Autoimmune diabetes is caused by selective loss of insulin-producing pancreatic β-cells. The main factors directly implicated in β-cell death are autoreactive, cytotoxic (islet-antigen specific) T-lymphocytes (CTL), and inflammatory cytokines. In this study, we have used an antigen-specific model of virally induced autoimmune diabetes to demonstrate that even high numbers of autoreactive CTL are unable to lyse β-cells by perforin unless major histocompatibility complex class I is upregulated on islets. This requires the presence of inflammatory cytokines induced by viral infection of the exocrine pancreas but not of the β-cells. Unexpectedly, we found that the resulting perforin-mediated killing of β-cells by autoreactive CTL is not sufficient to lead to clinically overt diabetes in vivo, and it is not an absolute prerequisite for the development of insulitis, as shown by studies in perforin-deficient transgenic mice. In turn, destruction of β-cells also requires a direct effect of γ-interferon (IFN-γ), which is likely to be in synergy with other cytokines, as shown in double transgenic mice that express a mutated IFN-γ receptor on their β-cells in addition to the viral (target) antigen and do not develop diabetes. Thus, destruction of most β-cells occurs as cytokine-mediated death and requires IFN-γ in addition to perforin. Understanding these kinetics could be of high conceptual importance for the design of suitable interventions in prediabetic individuals at risk to develop type 1 diabetes.

Type 1 diabetes is an autoimmune disease thought to be caused by the activation of autoreactive CD4 and CD8 effector cells that recognize islet self-antigens resulting in the destruction of >90% of the pancreatic insulin-producing β-cells (1,2). Because diabetes is often discordant in monozygotic twins carrying diabetes, susceptibility major histocompatibility complex (MHC) alleles (3,4), environmental factors, and/or infectious agents such as viruses have been implicated as the possible cause of diabetes (5). Cytotoxic (MHC class I restricted) T-lymphocytes (CTL) capable of killing target cells through secretion of perforin granules are one of the main components of most anti-viral immune responses. The participation of viruses in causing type 1 diabetes could be twofold. First, cross-reactivity that may involve molecular mimicry (6,7) between viral and self (islet) antigens could lead to direct perforin-mediated killing of β-cells. Alternatively or in addition, inflammatory cytokines secreted by such CTL and/or anti-viral CD4 lymphocytes could lead to γ-interferon (IFN-γ)–, tumor necrosis factor (TNF)-α–, and interleukin (IL)-1β–mediated death of islet cells (8,9). Historically, autoreactive CTL (10–12) FAS/FAS-ligand–mediated killing (13–17), as well as the direct effects of cytokines, (9,18–20) have been shown to destroy β-cells. However, not much is known about the precise relative contributions of these factors in vivo or their importance at different stages of type 1 diabetes pathogenesis. Previous studies have demonstrated the requirement for IFN-γ in type 1 diabetes and its role in upregulating MHC class I on β-cells in vivo (21–23). The direct β-cell toxicity of IFN-γ, as well as other cytokines such as TNF-α, has also been shown in vitro (9). CTL can rapidly produce these cytokines on activation (24), but their other task is to kill target cells expressing the appropriate MHC class I/peptide complex via the perforin granule exocytosis pathway (25). Various lines of evidence have implicated CTL in initiation, as well as effector phases, of type 1 diabetes pathogenesis (10–12,23,26). We reasoned that it would be of importance to further dissect their precise effector contributions in type 1 diabetes to judge the potential efficacy of antigen-specific interventions and precisely target CTL during the optimal susceptible phase(s) in type 1 diabetes pathogenesis.

Based on these considerations, our goal was to evaluate the relative contribution of perforin competent, lytic CTL, and
inflammatory cytokines are known to play an intricate role in enhancing type 1 diabetes in vivo (23). 4) This model appears to mimic many known aspects of human type 1 diabetes, such as the genetic influence, mononuclear infiltration of islets, and the production of islet autoantibodies caused by antigenic spreading preceding onset of clinical type 1 diabetes. RIP-LCMV mice express the glycoprotein of LCMV under control of the RIP selectively in their pancreatic β-cells. LCMV infection leads to activation of anti-viral CTLs that continue to attack islet cells expressing the viral transgene after they have eliminated the virus systemically and locally in the pancreas (27). Replicating LCMV is found in the pancreas and some islets (<2%) from 2–10 days postinfection, but pronounced infiltration and type 1 diabetes occur only later, at day 14 (31). The systemic CTL response to LCMV peaks between days 6 and 8 (32), which coincides with the first lymphocytes entering the islets. Thus, systemic elimination of LCMV infection and destruction of β-cells are two distinct immunologic processes that occur with different kinetics (hit-and-run event). From previous studies using MHC class I, CD8, or perforin-deficient RIP-LCMV mice, it was concluded that anti-self (viral) CTL destroy islets in RIP-LCMV mice (26,27), as well as NOD mice (33), through perforin-mediated killing. However, recent findings showing as many as 1 × 10^5 highly activated anti-LCMV CTL were unable to induce type 1 diabetes in RIP-LCMV recipients after adoptive transfer cast some doubt on this issue (31). In these experiments, LCMV CTL were activated with peptide-coated antigen presenting cells (APCs) free of infectious virus, and CTL precursor numbers reached after such transfers in vivo were equivalent to LCMV CTL numbers usually generated after acute infection (31) (see Research Design and Methods). We were interested in further investigating the relative contributions of perforin, inflammatory cytokines, and the local viral infection of the pancreas in β-cell destruction. The new mechanistic findings we present here could be of importance in shaping immune intervention strategies.

**RESEARCH DESIGN AND METHODS**

**Islet isolation.** Islets of Langerhans were isolated from mice, as previously described (34). In brief, the common bile duct was cannulated, and the pancreas was distended with 3 ml of Dulbecco’s modified Eagle’s medium containing 1.3 U/ml collagenase P (Boehringer Mannheim, Indianapolis, IN). 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, St Louis, MO) (35). The gradient was centrifuged with gradually increasing speed from 25–900g for 4 min, then at 800g for 10 min. Islets were aspirated from the media/gradient interface, washed, and hand picked, if necessary. The islets were dispersed into single cells with 0.2% trypsin (Calbiochem, La Jolla, CA), 10 mmol/l EDTA in Hank’s balanced salt solution (HBSS), and were allowed to recover in complete medium (Coombaugh Medical Research Laboratories, 7% fetal calf serum [FCS], 1% penicillin-streptomycin, and 1% glutamine) for 1 h before staining with monoclonal antibodies (mAb). To ensure the purity of the β-cell preparation, fluorescence-activated cell sorter (FACS)–sorted β-cells were fixed on immunohistochemistry slides and stained for intracellular insulin, as previously described (30). Antibodies used were a polyclonal guinea pig anti-insulin antibody (Dako, Carpinteria, CA) as primary antibody, and a biotinylated goat anti-guinea pig IgG-Ab (Vector Laboratories, Burlingame, CA) as secondary antibody. Of cells, 56% were β-cells on average, as evidenced by positive insulin stain, and >80% of cells in the final gated autofluorescent population were β-cells.

**Detection of MHC class I on β-cells.** Whole islets were trypsinized and isolated cells were counted. To stain MHC class I, we used 28-14-5 (PharMingen, La Jolla, CA), a biotinylated mouse IgG2a mAb directed against murine H-2D, which only cross-reacts with H-2B and H-2D. Secondary antibodies were as follows: goat F(ab)2, anti- rat IgG (H+L) peroxidase conjugated (Caltag Labs, Burlingame, CA), and phycoerythrin-conjugated streptavidin (Caltag Labs). Islet cell or spleen cell suspensions were incubated for 20 min on ice with monoclonal antibody diluted in Dulbecco’s phosphate-buffered saline (DPBS) containing 1% FCS. They were then washed and incubated for 15 min with an Fc receptor antibody as necessary. Finally, cells were suspended in DPBS with 1% FCS and 2.5 mg/ml propidium iodide to stain dead cells. Analysis was performed on a FACSort or FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) with Cell Quest software (Becton Dickinson). β-Cells were sorted on a FACStar™ (Becton Dickinson) on the basis of flavin adenine dinucleotide auto-fluorescence, according to the method described (37–39). Background levels were determined by staining MHC class I–deficient islets from B6−/− mice.

**Cytotoxicity assays.** CTL activity was measured in a 5–6 h in vitro 11Cr release assay (28). Briefly, to determine the amount of MHC-restricted CTL lysis, syngeneic or allogeneic target cells were either infected with LCMV-ARM (multiplicity of infection [MOI] = 1), or uninfected cells were coated with LCMV peptide (35–41 or 53–61 of NP, 105 U/ml), or uninfected BHK-21 (Clone 53b). LCMV CTL were activated with peptide-coated (10–5 mol/l) macrophages in the presence of T-cell growth factor (supernatant from ConA-stimulated rat splenocytes) containing IL-2 and irradiated syngeneic spleen feeder cells for 5–12 days (42). MC57 (H-2Kd) and Balb/Ct (H-2d) cells used as CTL targets were grown, as reported (28). When islet cells were used as targets, whole islets were isolated from collagenase-digested pancreata of at least three mice and labeled for 1 h at 37°C in the presence of 1 μCi of Cr⁶². After washing three times in glucose-free HBSS (Gibco, Gaithersburg, MD), islets were dispersed by trypsinization, washed again three times, and then plated at 1–2 × 10⁶ cells per well in 96-well flat bottom plates for the cytotoxicity assay. In some assays, cytokines (IFN-γ, TNF-α, and IL-1β) were added directly to the target cells at 1 μg/ml. When indicated, cytotoxicity assays were carried out over an extended time period of 20 h. Precursor frequencies of LCMV-specific CTL were determined, as described (43).

**Transgenic mice.** Generation and characterization of RIP-LCMV transgenic mice that develop type 1 diabetes after LCMV infection have been described (27,28,44). RIP-glycoprotein 34–20 (H-2b) transgenic mice, which express the viral glycoprotein on their β-cells, but not in any other organs, were used for this report (27). Transgenic mice expressing a dominant mutated form of the IFN-γ receptor selectively on their β-cells under control of the RIP (RIPΔγR) mice were described previously (39). Double transgenic mice expressing LCMV-glycoprotein as well as the ΔγR on their β-cells were generated by back-crossing RIP-ΔγR transgenics to RIP-LCMV–glycoprotein transgenics for 3–4 generations, and the resulting single transgenic, nontransgenic, or double transgenic littermates were used for all experiments. Routine screening for glycoprotein, as well as the ΔγR, was performed by polymerase chain reaction (PCR) (39,42). The cytokine or cytokine receptor–deficient mice were described and obtained from IFN-γ KO (45), IFN-α KO (Jackson Laboratories, Bar Harbor, ME), TNF-α KO (46), and IL-12 KO (Jackson Laboratories), and perforin KO B6 (Jackson Laboratories).

**Virus.** Virus stocks consisted of LCMV strain Armstrong (Clone 53b). LCMV was plaque-purified three times on Vero cells and stocks prepared by a single passage on BHK-21 cells (47).

**Blood glucose measurements.** Blood samples were obtained from the retro-orbital plexus of mice and plasma glucose concentration determined using a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA). Mice with blood glucose values higher than 300 mg% were considered diabetic (48).

**Secondary T-cell cultures and adoptive transfers.** Secondary LCMV-specific cell lines were generated by culturing LCMV immune spleenocytes harvested 45 days post-LCMV infection from LCMV-infected mice in the presence of LCMV H-2b or H-2d restricted LCMV peptides. All cultures were free of infectious virus as assessed by PCR and plaque assays. For adoptive transfer, 1 × 10⁶
LCMV-specific cultured lymphocytes were injected intravenously into naïve uninfected RIP-LCMV recipients irradiated nonlethally with 360 Rad (31). Histologic and immunohistochemical analysis of tissues. Tissues taken for histologic analysis were fixed in 10% zinc formalin and stained with hematoxylin and eosin. Immunohistochemical studies were carried out on 6–10 µm freshly frozen cryostat sections for immunostaining of islets to detect expression of MHC class I and II, B220, insulin, CD4, CD8, anti-CR51, anti-F4/80 (Clone A3–1), anti-MAA-1 (Clone M 170), anti-MHC class I (Clone M 1/42), and anti–class II (Clone M5/114), (PharMingen, San Diego, CA and Boehringer Mannheim). After washing in phosphate-buffered saline (PBS), the secondary antibody (biotinylated goat anti-rat or anti-mouse IgG; Vector Laboratories) was applied for 1 h. Color reaction was developed with sequential treatment using avidin–horseradish peroxidase conjugate (Boehringer-Mannheim, La Jolla, CA) and diaminobenzidine-hydrogen peroxide.

RNA analysis/RNAse protection assays. Whole pancreata were harvested 2, 7, 17, and 21 days after LCMV infection and immediately homogenized in 2 ml of Tri-reagent (Molecular Research Center) using a polytron homogenizer. Total RNA was extracted with chloroform followed by isopropanol precipitation and washing with ethanol. Twenty micrograms of total pancreatic RNA were used for hybridization with a 32P-labeled multitemplate set containing specific probes for IFN-γ and TNF-α provided by a commercial kit (Riboquant, mCK-3b; Pharmingen). The RNase protection assay was carried out according to the manufacturer’s guidelines. The resulting analytical acrylamide gels were scanned with a STORM-880 Phospho-Imaging System (Molecular Dynamics) and the intensity of bands corresponding to protected mRNAs was quantified with the ImageQuant image analysis software (Molecular Dynamics) using L32 as a reference gene.

Detection of the ΔYR transgene by FACS. Expression of the ΔYR transgene was measured on β-cells, spleen cells, and peripheral blood cells by flow cytometry. Biotinylated monoclonal antibody 9E10 directed against the C-myc epitope tag within the transgene (39) was incubated with cells followed by washing and incubation with PE-avidin.

RESULTS

Naïve β-cells are not lysed by perforin CTL, but by cytokines, unless they upregulate MHC class I. First, we tested the ability of CTL to kill β-cells via the perforin pathway in vitro. As shown in Table 1, syngeneic LCMV peptide–coated Cr51-labeled islet cells were not lysed to a significant extent by LCMV-CTL in vitro. No significant killing of H-2b islet cells was targeted independently of the LCMV peptide used to coat target (islet cells), whereas H-2b fibroblasts coated with such MHC class I–restricted LCMV peptides were lysed efficiently by LCMV-CTL (Table 1). The killing of islet cells but not fibroblasts by a combination of cytokines alone without CTL (IFN-γ, TNF-α, and IL-1β) (Table 1) occurred in the same experiment over a period of 20 h, during which CTL-mediated killing of islets was still not seen (20-h CTL assay) (data not shown). As expected, perforin-deficient effector CTL lysed neither fibroblasts nor β-cells. Thus, naïve β-cells are not susceptible to perforin-mediated CTL lysis, which can explain the lack of diabetes observed after adoptive transfer of activated LCMV-CTL into RIP-LCMV recipients (31).

LCMV infection of the pancreas induces MHC class I on β-cells. We previously described that MHC class I increases with age on NOD β-cells during the course of diabetes progression, and we hypothesized that a similar increase in MHC class I expression may be required for β-cell killing by LCMV-CTLs. We used FACS analysis (39) that applies stringent criteria for the identification of β-cells and tested MHC class I expression on β-cells from RIP-LCMV mice before the development of diabetes. Data are displayed in Fig. 1 and show a 10-fold increase of islet MHC class I in nontransgenic H-2b mice as soon as 2 to 3 days after infection and a >50-fold increase 7 days after LCMV infection. Interestingly, these maximal levels coincide with the systemic peak of anti-viral CTL activity, which is found ~7 days after infection (32). In nontransgenic mice, islet MHC expression follows a bell-shaped kinetic curve after LCMV, detectable as early as day 2 and receding to preinfection levels around day 18 to 21. In contrast, MHC class I levels remain elevated in RIP-LCMV–glycoprotein-transgenic mice beyond day 21, as determined in parallel experiments (Fig. 1). These transgenic mice develop profound insulitis beginning at 10–12 days postinfection (31).

**Table 1.** Perforin-mediated killing of β-cells by CTL in vitro only occurs after MHC class I is upregulated after LCMV infection in vivo.

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>Spont</th>
<th>LCMV</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected</td>
<td>LCMV</td>
<td>Peptide</td>
</tr>
<tr>
<td>B6 LCMV day 7</td>
<td>145/2,980</td>
<td>94/980</td>
<td>145/2,980</td>
</tr>
<tr>
<td>50:1 1</td>
<td>48 ± 9</td>
<td>47 ± 8</td>
<td></td>
</tr>
<tr>
<td>25:1 1</td>
<td>33 ± 7</td>
<td>30 ± 5</td>
<td></td>
</tr>
<tr>
<td>12:1 1</td>
<td>12 ± 5</td>
<td>13 ± 7</td>
<td></td>
</tr>
<tr>
<td>IFN-γ KO LCMV day 7</td>
<td>50:1 1</td>
<td>50 ± 13</td>
<td>52 ± 7</td>
</tr>
<tr>
<td>RIP-ΔYR LCMV day 7</td>
<td>50:1 1</td>
<td>55 ± 8</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>Perforin KO LCMV day 7</td>
<td>50:1 1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IFN-γ + TNF-α + IL-1β</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 2.** Monitoring of islet MHC expression in various LCMV-infected mice. The experiment was performed a total of five times. Where indicated, LCMV glycoprotein and NP MHC class I restricted peptides (see Research Design and Methods) were added at 10^{-5} mol/l to the islet cells 1 h before the assay. Fibroblasts were MC57 (H-2b) cells and were labeled as described in Research Design and Methods. Cytokine-killing values were obtained by adding TNF-α and IL-1β or IFN-γ at 1 µg/ml to islets, but no effector lymphocytes. These assays were performed over a period of 20 h, because cytokine-mediated killing of islet cells did not occur after 5 h.
which is the likely explanation for this persisting MHC upregulation. Thus, LCMV infection leads to MHC class I upregulation on β-cells as early as 2–3 days postinfection. Not direct viral infection of β-cells but anti-viral cytokines induce MHC class I on islet cells. We further investigated whether upregulation of MHC class I on β-cells was caused by a direct viral effect or LCMV-induced cytokines. When infected in vitro with LCMV for 48 h, islets did not upregulate MHC class I (Fig. 2), but after in vitro exposure to IFN-γ (1 ng/ml, 50-fold increase after 24–48 h) (data not shown) (39), MHC class I was induced. However, IFN-γ alone was unable to kill β-cells during this time period (Table 1).

IFN-γ is one of the main factors required for induction of MHC class I on β-cells. Consequently, LCMV-associated cytokine secretion was probably the principal cause for upregulation of MHC class I on β-cells. To dissect the relative contribution of several inflammatory cytokines to LCMV-induced MHC class I upregulation on β-cells, islets were harvested 5 or 6 days after LCMV infection from cytokine or cytokine-receptor–deficient mice (Fig. 3A). As evident from the summary analysis of data in Fig. 3B, IFN-γ–deficient mice only increased MHC class I on β-cells to a minimal extent (on average ~3-fold) after systemic LCMV infection compared with an up to 60-fold increase in wild-type mice. Transgenic mice with a dominant negative-mutated form of the IFN-γ receptor on their β-cells (RIP-ΔγR mice) (39) were still able to upregulate MHC class I to ~50% of wild-type levels (Fig. 3A and B), and MHC class I was slightly higher in littermates also expressing the LCMV-glycoprotein transgene (RIP-glycoprotein mice (RIP-LCMV–glycoprotein + transgenic mice was done in parallel for data shown in A). Upregulation of MHC class I occurs as early as 2 days postinfection (not shown) and reaches baseline levels in nontransgenic mice around day 21 (no islet infiltration), whereas RIP-glycoprotein transgenic mice continue to exhibit elevated MHC class I levels reflecting ongoing islet infiltration and destruction. At later time points, only very few islets were available in RIP-glycoprotein mice, reflecting onset of type 1 diabetes. One representative experiment is shown (4). The time-course study was performed at least twice for each time point with similar results. B shows the relative changes in MHC class I expression, allowing a statistical comparison between experiments. Background levels were indistinguishable from basal levels on uninfected islets stained with primary and secondary antibody (see RESEARCH DESIGN AND METHODS).
TNF-α and type I IFNs, may be able to partially compensate for the absence of IFN-γ. β-cells are lysed by perforin-CTL in vitro only if they upregulate MHC class I. We hypothesized that LCMV-induced class I should be sufficient to render β-cells susceptible to perforin-mediated lysis in vitro. Indeed, as shown in Table 2, when islets were harvested from nontransgenic mice 7 days after LCMV infection, coating with LCMV peptides and exposure to LCMV CTL led to substantial lysis. Similarly, islets harvested on day 7 post-LCMV from RIP-LCMV–glycoprotein+ transgenic mice were susceptible to CTL lysis even without peptide coating, indicating that a sufficient amount of transgenic LCMV-glycoprotein–derived peptides reached the surface in context with MHC class I. In contrast, islets harvested at day 7 from perforin-deficient mice and exposed to syngeneic CTL were not significantly lysed in the presence or absence of LCMV peptides. This is due to the fact that perforin-α-derived CTL populations cannot lyse any targets (Table 1), whereas islets from perforin-deficient mice are readily lysed by perforin competent CTL (Table 2). As expected and in agreement with our previous findings, MHC class I levels expressed on islets of IFN-γ–deficient mice (Fig. 3, Panel A) were insufficient to allow significant CTL lysis, whereas islets from other mice were all lysed by perforin CTL from LCMV-infected B6 mice in a dose-dependent manner (Table 2), which explains the resistance of RIP-glycoprotein+ transgenic mice to LCMV infection. Indeed, as shown in Table 2, when islets were harvested from nontransgenic mice 7 days after LCMV infection, coating with LCMV peptides and exposure to LCMV CTL led to substantial lysis. Similarly, islets harvested on day 7 post-LCMV from RIP-LCMV–glycoprotein+ transgenic mice were susceptible to CTL lysis even without peptide coating, indicating that a sufficient amount of transgenic LCMV-glycoprotein–derived peptides reached the surface in context with MHC class I. In contrast, islets harvested at day 7 from perforin-deficient mice and exposed to syngeneic CTL were not significantly lysed in the presence or absence of LCMV peptides. This is due to the fact that perforin-α-derived CTL populations cannot lyse any targets (Table 1), whereas islets from perforin-deficient mice are readily lysed by perforin competent CTL (Table 2). As expected and in agreement with our previous findings, MHC class I levels expressed on islets of IFN-γ–deficient mice (Fig. 3, Panel A) were insufficient to allow significant CTL lysis, whereas islets from other mice were all lysed by perforin CTL from LCMV-infected B6 mice in a dose-dependent manner (Table 2), which explains the resistance of RIP-glycoprotein+ transgenic mice to LCMV-induced type 1 diabetes when cross with IFN-γ–deficient mice (23). From these studies, it can be concluded that MHC class I levels increased on β-cells predominantly through virally induced IFN-γ before the β-cells can be killed by perforin-mediated CTL lysis.
TABLE 2
Uninfected islets ex vivo 7 days after LCMV (MHC<sup>hi</sup>)

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>B6/H-&lt;sup&gt;2b&lt;/sup&gt; GP&lt;sup&gt;-&lt;/sup&gt;</th>
<th>RFP-GP&lt;sup&gt;-&lt;/sup&gt;</th>
<th>RFP-ΔγR&lt;sup&gt;-&lt;/sup&gt;GP&lt;sup&gt;+&lt;/sup&gt;</th>
<th>IFN-γ KO</th>
<th>Perforin KO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spo/tot</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>B6 LCMV day 7</td>
<td>50:1</td>
<td>4 ± 2</td>
<td>78 ± 22</td>
<td>35 ± 15</td>
<td>65 ± 19</td>
</tr>
<tr>
<td>25:1</td>
<td>0</td>
<td>55</td>
<td>17 ± 3</td>
<td>34 ± 5</td>
<td>0</td>
</tr>
<tr>
<td>12:1</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>IFN-γ KO LCMV day 7</td>
<td>50:1</td>
<td>2 ± 1</td>
<td>18 ± 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFP-ΔγR LCMV day 7</td>
<td>50:1</td>
<td>33 ± 14</td>
<td>70 ± 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perforin KO LCMV day 7</td>
<td>50:1</td>
<td>96 ± 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ + TNF-α + IL-1β</td>
<td></td>
<td></td>
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</table>

Effector lymphocytes and various knockout mice with 1 × 10<sup>5</sup> pfu LCMV were used when MHC class I expression on islets as well as systemic anti-LCMV CTL activities were at their maximum (see Fig. 1). Note that, as assessed by immunohistochemistry using LCMV-NP–specific antibody 1.1.3 (54), <4% of the islets were directly infected with LCMV, which predominantly infects the exocrine pancreas. Therefore, islets are referred to as uninfected islets in the table. The experiment was repeated two times. LCMV peptides (glycoprotein-1, glycoprotein-2, and NP, all H-<sup>2</sup>-restricted, see RESEARCH DESIGN AND METHODS) were added simultaneously to islets just before the assay at 10<sup>−5</sup> mol/l. In parallel to each islet-cell cytotoxicity assay, MC57 (H-2<sup>b</sup>) fibroblasts were used as targets to ensure that effector splenocytes were showing equivalent LCMV CTL activities 7 days after LCMV infection (see Table 1).

FIG. 4. Type 1 diabetes is drastically reduced in RIP-LCMV transgenic mice expressing a dominant mutated IFN-γ receptor selectively on their β-cells. RIP-ΔγR transgenic mice were back-crossed to RIP-LCMV-glycoprotein transgenics for 3 or 4 generations and littermates were infected with 1 × 10<sup>6</sup> pfu LCMV. Similarly, RIP-glycoprotein mice were crossed with perforin-deficient mice (Jackson Laboratories, B6), and the F<sub>1</sub> were intercrossed resulting in perforin<sup>−/−</sup> or perforin<sup>+</sup> RIP-glycoprotein F2 offspring. Note that the genetic background of the perforin-deficient B6 line, as opposed to the perforin-deficient SV129 line, does not interfere with diabetes incidence in RIP-LCMV mice that are perforin<sup>−/−</sup> or perforin<sup>+</sup> (43). Some perforin-deficient mice (30%) survived LCMV infection long enough for a 3 month observation period, as shown previously (43). Blood glucose was measured weekly, and mice with blood glucose values > 300 mg/dl were considered diabetic. Note that the F<sub>1</sub> backcross single transgenic RIP-glycoprotein<sup>−/−</sup> mice have a slightly higher incidence than the F<sub>2</sub> backcrosses, which is probably because of non-MHC background genes from RIP-ΔγR transgenic mice that negatively influence type 1 diabetes in RIP-glycoprotein lines. Statistical analysis (Fisher’s exact test) using InStat software (GraphPad software, San Diego, CA) showed significant differences between perforin<sup>−/−</sup> and perforin<sup>+</sup> RIP-glycoprotein mice as well as ΔγR expressing versus nonexpressing RIP-glycoprotein mice.

In vivo destruction of most β-cells occurs requiring γ-IFN but not perforin. We further investigated the issue of whether or not all β-cell death in our transgenic model could be attributed to perforin-mediated killing in vivo. We expected that the answer would be yes, because islets were exquisitely sensitive to CTL killing as shown in Table 2. IFN-γ alone did not lead to significant islet cell lysis after 24–48 h in vitro, whereas a combination of IFN-γ, TNF-α, and IL-1β did (Table 2).
but an essential role for IFN-γ in mediating β-cell death in RIP-LCMV mice in synergy with other cytokines.

Finally, we tested whether pancreatic levels of IFN-γ would parallel disease development comparing diabetic RIP-glycoprotein+ transgenic mice with nondiabetic RIP-glycoprotein littermates. Indeed, as shown in Fig. 5, IFN-γ mRNA levels remained elevated up to 21 days after infection only in RIP-glycoprotein+ mice but not in nondiabetic littermates. In contrast, TNF-α mRNA levels dropped at 21 days postinfection to levels found at 2 days postinfection in both nondiabetic RIP-glycoprotein+ and RIP-glycoprotein+ transgenic mice. Thus IFN-γ, more than TNF-α, is present in pancreata of diabetic mice, which together with the data from Fig. 4 identifies it as a key mediator of β-cell destruction in the RIP-LCMV model. TNF-α probably plays a synergistic role in MHC class I induction and β-cell destruction more in the earlier phases of insulitis, when it is found at higher levels in the pancreas after LCMV infection.

DISCUSSION

Based on our present studies, the following scenario emerges for the pathogenesis of type 1 diabetes in RIP-LCMV–glycoprotein mice. LCMV infection of the pancreas induces cytokines such as type I and II IFNs, as well as TNF-α (24,32), that lead to upregulation of MHC class I on β-cells as early as 2 days postinfection. The probable early source (2–4 days postinfection) for these cytokines are natural killer cells (52), as well as activated APCs, but not LCMV-specific CD4 or CD8 lymphocytes that are not found at this early stage in the pancreas (31). In the second phase (around day 7), a few autoreactive (anti-LCMV) lymphocytes reach the pancreas and islets, which mainly produce IFN-γ and TNF-α leading to further increase of MHC class I on β-cells. According to our in vitro findings, islet cells can be directly lysed at this stage in a perforin-dependent manner (Table 1). Based on our present findings (Table 1 and Fig. 4) (26), perforin-dependent killing is mainly important only during this triggering but not during the effector phase of type 1 diabetes and is not an absolute requirement (Fig. 4). However, complete destruction of islets takes place after LCMV is eliminated from the host (7–10 days postinfection) around day 14 and requires a direct effector mechanism essentially involving IFN-γ to kill β-cells (Fig. 4). This does not necessarily imply that the final effector phase is independent from self-antigens, because LCMV-specific lymphocytes and T-cells reactive to other auto-antigens are the probable source of the IFN-γ. Further, synergistic effects with other cytokines are likely required, because β-cells die in vitro after exposure to a combination of cytokines including IFN-γ but not IFN-γ alone (Table 1). Others have shown that type 1 diabetes can be induced by CTL in NOD mice (10,15). Interestingly, type 1 diabetes in the NOD mouse model appears to not require IFN-γ to kill β-cells in the effector phase (39) but perforin-competent CTL (10,15,24,51). It is somewhat surprising to observe such opposite scenarios in two animal models, especially because initial predictions attributed β-cell death to perforin CTL in RIP-LCMV mice (28,44). The situation in the NOD mice is still not completely clear because both autoreactive CD8 and -CD4 lymphocytes alone can cause disease in adoptive transfers (29). Thus, the current findings should raise the awareness for redundant pathways in β-cell destruction, which should be taken into account when developing targeted intervention strategies for human type 1 diabetes.

Our observations are in agreement with the previous finding that destructive insulitis in the final effector phase of type 1 diabetes in RIP-LCMV mice is characterized by a
predominance of IFN-γ over IL-4 (48). This explains why, even at this late stage, immune regulatory approaches can still prevent type 1 diabetes (36). For example, regulatory lymphocytes induced in response to oral insulin can change the cytokine profile in islets toward T-helper 2 (IL-4) when induced rather late during pathogenesis (36). Perforin–competent committed effector CTLs in the islets are alone insufficient to lead to complete and rapid destruction, which leaves the potential for intervention even in situations with pronounced insulinitis in prediabetic individuals. It is interesting to note that perforin-deficient CTLs did not induce any lysis of islets in vitro (Table 1) but some diabetes in vivo (Fig. 4). This is likely due to their ability to produce large amounts of TNF-α and IFN-γ by significantly increased numbers of LCMV-effector CTLs in vivo (43). The probable reason for the lack of killing in vitro is that within the time of the CTL assay (5–20 h) sufficient cytokine amounts are not produced. Indeed, longer incubation period (3–6 days) of perforin-deficient CTL lines with APCs lead to sufficiently high amounts of cytokines in culture supernatants (43) that, when transferred, could lyse β-cells in vitro (data not shown). Therefore, it is plausible that some perforin-deficient RIP-LCMV mice actually did develop type 1 diabetes (Fig. 4), which is probably mediated by cytokines.

In conclusion, pathogenesis of type 1 diabetes is multifactorial and involves a complex network of cytokines, autoreactive CTLs, and MHC class I induction on β-cells, even in a model formerly thought to be as simplistic as the RIP-LCMV model of virally induced diabetes (27,28,44). We show here that after the breaking of peripheral autoreactive CTL unresponsiveness, islet-cell death still depends on a multitude of factors. One is the requirement of direct viral presence in the target organ that leads to APC activation as well as IFN production, which in turn leads to MHC class I upregulation on β-cells. Although islet cells are in this way sensitized to lysis by autoreactive CTLs, type 1 diabetes does not develop in the presence of decreased IFN-γ signaling on β-cells. Importantly, the trigger level for IFN-γ–mediated MHC class I induction appears to be lower than that for β-cell death, indicating that potentially different pathways are used. Our finding suggests that activated autoreactive perforin-competent CTLs alone are insufficient to cause disease, and diabetes can develop in their absence, when sufficiently high amounts of IFN-γ are generated for a prolonged period of time and/or the target organ has undergone some preceding damage. Causative agents, such as viruses, should therefore be directly detectable in an autoimmune-affected pancreas at some point of time before clinically overt type 1 diabetes. The fact that cytokines can be involved as final effectors, killing β-cells as bystanders, might explain why transfers of CD4, as well as CD8 lymphocytes, can cause type 1 diabetes in NOD mice. Also, this fact should allow targeting of cytokines in preventive approaches for type 1 diabetes in prediabetic individuals with already ongoing islet destruction.

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