Adenovirus E3 MHC inhibitory genes but not TNF/Fas apoptotic inhibitory genes expressed in β cells prevent autoimmune diabetes

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To mimic events and molecules involved in type 1 insulin-dependent diabetes mellitus (T1D), we previously designed a transgenic (tg) mouse model where the viral nucleoprotein (NP) gene of lymphocytic choriomeningitis virus (LCMV) was expressed in the thymus to delete high affinity antiself (virus) T cells and in insulin-producing β cells of the islets of Langerhans. Such tg mice, termed RIP-LCMV, fail to spontaneously develop diabetes. In contrast, when these mice are challenged with LCMV, they develop diabetes as they display hyperglycemia, low to absent levels of pancreatic insulin, and abundant mononuclear cell infiltrates in the islets. However, expressing the adenovirus early region (E3) gene in β cells along with the LCMV transgene aborted the T1D. The present study utilizes this combined tg model (RIP LCMV × RIP E3) to define the requirement(s) of either pro-apoptotic TNF and Fas pathways or MHC class I up-regulation on β cells for virus-induced T1D. Inhibitors to either pathway (TNF/Fas or MHC class I) are encoded in the E3 gene complex. To accomplish this task either the E3 region encoding the inhibitors of TNF and Fas pathways or the region encoding gp-19, a protein that inhibits transport of MHC class I molecules out of the endoplasmic reticulum were deleted in the RIP LCMV × RIP E3 model. Thus only the gp-19 is required to abort the virus-induced T1D. In contrast, removal of TNF- and Fas-pathway inhibitory genes had no effect on E3-mediated prevention of T1D.

adenovirus E3 regulatory region | Type 1 diabetes

Type 1 diabetes (T1D) results from destruction of insulin-producing β cells located in the pancreatic islets of Langerhans. Most often destruction of β cells occurs over a prolonged period, and disease is characterized by hyperglycemia, hypoinsulinemia, and mononuclear cell infiltration in the islets. To dissect the molecular and biologic events involved, we created a transgenic (tg) mouse model of T1D inducible by virus infection, where a unique protein of that virus was expressed in the thymus and in the β cells of the islets of Langerhans. As the virus transgene was passaged in the germ line of progeny mice, the mouse immune system treated the viral protein as a self antigen. Such unmanipulated mice had a negligible spontaneous incidence of T1D of less than 1% over their normal life span (1, 2). However, when immunologic tolerance to the virus (self) protein was broken by infection with the virus or a different virus that shared cross-reactive T cell epitopes, diabetes developed with an incidence of >90% within 1 to several months depending on the host's genetic background (1–5). Such mice displayed the triad of hyperglycemia, hypoinsulinemia, and mononuclear cell infiltrates in the islets.

The expression of the virus (self) transgene in the thymus allowed the removal (negative selection) of potential high affinity antiviral (self) T cells (6). This created a scenario closely mimicking human diabetes, where a time-lapse component was required from the initiation of breakage of tolerance until clinical manifestation of T1D. Thus, this model of slow-onset diabetes, termed here RIP-lymphocytic choriomeningitis virus (LCMV), was characterized by the generation of low affinity and low avidity antiself (viral) CD8 cytotoxic T cells (CTLs) that depended on CD4 T cells help to cause diabetes and contrasted to rapid-onset T1D models, where the viral transgene was only expressed in β cells, and after virus infection, T1D developed within 10–14 days as such mice generated higher affinity and avidity CD8 T cells, which did not require CD4 T cell help to cause diabetes (2, 7). Additional studies with the slow-onset T1D model using deletion or inhibition techniques combined with complementation revealed that virus infection of the islets was associated with a variety of locally released cytokines and chemokines as IL-10, IFN-γ, TNF-α, and IP-10, which combined with the antiviral (self) T cells were associated with T1D (4, 8–12). However, which of these factors was absolutely necessary for causing diabetes as compared to assisting or amplifying the disease was not clear.

To begin to sort out the various genes and their products that might play commanding roles in T1D, we earlier expressed the adenovirus early region (E3) in the β cells of the islets along with the viral (self) transgene (13). The adenovirus E3 contains an immunoregulatory gene that encodes a 19-kDa glycoprotein (gp 19) that inhibits the transport of class I major histocompatibility complex (MHC) molecules out of the endoplasmic reticulum (14). The E3 also possesses a cluster of genes that encode for three protein inhibitors of the cytolytic functions of tumor necrosis factor α (TNF-α) and Fas (14). When the adenovirus E3 transgene was expressed in β cells, it prevented T1D induced by infection in either the fast- or slow-onset T1D model (13). These clear results paved the way to address the question as to whether MHC expression on β cells and function of TNF-α and FAS were both necessary to cause T1D or, in contrast, was expression of just one of these factors required. To select between these possible scenarios, we deleted either the gene encoding the MHC I inhibitory gp 19 (Δ704) or removed the adenovirus E3 gene complex that encodes a 14.7-kDa protein as well as the heterotrimer of two proteins (10.4 and 14.5 kDa) that inhibits TNF-α and Fas activity (Δ309). Our results indicate that removal of the gene that inhibited MHC class I cell surface expression resulted in increased MHC class I expression on islets during diabetogenesis and restoration of T1D susceptibility as manifested by hyperglycemia and mononuclear cell infiltration. This occurred in the presence of the TNF-α and Fas inhibitory E3

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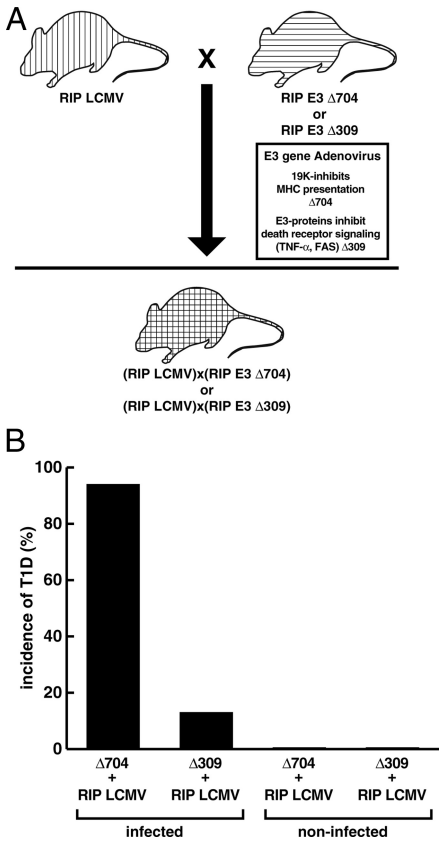

Fig. 1. Incidence of T1D in double-transgenic RIP LCMV × RIP E3-deletion mice. (*A*) A cartoon of the transgenic model used. (*B*) Results showing that following LCMV infection (1×10^5 PFU i.p.) RIP LCMV × RIP E3 Δ704 had an incidence of T1D of 94% while RIP LCMV × RIP E3 Δ309 had an incidence of 13%. However, RIP LCMV tg mice with either Δ704 or Δ309, failed to develop diabetes when not infected with virus. The graph records the cumulative incidence of T1D over a 4-month observation period (see *Materials and Methods* section for details).

genes. In contrast, deletion of the TNF-α and Fas inhibitory E3 genes, but preventing the expression of MHC class I molecules on β cells failed to be permissive for T1D.

Results

The MHC-Inhibitory gp 19, But Not the Antiapoptotic 14.7-, 10.4-, or 14.5-kDa Proteins, Is Essential for Prevention of Virus-Induced T1D in RIP LCMV × RIP E3 tg Mice. Fig. 1*A* displays a cartoon of the double tg model used. Fig. 1*B* indicates that T1D occurred in RIP LCMV × RIP E3 Δ704 tg mice, in which only the gene inhibiting MHC class I cell surface expression was removed. Diabetes, defined as a blood sugar over 250 mg/dL, was found in three of 15 mice by 20–28 days post-LCMV challenge with the remainder becoming diabetic 56 days postinfection. By contrast, removal of the gene complex that inhibits TNF-α and Fas pathways in β cells (Δ309) but retention of the gene that inhibits MHC class I expression on β cells still resulted in prevention of T1D (Fig. 1*B*) over the 4-month observation period. As anticipated, all RIP LCMV × RIP E3 Δ704, RIP LCMV × RIP E3 Δ309, and RIP LCMV tg mice failed to develop diabetes when not infected with virus.

Fig. 2 shows the maximal blood glucose level in individual mice followed over the 4-month observation period. Once the blood glucose level reached 350 mg/dL or higher, mice were killed. One RIP LCMV × RIP E3 Δ309 mouse of the eight inoculated with LCMV (13%) developed hyperglycemia and

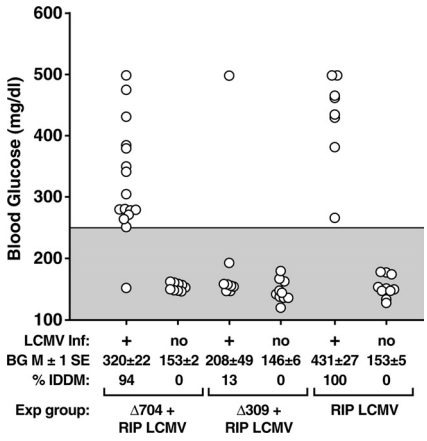

Fig. 2. Glycemia in double-transgenic RIP LCMV × RIP E3 deletion mice. The shaded area records normal blood glucose level + 2 SD. Each dot records the highest level of blood glucose for individual mice over the 4-month observation period. When levels reached 350 mg/dL or higher mice were killed. Differences between RIP LCMV × RIP E3 Δ704 tg mice and RIP LCMV × RIP E3 Δ309 tg mice are highly significant. $P < 0.001$.

displayed mononuclear infiltrates in its islets of Langerhans. In contrast, 15 of 16 (94%) RIP LCMV × RIP E3 Δ704 mice developed T1D.

RIP LCMV × RIP E3 Δ704 tg Mice That Developed T1D Displayed High Levels of MHC Class I Glycoproteins on β Cell Surface and Mononuclear Cell Infiltrates into the Islets of Langerhans. The next series of experiments isolated islet cells from the pancreas of RIP LCMV × RIP E3 Δ704 and RIP LCMV × RIP E3 Δ309 tg mice 6 days after LCMV infection. Individual β cells were obtained as described (15), stained for surface expression of MHC class I Db using monoclonal antibody (mAb) HB27 and analyzed by FACS. As shown in Fig. 3, MHC expression was dramatically enhanced on β cells from RIP LCMV × RIP E3 Δ704 over background MHC class I levels during diabetogenesis. By contrast, MHC class I levels in RIP LCMV × RIP E3 Δ309 mice that still expressed the MHC inhibitory gp 19 showed negligible to minimal MHC class I expression on β cells.

RIP LCMV × RIP E3 Δ704 tg mice that developed hyperglycemia also demonstrated infiltration of mononuclear cells into the islets (Fig. 4). Infiltration was restricted only to islets in the pancreas. In contrast, negligible mononuclear cell infiltrates occurred in islets from RIP LCMV × RIP E3 Δ309 tg mice, and the integrity of the islet structure was maintained.

Discussion

T1D occurs when insulin-expressing β cells in the islets of Langerhans in the pancreas are destroyed, most often by an autoimmune inflammatory process. This destruction is complex and associated with multiple factors including chemokines that signal or assist in directing killer T cells that are responsible for killing β cells to the target, molecules on or secreted by both target cells and effector T cells that play roles in the recognition and killing process, and the target β cells and effector T cells themselves (3, 6, 8–12, 16, 17). Previous studies have identified cytokine-mediated pathways involving TNF, IL-1β, and IFN-γ (12, 15, 18, 19) as well as Fas-mediated (20, 21) and perforin-mediated killing (22, 23) of importance for β cell demise in various animal models. Of importance is that the main cell type found in human islet infiltrates is the CD8 lymphocyte (24, 25), which can secrete TNF and IFN-γ and kill target cells using Fas and perforin pathways. Furthermore, CD8 cells are excellent

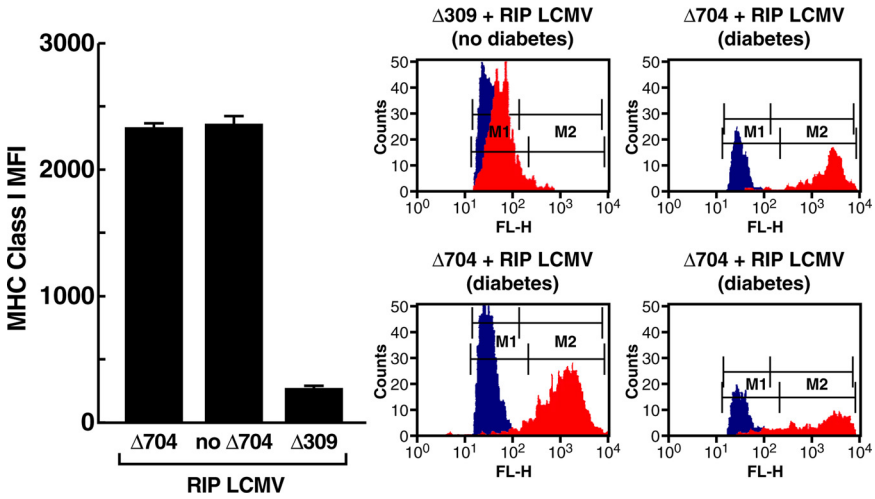

Fig. 3. Induction of MHC class I on β cells of RIP LCMV × RIP E3 deletion mice during diabetes development. Islet β cells were isolated from mice 6 days following induction of diabetogenesis with LCMV infection. MHC class I levels were determined by FACS. Panel on left records mean + SD of four mice/group. MFI, mean fluorescent index. Four panels on the right reflect data for individual mice. Blue records baseline (control) level, while red records expression of MHC I on β cells following LCMV infection.

antiviral effectors, and viral proteins were recently identified in human β cells in several studies (26, 27).

The well structured RIP LCMV model of virally induced T1D seemed an ideal tool to sort out the relative contribution of these various pathways in causing diabetes (1, 2, 4). For these studies, we expressed the self (viral) autoimmune diabetes antigen [transgene-encoding LCMV nucleoprotein (NP)] in β cells along with the adenovirus E3 genes that contained deletions of either the gene encoding the inhibitor that aborts MHC expression on target β cell surfaces or the genes encoding inhibitors of TNF-α and Fas pathways. This allowed us to clearly define the presence of MHC class I molecules on β cell surface as a necessary and essential factor in the initiation of T1D in this model. Thus, while 15 of 16 RIP LCMV × RIP E3 Δ704 tg mice infected with LCMV developed T1D, only one of eight RIP LCMV × RIP E3 Δ309 did. Our finding of an absolutely essential role for the expression of MHC class I molecules on the target β cell surface is supported by earlier studies in RIP LCMV tg mice in which the IFN-γ gene was deleted (12). Such mice when infected with LCMV displayed the generation and expansion of a normal and robust T-cell response, but T1D did not occur. These T cells displayed normal killing activity ex vivo and migration to the target tissue, but MHC expression on islet cells was lacking. In additional studies using the tg model of more rapid-onset diabetes, where the self (viral) antigen is expressed only in the islet and not in the thymus to allow positive selection of high affinity and avidity T cells, a MHC class I-restricted blocking peptide, SMIENLEYM, that bound at high affinity to MHC class I H-2Db molecules and blocked killing of H-2Db target cells in vitro by CTL also blocked T1D in the RIP LCMV fast-onset diabetes model (28–30). This abortion of T1D occurred in the presence of a robust generation and expansion of effector T cells in vivo and their migration to the islets.

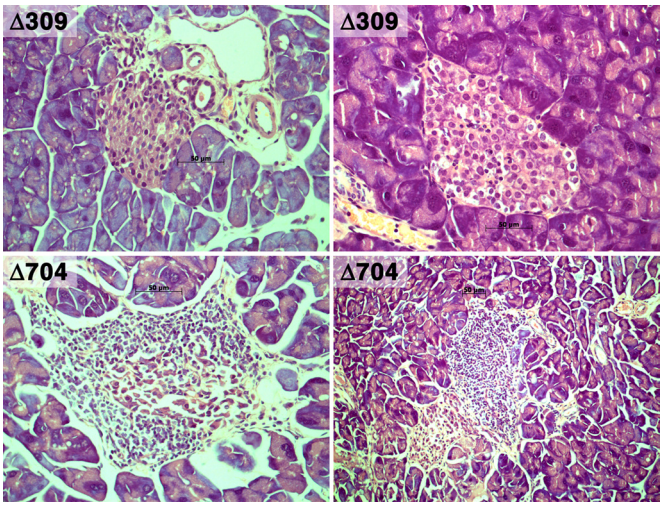

Fig. 4. Histological analysis of pancreas from RIP LCMV × RIP E3 deletion mice when their blood glucose levels exceeded 350 mg/dL (Δ704) or with normal blood glucose levels (Δ309) 4 months post-LCMV infection. Note marked mononuclear cell infiltration into islets of LCMV-infected RIP LCMV × RIP E3 Δ704 transgenic mice accompanied by abnormal islet anatomy. In contrast, LCMV infection of RIP LCMV × RIP E3 Δ309 mice resulted in preservation of islet structure and negligible to minimal mononuclear infiltrates.

Most RIP LCMV × E3 Δ309 tg mice failed to develop T1D. Thus the presence of intact Fas and TNF-α pathways in the target β cells was not essential for T1D. Other molecules like IP-10, IFN-γ, perforin, IL-1, IL-2, and IL-12 and some other cytokines or chemokines have been reported to play a role in T1D. For example, IP-10 can direct autoreactive T cells to the target area, and IFN-γ can enhance MHC expression. Expression of MHC on β cell surface is known to be a prerequisite for CD8 CTL-mediated killing (15). In conjunction with the proof in the present study that MHC class I is required for virally mediated diabetes in the RIP-LCMV model, previous observations that had attributed important roles for IFN-γ (12, 15), IP-10 (8, 9), and perforin (22, 23) in this model make absolute sense. In addition, several investigators have found strong up-regulation of MHC class I on human β cells in T1D patients and to a lesser extent on healthy controls (24, 26, 31). Since MHC can also be present in human islets in the absence of immune infiltrates (32), a viral cause has to be taken into account, which is supported by recent immunogenetic studies (33, 34).

Animal models (including tg models) are helpful in dissecting various parameters in pathogenesis but may only mimic selected events. That is, the model used may vary in the essential molecules involved. In our tg model of virus-induced T1D, using selected E3 gene deletions implicated gp 19 as essential and not the proteins inhibiting the Fas or TNF-α pathways. Opposite

results occurred when the same RIP-E3 constructs were used in the NOD model of spontaneous diabetes (35). In this case, the antiapoptotic 10.4-, 14.5-, and 14.7-kDa proteins were found to be essential in decreasing the incidence of T1D while gp 19 was not (35). The reasons for these differences are not clear but highlight the need to be cautious in expanding experimental results to a general conclusion when different models are used. The key issue will be to define which model best mimics human T1D. Recent reports (26, 27, 30) suggest that a strong case may be made for the contribution of viruses to T1D.

Viruses have been implicated as one of the initiating factors in T1D in addition to genetics and autoimmunity. While genetic influences are clear, the finding that T1D is discordant in approximately 50% of identical twins when the diagnosis of one twin was made before 40 years of age provides compelling evidence that environmental factors are important in the disease (36). We (1, 2) and others (7) have established a virus-dependent portrait of T1D by expressing a virus (self) gene in islet β cells. In conjunction with evidence of up-regulated MHC class I molecules on human β cells (24), coupled with recent evidence of enteroviral proteins (26, 27) in some islets, raises the possibility that viruses may contribute to the pathogenesis of T1D in at least some cases by leading to the release of interferons that would up-regulate MHC proteins on β cells thus rendering them susceptible to attack by autoreactive CTL. Indeed all of the ingredients are present in human islets, including predominance of CD8 T cells in islet infiltrates. Finally, our results refocus attention for a potential role for virus interplayed with genetics as contributing to T1D and support earlier reports of the isolation of an enterovirus (coxsackie B4) from the pancreas of a child with diabetic ketoacidosis and inoculation of mice with that human isolate causing diabetes (37) and studies in nonhuman primates showing that under certain circumstances coxsackie virus altered glucose homeostasis (38).

Materials and Methods

Transgenic Lines. Transgenic lines, generation and characterization of the RIP LCMV, RIP E3 Δ704 or RIP E3 Δ309 tg lines have been described (1, 2, 13). Briefly, the RIP LCMV line (RIP NP 25-13) (H-2b: C57BL/6) tg mice express the LCMV Armstrong (ARM) viral NP in the thymus and pancreas but not other tissues (1, 2). The immunodominant H-2Db-restricted NP epitope is amino acids 396–402, and expression in the thymus in H-2b mice aborted the generation of high affinity high avidity LCMV NP-specific CTL response (6) and any alternative subdominant NP CTL responses upon primary challenge with LCMV. Administration of LCMV ARM resulted in T1D in >90% of these tg mice by 2–4 months. The RIP E3 Δ704 and RIP E3 Δ309 constructs were also injected into H-2b (C57BL/6) mice. RIP LCMV tg mice were bred to E3 Δ704 or E3 Δ309 tg mice and mice bearing double-tg detected by PCR (1, 2, 13) were used for experiments when 8–10 weeks old.

Virus. Virus used was plaque purified LCMV ARM Clone 53b. The origin, sequence, and quantication of virus by plaque assay have been described (1, 2, 5, 6). Virus was administered as 1×10^5 PFU i.p. per mouse.

Blood Glucose Measurements. Blood samples were obtained from the retro-orbital plexus of mice before administrating virus or vehicle and then every 14 days for 4 months. Plasma glucose concentration was determined using One-Touch Ultra (LifeScan) as reported (8–10, 12).

Histologic Analysis of Tissues. Tissues taken for histologic examination were fixed in 10% zinc-formalin, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin.

Isolation of Pancreatic Islet Cells. Islets of Langerhans were isolated from mice, as previously described (15). In brief, the common bile duct was cannulated, and the pancreas was distended with 3 mL Dulbecco's modified Eagle's medium containing 1.3 U/mL collagenase P (Roche Molecular Biochemicals). Pooled pancreata were digested at 37 °C for 20 min and were then disrupted by shaking. Islets were purified on Histopaque-1077 density gradients (Sigma). The gradient was centrifuged with gradually increasing speed from 25–800 $\times$ *g* for 4 min, then at 800 $\times$ *g* for 10 min. Islets were aspirated from the media/gradient interface, washed, and handpicked, if necessary. The islets were dispersed into single cells with 0.2% trypsin (Calbiochem), 10 mmol/L EDTA in Hanks' balanced salt solution (HBSS), and were allowed to recover in complete medium (7% FCS, 1% penicillin/streptomycin, and 1% glutamine; Connaught Medical Research Laboratories) for 1 h before staining with mAbs. To ensure the purity of the β cell preparation, fluorescence-activated cell sorted (FACS) β cells were fixed on immunohistochemistry slides and stained for intracellular insulin, as previously described (15). Antibodies used were a polyclonal guinea pig anti-insulin antibody (Dako) as primary antibody, and a biotinylated goat anti-guinea pig IgG-Ab (Vector Laboratories) as secondary antibody. Of the isolated islet cells, on average 56% were β cells, as evidenced by positive insulin stain, and >98% of cells in the final gated autofluorescent population were β cells.

Detection of MHC Class I on β Cells. Whole islets were trypsinized, and islet cells were counted. To stain MHC class I, we used 28–14-8 (BD), a biotinylated mouse IgG2a mAb directed against murine H-2Db, which only cross-reacts with H-2Ld and H-2Dq. Secondary antibodies were as follows: goat F(ab)2, anti-rat IgG(H+L) phycoerythrin-conjugated (Caltag Labs), and phycoerythrin-conjugated streptavidin (Caltag Labs). Islet cell or spleen cell suspensions were incubated for 20 min on ice with mAb diluted in Dulbecco's PBS (DPBS) containing 1% FCS. They were then washed and incubated for 15 min with a secondary antibody as necessary. Finally, cells were washed and resuspended in DPBS with 1% FCS and 2.5 μg/mL propidium iodide to stain dead cells. Analysis was performed on a FACSort or FACScalibur flow cytometer (Becton Dickinson) with Cell Quest software (Becton Dickinson). β Cells were sorted on a FACStarplus (Becton Dickinson) on the basis of flavin adenine dinucleotide autofluorescence, according to the method described (15). Background levels were determined by staining MHC class I-deficient islets from $\beta 2m^{-/-}$ mice.

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