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 antihuman (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 (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 antiviral (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 cytocidal 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 diabetes 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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inoculated with LCMV (13%) developed hyperglycemia and killed. One RIP LCMV mice followed over the 4-month observation period. Once the LCMV tg mice failed to develop diabetes when not infected with expression on the gene complex that inhibits TNF- becoming diabetic 56 days postinfection. By contrast, removal of 15 mice by 20–28 days post-LCMV challenge with the remainder defined as a blood sugar over 250 mg/dL, was found in three of MHC class I cell surface expression was removed. Diabetes, LCMV over the 4-month observation period. As anticipated, all RIP LCMV 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.

**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 displays a cartoon of the double tg model used. Fig. 1B 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. 1B) 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

**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
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 in 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, SMIEFLNVY, 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.

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 TID 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 determine 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.

Virologists 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 enterviral 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 reocess attention for a potential role for virus interplayed with genetics as contributing to T1D and support earlier reports of the isolation of an enterovirus (cossackie 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 cossackie virus altered glucose homeostasis (38).

Materials and Methods

Transgenic Lines. Transgenic lines, generation and characterization of the RIP LCMV, RIP E3Δ704 or RIP E3Δ309tg t 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 tg mice abrogates 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 E3Δ309 tg mice and bearing double-tg detected by PCR (1, 2, 13) were used for experiments when 8–10 weeks old.

Virus. Virus was used was plaque purified LCMV ARM Clone 53b. The origin, sequence, and quantification of virus by plaque assay have been described (1, 2, 5, 6). Virus was administered as 1 × 10⁵ PFU i.p. per mouse.


