% incidence), when disease does occur it has been accompanied by profound insulitis, suggesting an autoimmune pathogenesis (our unpublished observation). Furthermore, the RIP-mCD80β mice are highly susceptible to insulin-dependent diabetes mellitus (IDDM) under circumstances that do not induce IDDM in nontransgenic (NT) mice. For instance, mice that express both the transgene-encoded gp of the lymphocytic choriomeningitis virus (LCMV-gp) specifically on their β cells and a transgene-encoded TCR specific for an epitope of that LCMV-gp (so called RIP-LCMV-gpβ−/− × TCRβ−/− “double transgenic” mice) never spontaneously develop diabetes (12). Strikingly however, breeding RIP-LCMV-gpβ−/− × TCRβ−/− with RIP-mCD80β to produce triple transgenic mice invariably resulted in spontaneous IDDM onset by...
Materials and Methods

Mice

The experiments described in this study were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animals Resources, National Research Council, DHHS, Pub. No. NIH 86-23 (1985)).

The FVB/N (H-2b) strain of mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) or bred in house (Laboratory Animal Medicine and Science Division of the Naval Medical Research Center). C57BL/6 mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in house (Laboratory Animal Medicine and Science Division of the Naval Medical Research Center). (Ontario Cancer Institute, Toronto, Canada) (19), and the RIP-LCMV-gp transgenic line was provided by P. Ohashi (Ontario Cancer Institute) (12).

RIP-mCD80 mice were originally obtained from T. Mak (Ontario Cancer Institute, Toronto, Canada) (19), and the RIP-mCD80 transgenic line has been described (10, 11). Here we provide further evidence that MLDS-induced diabetes in RIP-mCD80 mice is T cell mediated and Ag specific. When the RIP-mCD80 mice were compared with their NT littermates, both naive mice and mice studied immediately following MLDS administration displayed indistinguishable β cell mass as reflected by pancreatic insulin content. Moreover, breeding RIP-mCD80 mice to CD28−/− mice demonstrated that CD28−/− mice were markedly resistant to both MLDS-induced diabetes and insulinosis regardless of whether the β cells of the mouse expressed mCD80. Finally, we demonstrate autoantigen-specific, MHC class I-restricted CD8+ T cells among the islet infiltrate suggesting that, in the presence of ectopically expressed CD80, STZ gave rise to a classical Ag-specific immune response in the pancreatic islets that ultimately manifests as IDDM.

Induction of diabetes

STZ, provided by Dr. Martin D. Meglasson (Upjohn, Kalamazoo, MI) or purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany), was administered to the mice by injecting 20 mg/kg body weight i.p. for five consecutive days. As previously reported, this regimen led to diabetes in female RIP-mCD80 mice with a mean onset at day 60 (range 26–100 days) (11). In other experiments we used STZ at 30 mg/kg body weight, which resulted in an earlier IDDM onset in RIP-mCD80 CD28−/− mice (female FVB/N, H-2b; 31 ± 16.6 days; female F3, H-2b/c; 25.8 ± 10.2 days; male F3, H-2b/c; 15.8 ± 3.9 days). In all cases STZ was dissolved in a citrate buffer (pH 4.2), sterile filtered, and injected within 10 min of dissolution (22). In other studies, diabetes was induced by immunizing RIP-mCD80/LCMV-gp mice with a single i.p. injection of synthetic IFN-γ-pretreated fibroblast cell lines loaded with the LCMV-gp-derived, immunodominant, H-2Db-restricted CTL epitope gp33 (aa 33–41, KAVYNFATM; Ref. 23). The Dα-restricted, LCMV-nucleoprotein (LCMV-np) epitope np396 (aa 396–404, FPQPNGQF) was used as a control. All peptides were synthesized and purchased from Interactiva (Ulm, Germany). Blood glucose (BG) and urine glucose levels were monitored using the HexoCue test kit (HexoCue, Angelholm, Sweden) and Ketos-Diastix (Bayer, Elkhart, IN), respectively.

Pancreatic insulin content determination

Pancreata from euthanized mice were homogenized, and protein was extracted for 4 h at 4°C in acid-isethionate. Proinsulin and insulin concentrations in the extract were measured using a competitive ELISA as previously described (24). Briefly, plates were coated with rabbit anti-guinea pig Ig second antibody (Organon Teknika, Durham, NC) followed by incubation with guinea pig anti-human insulin Ab (Caltag, South San Francisco, CA). Following two washing steps, various extract dilutions or insulin standards (Linco Research, St. Louis, MO) were mixed with a constant concentration of HRP-conjugated rat insulin (Organon Teknika) for 4 h at room temperature or at 4°C overnight, before competitive capturing was allowed for 3 h. After washing five times, SigmaFAST OPD tablets (Sigma, St. Louis, MO) were used as substrate. Results were analyzed using a Ceres 900 C ELISA-reader with KC3 software (Bio-Tek Instruments, Winooski, VT). Pancreatic extract total protein was measured by a protein assay (Bio-Rad, Richmond, VA) according to the manufacturer’s recommendations using BSA standards (Bio-Rad).

Immunohistochemistry and mAbs used

Pancreata were snap frozen in isopentane. Cryostat sections (5–6 μm) were obtained and processed using standard procedures. Briefly, after acetone fixation for 15 min at 20°C, 8 min, sections were air-dried for at least 30 min and rehydrated in wash buffer (0.5% Tween 20 (Sigma) in PBS (Dige Diagnostics, Beltsville, MD)). Immunolabeling was performed for 30 min at 20°C with appropriately diluted anti-CD4 (K 1.5, rat IgG2a) and anti-CD8 (53-6.72, rat IgG2a) primary mAbs (American Type Culture Collection (ATCC), Manassas, VA). Sections were then stained 30 min with an appropriate secondary Ab-HRP (Dako, Carpinteria, CA). All sections were washed three times in wash buffer before individual staining steps. Color was developed using diaminobenzidine (DAB)/H2O2 (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin according to standard procedures. The following mAbs and second-step reagents were used for FACS staining and were obtained from PharMingen-Europe (Hamburg, Germany) unless indicated otherwise: CD3-biotin, CD4-PE (Caltag, South San Francisco, CA), CD8-biotin, B220-FITC, DX-5-FITC, streptavidin-PE, and streptavidin-PerCp (Becton Dickinson, Heidelberg, Germany).

Islet isolation

Islets of Langerhan were isolated from euthanized mice as previously described (25). Briefly, the common bile duct was clamped distal to the pancreatic duct junction at its duodenal insertion. The proximal common bile duct was then cannulated using a 27-gauge needle, and the pancreas was removed by retrograde injection of 6 ml of cold collagenase solution (0.5 mg/ml; Sigma) in PBS (Life Technologies, Eggenstein, Germany). Pancreatic tissue was collected and subjected to a 32-min digestion at 37°C in a water bath. Subsequently, pancreatic tissue was mechanically disassociated by repeated gentle aspiration through a 14-gauge needle, then filtered through a sterile 0.8-mm pore size mesh. Islets in the digested pancreata were enriched by Ficol-Hypaque centrifugation (20 min, 1100 × g, specific gravity 1.077 g/ml; Bioschroth, Berlin, Germany) and washed twice. Islets were hand-picked twice for final purification.

Phenotypic and functional analysis of islet infiltrating lymphocytes (ILL)

Purified infiltrated islets were dissociated into single cell suspensions by incubation in calcium-free PBS for 5 min at 37°C, then were subsequently rested overnight in culture medium (α-MEM, containing 10 mM HEPES buffer, 2 mM l-glutamine, 0.05 mM l-L-ME, 100 μg/ml streptomycin (Life Technologies), and supplemented with 10% FCS (Bio- technologies, plus 1 ng/ml IL-2 (R&D Systems, Wiesbaden, Germany)). The next day, islet suspension cells were stimulated with peptide-loaded EL-4 target cells (H-2b, ATCC) in the presence of 1 μM monensin (Fluka, Neu-Ulm, Germany). In addition to gp33 and np396, we also used a subdominant LCMV-gp CTL epitope, gp276 (278–286, SVGNPGYGCQ) (26), and an epitope derived from bovine insulin B chain, InsB37 (aa 7–15, CGSHLVEAL) as reported (27). To estimate the maximal frequency of responding cells, positive controls were stimulated with 5 ng/ml PMA plus 500 ng/ml ionomycin (both obtained from Sigma). After 4 hr, cells were washed twice in PBS containing 1% BSA (Serva, Heidelberg, Germany),...
and surface stained for 20 min at 4°C. After two more washes in calcium-free PBS, cells were fixed in 2% paraformaldehyde for 30 min at room temperature in the dark. Following two additional washes with PBS, cells were incubated in 100 μl saponin buffer (0.5 mg/ml in PBS/2% BSA; Fluka) containing the appropriate cytokine mAbs for 15 min at room temperature in the dark. Finally, cells were washed once with saponin buffer, resuspended in FACS sample buffer (PBS/1% BSA), and were immediately analyzed on a FACS Calibur (BDIS, Heidelberg, Germany). FACS analyses were performed using either CellQuest Software (BDIS) or the personal computer shareware WinMDI (J. Trotter, The Scripps Research Institute, La Jolla, CA).

Results

**RIP-mCD80 expression on pancreatic β cells does not affect insulin content in either naive mice or in mice recently given MLDS**

RIP-mCD80+ mice in our colony do spontaneously develop fatal diabetes, but with a strikingly low frequency of 6.8% (median age of onset: 47 wk, Ref. 11, and our unpublished observation). We reasoned that the exquisite sensitivity to MLDS-induced diabetes displayed by the RIP-mCD80+ mice compared with NT littermates could reflect compromised β cell number or function in the untreated transgenic mice. Therefore, we determined the relative pancreatic insulin content in RIP-mCD80+ and NT littermates before and after MLDS treatment. The relative insulin/total protein content has been used as an appropriate measurement of overall β cell mass (28). As is shown in Fig. 1 (left and middle), we detected no difference between RIP-mCD80+ and NT mice before and 3 days after the completion of MLDS induction (experimental day 8) using the 20 mg/kg dosage. Consistent with earlier reports (29), we did observe that MLDS treatment caused a substantial depletion of pancreatic β cell mass such that the insulin content was diminished by ∼50% at day 8 (Fig. 1, middle). After this initial drop, whereas NT mice remained euglycemic, the RIP-mCD80+ mice manifested an inexorable decline in the insulin content as demonstrated by the substantially depleted insulin content by day 77 (Fig. 1, third pair of columns). At this point, two of three (67%) RIP-mCD80+ mice were hyperglycemic, showing typical diabetes symptoms such as elevated glyceremia (>15 mM), glycosuria, polyuria, weight loss, ketonuria, and eventual death. Controls included mice given a single high dose STZ injection (200 mg/kg), which induced insulin depletion and IDDM within a few days (Fig. 1). We conclude that the β cell mass as estimated by the insulin/total protein ratio of RIP-mCD80+ mice and NT littermates does not significantly differ in naive mice and in the early phase following MLDS induction. Subsequently, RIP-mCD80+ mice but not NT littermates do experience a gradual loss of β cell function.

**Insulitis and subsequent depletion of β cells is dependent on CD28**

Pancreatic histological analysis following MLDS is characterized by an intense infiltrate consisting predominantly of CD4+ and CD8+ T cells and B lymphocytes. To address the role of CD28, RIP-mCD80+ mice (H-2b) were crossed twice with CD28-deficient (CD28−/−, H-2b) animals to obtain CD28−/− mice (F2 generation). Following the administration of MLDS, female (Fig. 2a) and male mice (Fig. 2b) were monitored for the onset of glycosuria and fasting hyperglycemia to detect IDDM onset. Although others have reported differences in the MLDS susceptibility attributable to differences in genetic background (22), we did not observe any significant differences when comparing female RIP-mCD80+ mice of an inbred FVB/N strain (H-2b) with the variously mixed proportions of H-2a and H-2b genetic backgrounds of the F2 (CD28−/− × RIP-mCD80+ ) offspring (Figs. 2 and 3, and data not shown). All RIP-mCD80+ transgenic mice with T cells expressing CD28 developed IDDM between 15 and 62 days (average 24 days) after MLDS induction (30 mg/kg regimen), and there was no obvious difference between mice carrying one normal CD28 allele (CD28+/−) as opposed to having both (CD28+/+). Some NT males, but not females, developed mild intermittent glycosuria (Fig. 2b), presumably reflecting the well-known sex hormone-related increased susceptibility to MLDS among male mice (22, 30). However, the most striking finding was that in the absence of CD28 there was no evidence that transgene-mediated β cell mCD80 expression heightened the susceptibility to MLDS-induced β cell destruction and IDDM.

We next considered the possibility, that CD28−/− mice carrying the RIP-mCD80 transgene may have mild but still significant hyperglycemia, indicating a partial susceptibility to MLDS. To test this possibility, we measured the fasting BGlc level regularly after MLDS; the highest BGlc measured is shown in Fig. 3. Mice that expressed both wild-type (WT) CD28 (either CD28+/+ or CD28−/−) and the transgene-encoded β cell mCD80 developed hyperglycemia (33.5 ± 4.1 and 36.4 ± 4.9 mM for females and males, respectively), whereas the BGlc levels of female NT mice remained entirely normal (9.6 ± 1.7 mM). Although some NT males developed mild hyperglycemia (13.3 ± 5.4 mM), this was independent of CD28 expression (13.3 ± 5.4 and 12.9 ± 3.6 mM for CD28+/+ and CD28−/−, respectively), and β cell CD80 co-expression had no effect on MLDS susceptibility in CD28−/− mice. Following MLDS, the BGlc levels of RIP-mCD80+CD28−/− and WT mice were comparable (females: 9.6 ± 1.7 vs 9.4 ± 1.3 mM; males: 13.3 ± 5.4 vs 18.7 ± 9.4 mM, RIP-mCD80+CD28−/− vs NT, respectively).

Previously we reported the appearance of a limited but significant lymphocytic infiltration in both NT and in RIP-mCD80+ mice 10–20 days after MLDS administration (11). This infiltrate is temporary in the NT mice but becomes much more extensive and

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**FIGURE 1.** Pancreatic insulin content after MLDS induction. Pancreata from RIP-mCD80+ and NT mice were taken from naive animals or experimental animals 3 and 72 days (experimental days 8 and 77, respectively) after administering MLDS at 20 mg/kg × 5 days. Two of the three RIP-mCD80+ mice were hyperglycemic by day 77; all other mice were normoglycemic. As a control, pancreata from high-dose STZ-treated animals were taken 7 days after a single high dose injection when all mice were hyperglycemic and had glycosuria for at least 2 days. The relative pancreatic insulin content represents the ratio of insulin and total protein concentrations (ng/mg). The values given are the average ± SE from NT and RIP-mCD80+ mice, respectively, as follows: untreated (4, 2), MLDS-d8 (3, 3), MLDS-d77 (3, 3), and high dose STZ (2, 2).
FIGURE 2. Influence of CD28 and CD80 transgene expression on the incidence of diabetes after MLDS induction. Groups of four to eight female (a) and male mice (b) with the indicated CD28 and RIP-mCD80 genotypes were monitored for glycosuria after MLDS induction (30 mg/kg × 5 days). Animals were considered diabetic when glycosuria occurred for three consecutive determinations within a week. Female FVB/N mice (H-2b) (a) were included to control for variations in the MLDS susceptibility attributable to the variously mixed MHC backgrounds in the F2 (RIP-mCD80+/CD28−/−) mice. Cumulative data are shown from two independent experiments.

destructive in the RIP-mCD80+ mice. The immunohistological evaluation of islet infiltration from consecutive pairs of pancreatic sections obtained from MLDS-treated H-2b mice is illustrated in Fig. 4 as follows: NT/CD28+/− mice (a and b), RIP-mCD80+/CD28+/+ mice (c and d), NT/CD28−/− mice (e and f), and RIP-mCD80+/CD28−/− mice (g and h). Although none of the mice analyzed were diabetic 11 days after completion of the MLDS treatment, the WT mice (NT/CD28+/+) showed significant CD4+ and CD8+ T cell peri-insulitis (Fig. 4b), whereas the RIP-mCD80+/CD28+/+ have already developed a more intense T cell infiltrate (Fig. 4d). Closer examination of these infiltrating cells revealed a high CD8+/CD4− T cell ratio in the RIP-mCD80+/CD28+/+ mice, whereas the infiltrate in the NT/CD28−/− mice consisted of equivalent CD4+ and CD8+ T cell numbers (data not shown). Most strikingly, pancreatic sections from CD28−/− mice did not harbor a substantial infiltrate whether or not the islets from those mice expressed the transgene-encoded mCD80 (Fig. 4, e–h). We have semiquantified these data, as is shown in Table I, by scoring T cell infiltration as estimated by combined staining with anti-CD4 and anti-CD8 mAb. Virtually all RIP-mCD80+/CD28+/+ mouse islets had a significant T cell infiltrate (49 of 50 islets examined) and many of them were severely affected. In contrast, pancreatic sections from NT/CD28−/− mice generally displayed a lower insulitis prevalence (41 of 69 islets examined), and affected islets tended to have a less extensive, mostly peri-insulitic infiltrate. Most notably, CD28−/− mice demonstrated little evidence of infiltrate at all. Sections revealed only a few T cells at the periphery of a minority of the islets (8/53). Thus we conclude that CD28−/− mice are protected from MLDS-induced IDDM, even in the presence of transgene-encoded mCD80, due to the abrogation of lymphocytic infiltrate formation.

MLDS induces autoantigen-specific, IFN-γ-secreting, CD8+ T cells among the IIL

MLDS-induced IDDM in RIP-mCD80+ mice may involve “ bystander” destruction of pancreatic β cells by inflammatory cytokines or nonspecific cell-mediated cytotoxicity (31). That is, the presence of transgene-encoded costimulatory CD80 molecules in the inflamed islets may account for much of the organ destruction that leads to diabetes, similar to the mechanism described for activation of virus-specific memory CTL (32). Therefore, we investigated whether IIL from MLDS-induced insulitic lesions were capable of responding specifically to autoantigenic epitopes. Intracytoplasmic cytokine staining was used to detect self-peptide-reactive, IFN-γ-secreting, CD8+ TIL, the main T effector subset in this model (11). To this end, RIP-mCD80+ FVB/N mice were backcrossed for nine generations onto the C57BL/6 background before they were bred to the RIP-LCMV-gp+ transgenic mouse (H-2b) encoding one dominant and one subdominant, H-2Db-restricted CTL epitope (12). H-2b mice expressing CD80 or CD80 plus LCMV-gp in their pancreatic islets were found to be equally

FIGURE 3. Influence of CD28 and CD80 transgene expression on the extent of hyperglycemia after MLDS induction. The mice from the experiments shown in Fig. 2 were also monitored weekly for the extent of hyperglycemia. The values shown represent the highest over all BGlc reading from each individual mouse obtained after a fasting period of at least 4 h.

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susceptible to MLDS treatment; both overall incidence and kinetics of disease development did not differ significantly from the FVB/N strain (H-2q) discussed above (data not shown). Islets from both groups of four to five MLDS-treated mice each were isolated immediately after the first mouse of that group first scored urine glucose positive (generally around experimental day 20–28 following MLDS treatment), allowing us to obtain heavily infiltrated islets from a group of mice just before disease onset. Among the groups, severely infiltrated islets contained roughly equivalent relative frequencies of CD8^+^, CD4^+^, and B cells (Fig. 5, left, and Table II). Interestingly, we quite regularly detected a CD8 and CD4 double-positive subset not typically found in the periphery of these mice (Fig. 5, and data not shown). These double-positive cells accounted for ~10% of all cells collected in the lymphocyte gate. We did not detect NK cells using the pan NK cell marker DX-5 (Table II, and data not shown).

To analyze their functional responsiveness and Ag specificity, IIL were stimulated with EL-4 stimulators (H-2b), loaded with either control peptide (or PBS) or with epitopes known to be encoded by the LCMV gp transgene (26). This approach not only could demonstrate islet specificity, but also allowed us to selectively test for recognition of putative epitopes encoded by the gp transgene and expressed in gp-transgenic β cells. Thus, only T cells from RIP-mCD80^-^LCMV-gp^-^ but not RIP-mCD80^+^ mice would be expected to be primed by LCMV-gp CTL epitopes. As is illustrated in Fig. 5, stimulating IIL with PMA + ionomycin (positive control) resulted in IFN-γ secretion by a large proportion of CD8^+^ IIL, whereas an irrelevant peptide (np396) yielded only a few IFN-γ-producing cells (Fig. 5, control). Most strikingly, stimulating RIP-mCD80^-^ LCMV-gp^-^-derived IIL with the autoantigenic CTL epitope (gp33)-loaded EL-4 cells resulted in IFN-γ production from a substantial proportion of CD8^+^ IIL. In contrast, IIL harvested from RIP-mCD80^+^ mice (i.e., those lacking β cell LCMV-gp) revealed a background level frequency of gp33-specific IFN-γ producers (Fig. 5, gp33). Data from two independent experiments, each using highly purified and pooled pancreatic islet suspension cells from four to five RIP-mCD80^-^ (Expt. 1 and 2) and RIP-mCD80^+^ LCMV-gp^-^ mice (Expt. 3 and 4) are summarized in Table II. Consistent with earlier results suggesting a dominant role for the CD8^+^ T cell subset in the pathogenesis of diabetes in RIP-mCD80^-^ mice, CD8^+^ cells always constituted the major lymphocytic subset isolated from prediabetic, destructive
There is some variability in the extent of both spontaneous and have been due to residual islet Ags present in the islet suspensions. The data in Table II have been calculated to represent the fraction of both overall and, in parentheses, PMA + ionomycin-stimulated CD8\(^+\) IIL, IFN-\(\gamma\) secretion by unstimulated (Expt. 1) IIL, or IIL stimulated by irrelevant epitopes (Expt. 2–4) was clearly detectable (range 1.1 and 4.5%; 6.6–14.3%) and was comparable in both RIP-mCD80\(^+\) and RIP-mCD80\(^+\)LCMV-gp\(^+\) double-transgenic mice. This background IFN-\(\gamma\) secretion may have been due to residual islet Ags present in the islet suspensions. There is some variability in the extent of both spontaneous and PMA + ionomycin-stimulated IFN-\(\gamma\) secretion among CD8\(^+\) IIL, which may reflect alterations in the overall immune reactivity due to losses of \(\beta\) cell Ags as the mice progress toward IDDM. Because it has been very difficult to precisely match the extent of insulitis and the projected disease manifestation of prediabetic mice, there may be substantial differences in the immune reactivity among IIL analyzed in Table II. In fact, IIL isolated from diabetic mice seemed to have lost Ag and mitogen responsiveness among IIL analyzed in Table II. In fact, IIL isolated from diabetic mice was clearly detectable (range 1.1 and 4.5%; 6.6–14.3%) and was comparable in both RIP-mCD80\(^+\) and RIP-mCD80\(^+\)LCMV-gp\(^+\) double-transgenic mice. This background IFN-\(\gamma\) secretion may have been due to residual islet Ags present in the islet suspensions. There is some variability in the extent of both spontaneous and PMA + ionomycin-stimulated IFN-\(\gamma\) secretion among CD8\(^+\) IIL, which may reflect alterations in the overall immune reactivity due to losses of \(\beta\) cell Ags as the mice progress toward IDDM. Because it has been very difficult to precisely match the extent of insulitis and the projected disease manifestation of prediabetic mice, there may be substantial differences in the immune reactivity among IIL analyzed in Table II. In fact, IIL isolated from diabetic mice seemed to have lost Ag and mitogen responsiveness substantially (data not shown). However, most importantly, stimulating IIL from RIP-mCD80\(^+\)LCMV-gp\(^+\) mice with the LCMV-gp-derived, immunodominant, H-2K\(\beta\)-restricted CTL epitope (gp33) elicited a substantially increased IFN-\(\gamma\) response, accounting for as much as one-fourth to one-third that resulting from PMA + ionomycin stimulation.

These data suggested that insulitis and diabetes could possibly be induced by gp33-specific immunization in RIP-mCD80\(^+\)LCMV-gp\(^+\) mice in the absence of MLDS. As shown in Table II (Expt. 5), we found that to be the case (one representative experiment of four is shown). Surprisingly, diabetes so induced did not lead to a higher frequency of gp33-specific CD8\(^+\) IIL in prediabetic mice (K. P., unpublished observation). Moreover, we did detect minor CTL responses toward the subdominant LCMV-gp epitope (gp276) as well (Table II). However, we did not detect responses toward a H-2K\(\beta\)-restricted CTL epitope derived from the bovine insulin B chain (InsBp7) reported to elicit H-2K\(\beta\)-restricted CTL responses due to cross-reactivity with a mouse insulin epitope (27). Most notably, in the absence of LCMV-gp, IIL displayed no significant increase in IFN-\(\gamma\) secretion upon gp33 stimulation.

We conclude that MLDS gives rise to potent autoantigen-specific CTL, which accumulate in the islet infiltrate and are critically involved in \(\beta\) cell destruction. Furthermore, the heavy islet infiltrate observed in both MLDS-induced and Ag-immunized RIP-mCD80\(^+\)LCMV-gp\(^+\) mice appeared comparable with regard to cellular subset composition and Ag responsiveness.

### Discussion

Although administration of multiple low doses of the \(\beta\) cell toxin STZ has been used successfully to induce IDDM in susceptible mice (34–36), the model discussed herein uses a STZ dose and a transgenic mouse strain that is unique. For instance, whereas male mice of certain strains are known to be susceptible to STZ using a 40–50 mg/kg \(\times\) 5 day regimen, females are generally regarded to be resistant under the same conditions (30). We have demonstrated profound MLDS-induced diabetes susceptibility of RIP-mCD80\(^+\) but not NT males and females of the FVB/N and C57BL/6 strains using doses as low as 20 mg/kg, and we noted a substantially accelerated disease onset at 30 mg/kg. Using the higher dose regimen in both CD28 WT and CD28-deficient NT male littermates, we
observed few mice with a tendency to develop transient episodes of mild glycosuria and hyperglycemia, although none of them developed progressive IDDM or died within the 100-day observation period. We cannot distinguish whether the apparently different STZ susceptibility we have observed in male vs female mice is secondary to differential STZ susceptibility (30), genetic background (37), or rather reflects the higher absolute dose of STZ given to the generally heavier male mice. Thus, we recognize that MLDS-induced transient, mild diabetes in NT male mice is probably not immune mediated. In any case, transgene-encoded β cell mCD80 expression, in the presence of T cells expressing CD28, profoundly heightens susceptibility to MLDS-induced IDDM in both male and female mice. Therefore, this model overcomes the sex-related and genetic background limitations of the low dose STZ-based autoimmune diabetes model.

STZ is well known to have β cell-toxic properties as reflected by the almost complete insulin depletion within 3–4 days following a single injection of high dose STZ (200 mg/kg). However, the data presented herein suggest that it is very unlikely that STZ caused IDDM in the RIP-mCD80β-transgenic mouse model system via direct β cell toxicity. For instance, STZ has a short biologic half-life (38) and yet diabetes does not develop in most mice for several weeks after MLDS induction. Likewise, data presented in Fig. 1 show comparable, although diminished, amounts of insulin (i.e., β cell mass) remaining 3 days after completion of MLDS treatment in both RIP-mCD80β and NT mice, at a time when there was very limited or no lymphocytic infiltrate detectable. Rather than direct β cell toxicity from the STZ, we have proposed that the data support a T cell-mediated process in RIP-mCD80β mice. Data supporting a T cell-mediated process include the prolonged period of clinical latency following MLDS induction, the development of an intense lymphocytic infiltrate, and inhibition of IDDM by administration of anti-T cell mAbs (especially anti-CD8 mAbs) and anti-CD80 mAb (11).

We now add additional evidence to favor a MLDS-induced cellular immune response resulting in IDDM. That is, RIP-mCD80βCD28−/− mice are resistant to MLDS-induced IDDM. Thus, consistent with earlier reports by Green et al. (39), in the absence of CD28 there is neither CTLA-4 (CD152) nor any other, as yet unknown T cell surface molecule capable of transmitting CD80-mediated signals to facilitate β cell-specific T cell responses. Although MLDS failed to induce IDDM in WT mice, their islets were reproducibly infiltrated by T cells. Interestingly, CD28−/− mice were not only protected from IDDM but they also displayed little or no infiltrate. To our knowledge, CD28−/− mice bred onto the FVB/N genetic background have not been carefully studied. Therefore, we cannot rule out the possibility that the variously mixed (C57BL/6 × FVB/N) F₂ genetic background of our mice has affected the results obtained, and hence limits potential interpretations. Nevertheless, our data suggested an important role for T cells in the pathogenesis of MLDS-induced IDDM by interaction of CD28 with its ligands in the pancreatic islets of both transgenic and WT mice, and are consistent with the report by Herold et al. (40) who observed, using a higher STZ dose (40 mg/kg), that otherwise genetically susceptible NT males are resistant to STZ in the absence of CD28.

The lack of infiltrate in the CD28−/− mice may be due to the failure of CD28-deficient T cells to secrete inflammatory mediators necessary to generate an environment for the development of a mature T cell response. To date, few studies have directly or indirectly examined the capacity of CD28−/− T cells to form adequate inflammatory foci. For instance, CD28−/− T cells were found to be only slightly less effective at allogeneic skin graft rejection (41) (B. Saha, unpublished observation) and efficiently rejected syngeneic tumors in immunized hosts (42). In a schistosomiasis model, comparable granuloma formation has been observed in CD28−/− mice (43), although CD28 deficiency disrupted lymph node germinal center formation (44, 45). Two other models of organ-specific autoimmune disease depended on functional CD28: spontaneous development of experimental autoimmune encephalomyelitis (EAE) in myelin basic protein-specific TCR-transgenic mice (46), and induction of collagen-induced arthritis (47). Interestingly, and in obvious contrast, the absence of the CD28 receptor in the NOD mouse exacerbates islet destruction and diabetes (48). Taken together, these studies fail to conclusively demonstrate a fundamental inability for CD28−/− T cells to mount a local infiltrative immune response. Thus, prevention of MLDS-induced insulitis and subsequent diabetes in CD28−/− mice presumably reflected the importance of costimulatory ligand CD80 (or CD86) availability rather than the inability of T cells to infiltrate the islets.

Numerous studies have shown that both β cell-specific CD4+ (49, 50) and CD8+ T cells (51, 52) can induce diabetes in adoptive T cell transfer studies. Nevertheless, it has not been resolved whether β cells are destroyed by a cognate interaction of autoantigen-specific T cells or rather by bystander mechanisms such as cytokine-mediated toxicity or unspecific cell-mediated cytotoxicity. It appears that CD8+-mediated islet destruction tends to be Ag specific, whereas CD4+ T cells act by facilitating an inflammatory milieu that in turn is toxic to the β cells (53). In support of this, we...
have demonstrated by immunohistochemistry and by FACS the presence of a substantial number of autoantigen-specific CD8\(^+\) IIL in the inflamed islets of MLDS-treated, prediabetic mice. Moreover, the CD8\(^+\) Ag specificity (based on autoantigen-specific epitope recognition) and the CD4 independence of MLDS-induced diabetes (11) suggest that islet-specific CD8\(^+\) T cells may in fact be directly primed by insulin-producing \(\beta\) cells, thereby circumventing the requirement for cross-priming, which has been found to critically involve host APC and CD4\(^+\) T cells (54). Remarkably, activated CD8\(^+\) T cells recognized a single self-epitope (Fig. 5, and Table II). This phenomenon of epitope dominance has been known from virus infection models, where CTL responses frequently focus on one or very few dominant epitopes. Subdominant epitopes will give rise to a functionally competent CTL response only in the absence of the dominant epitope (55, 56). In the prediabetic stage, such epitope dominance appears not to result from the method used to induce insulin (MLDS or autoantigenic epitope immunization), but rather from local regulatory mechanisms operating during the inflammatory processes. Thus, specific CTL recognition of islet autoantigens plays an important role in the pathogenesis of MLDS-induced diabetes in RIP-mCD80\(^+\) mice. We have been unable to adoptively transfer disease using spleen or lymph node cells from diabetic to naive RIP-mCD80\(^+\) or NT littermates (data not shown). Ongoing studies indicate that the failure of the costimulatory pathway in the pathogenesis of RIP-mCD80\(^+\) mice.

In conclusion, we now provide additional evidence that MLDS-induced IIL diabetes in RIP-mCD80\(^+\) mice is T cell dependent, requires the T cells to express CD28, and that the model results in islet Ag-specific T cell activation. The fact that we and others are finding costimulatory ligands on other than professional APCs, and that B7 family receptor expression can be dynamically regulated, suggests that the role of the costimulatory pathway in the pathogenesis of autoimmune illness warrants further exploration. Indeed, several reports now suggest that CTLA4 (CD152) polymorphisms are an important risk factor for human IDDM (57) and perhaps other autoimmune endocrine disorders. Thus MLDS-induced diabetes in RIP-mCD80\(^+\) mice may be a suitable model to study immunopathogenesis or therapeutic approaches, if inappropriate costimulation forms the basis of development of IDDM or other T cell-mediated autoimmune disorders.

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