Virus-Induced Autoimmune Diabetes

Most β-Cells Die Through Inflammatory Cytokines and Not Perforin From Autoreactive (Anti-viral) Cytotoxic T-Lymphocytes

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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. *Diabetes* 49:1801–1809, 2000

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APC, antigen-presenting cell; CTL, cytotoxic T-lymphocyte; DPBS, Dulbecco's phosphate-buffered saline; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; IFN-γ, γ-interferon; IL, interleukin; LCMV, lymphocytic choriomeningitis virus; mAb, monoclonal antibody; MHC, major histocompatibility complex; MOI, multiplicity of infection; PCR, polymerase chain reaction; RIP, rat insulin promoter; TNF, tumor necrosis factor.

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 in an antigen-specific model of autoimmune diabetes. We chose the well-established rat insulin promoter (RIP)–lymphocytic choriomeningitis virus (LCMV) model (27,28) over the NOD mouse model (1,26,29,30), because it offered the following advantages. *1*) The impact of a viral infection, in addition to genetic factors, is taken into account, and the initiating self-antigen is well defined and the time point of disease induction can be chosen experimentally. *2*) The participation of autoreactive CTL has been proven. *3*) 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 islet 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 1×10^8 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 25–800*g* for 4 min, then at 800*g* 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 Hanks' balanced salt solution (HBSS), and were allowed to recover in complete medium (Connaught 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 (36). 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 the isolated islet cells, 56% were β-cells on average, 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 (PharMingen, La Jolla, CA), a biotinylated mouse IgG2a mAb directed against murine $H\text{-}2D^b$, which only cross-reacts with $H\text{-}2L^d$ and $H\text{-}2D^q$. Secondary antibodies were as follows: goat F(ab)2, anti-rat IgG(H+L) phycoerythrin 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 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, San Jose, CA) with Cell Quest software (Becton Dickinson). β-Cells were sorted on a $FACStar^{plus}$ (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 $\beta2m^{-/-}$ mice.

Cytotoxicity assays. CTL activity was measured in a 5- to 6-h in vitro ^{51}Cr 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 peptides glycoprotein 33–41 (KAVYNFATC), glycoprotein aa 276–286 (SGVENPGGYCL), NP 396–404 (FQPQNGQFI), all $H\text{-}2^b$ restricted (40), or NP 118–126 (RPQASGVYM) restricted by $H\text{-}2^d$ (41). Assays used splenic lymphocytes harvested 7 days after primary LCMV infection (10^5 pfu i.p.) at effector to target ratios of 50:1, 25:1, and 12:1, or CTL clones and secondary CTL lines at ratios of 10:1 and 5:1. To determine CTL activity after secondary stimulation, spleen cells harvested from mice 30–180 days after primary inoculation with 1×10^5 pfu LCMV i.p. were incubated with MHC-matched, irradiated, LCMV-infected, or 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 ($H\text{-}2K^bD^b$) and Balb/Cl7 ($H\text{-}2^d$) 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 mCi of Cr^{51}. After washing three times in glucose-free HBSS (Gibco, Gaithersburg, MD), islets were dispersed by trypsinization, washed again three times, and then plated at $1\text{–}2 \times 10^4$ 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 1μg/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 ($H\text{-}2^b$) transgenic mice, which express the viral glycoprotein from LCMV strain Armstrong only in the β-cells of their islets and 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 (RIP-ΔγR 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 before and obtained from IFN-γ KO (45), IFN-α/β receptor 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 Accucheck III (Boehringer Mannheim). 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 cultivating LCMV immune splenocytes harvested 45 days post-LCMV infection from LCMV-infected mice in the presence of LCMV $H\text{-}2^b$ or $H\text{-}2^d$ restricted LCMV peptides. All cultures were free of infectious virus as assessed by PCR and plaque assays. For adoptive transfer, 1×10^8

LCMV-specific cultured lymphocytes were injected intravenously into naive uninfected RIP-LCMV recipients irradiated nonlethally with 300 Rad (31).

Histologic and immunochemical analysis of tissues. Tissues taken for histologic analysis were fixed in 10% zinc formalin and stained with hematoxylin and eosin. Immunochemical studies were carried out on 6–10 µm freshly frozen cryomicrotome sections for immunostaining of islets to detect expression of MHC class I and II, D^b, insulin, CD4, CD8, B220, F4/80. Primary antibodies were applied for 1 h. These consisted of rat anti-mouse CD4 (Clone RM 4–5), anti-CD8 (Clone 53–6.7), anti-B220 (Clone RA3 6B2), anti-F4/80 (Clone A3–1), anti–MAC-1 (Clone M 1/70), 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 ^{32}P-UTP 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 gel was scanned with a STORM-860 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 ΔγR transgene by FACS. Expression of the ΔγR 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

Naive 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 Cr^{51}-labeled islet cells were not lysed to a significant extent by LCMV-CTL in vitro. No significant killing of H-2^b islet cells was detected independently of the LCMV peptide used to coat target (islet cells), whereas H-2^b 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-2^b 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

		Fibroblasts (MHC^{hi}) MC57/H-2^b			Islets ex vivo, uninfected mice (MHC^{lo}) B6/H-2^b		
		Uninfected	LCMV	Peptide	Uninfected	LCMV	Peptide
Effector cells	Spo/tot	145/2,980	94/980	145/2,980	456/2,480	32/340	456/2,480
B6 LCMV day 7	50:1	0	48 ± 9	47 ± 8	10 ± 4	12 ± 8	10 ± 4
	25:1	0	33 ± 7	30 ± 5	6 ± 1	10 ± 2	9 ± 4
	12:1	0	12 ± 5	13 ± 7			
IFN-γ KO LCMV day 7	50:1	0	50 ± 13	52 ± 7	3 ± 1	9 ± 4	
RIP-Δγ LCMV day 7	50:1	0	55 ± 8	45 ± 4			
Perforin KO LCMV day 7	50:1	0	0	0	8 ± 5	ND	12 ± 3
IFN-γ		2	0	0	4 ± 2		
IFN-γ + TNF-α + IL-1β		0	0	0	62 ± 4		

Effectors are H-2^b splenocytes harvested 7 days after LCMV infection containing up to 1:3 LCMV-antigen–specific CD8 lymphocytes (53) and used with various knockout mice with 1×10^5 pfu LCMV at an effector target ratio of 50:1. Data shown represent specific Cr^{51} release. Spontaneous Cr^{51} release of islet cells was <30% of total release for all experiments shown (5-h values) and <50% for the 20-h cytokine values on display. Because dispersed islet cells do not retain Cr^{51} very well, these spontaneous/total ratios are the best that were achievable, which allowed for a sufficient and reproducible experimental read-out range. All mice used were H-2^b. SE is given for each group and 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-2^b) 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.

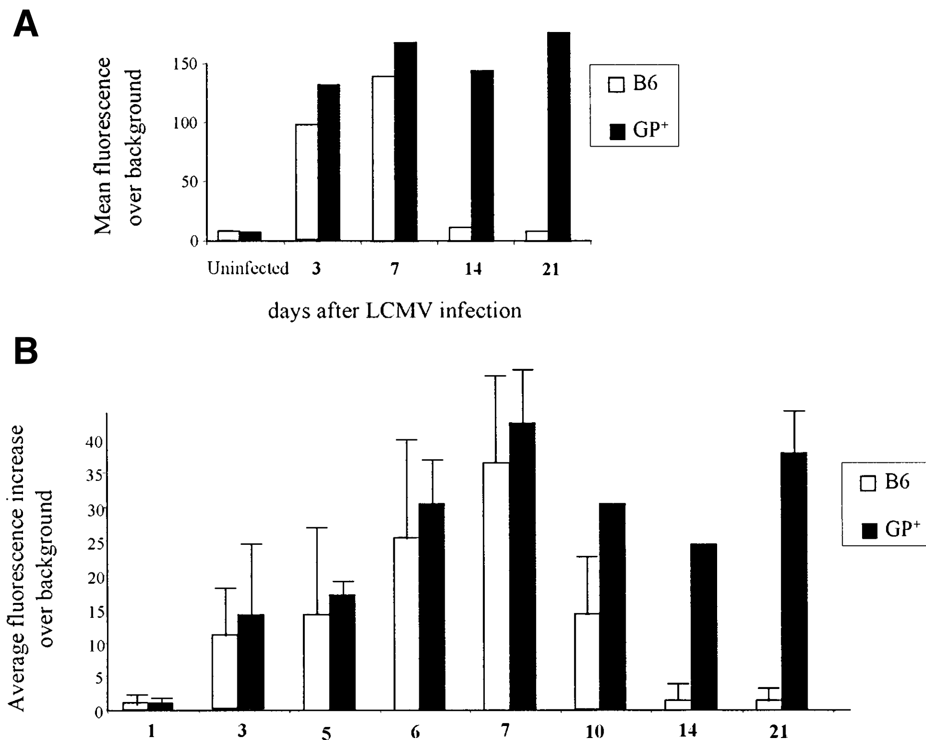

FIG. 1. Time course of MHC class I induction in nontransgenic and RIP-LCMV- glycoprotein transgenic mice after LCMV infection. Islet cells were isolated as described in RESEARCH DESIGN AND METHODS from mice infected or uninfected with LCMV (1×10^5 pfu i.p.). Briefly, islet β-cells were identified by FACS in forward and side scatters, as well as by autofluorescence, and all dead cells were gated out through identification with propidium iodide, FL-3. MHC staining signal was detected in FL-2. Staining to compare each time point between nontransgenic (B6) and 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 (*A*). 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).

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 µg/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. 3*A*). As evident from the summary analysis of data in Fig. 3*B*, 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. 3*A* and *B*), and MHC class I was slightly higher in littermates also expressing the LCMV-glycoprotein transgene (RIP-glycoprotein ΔγR, Fig. 3*A*). Finally, compared with wild-type mice, type I IFN receptor–deficient mice (IFNα/β receptor KO) generated 40% of MHC class I levels on β-cells after LCMV infection, whereas TNF-α or IL-12–deficient mice generated up to 50% (Fig. 3*B*). In summary, these experiments identify IFN-γ as one of the key factors required for upregulating MHC class I on β-cells after LCMV infection, whereas type I IFNs contribute to a somewhat lesser extent and some synergistic effects of TNF-α and IL-12 are observed, which could also reflect lower overall IFN-γ in such mice after LCMV infection (49,50). The relatively high levels of MHC class I on β-cells of RIP-ΔγR mice are possibly explained by the fact that these mice are heterozygous but not homozygous for the dominant mutated IFN-γ receptor. Heterozygous transgenic mice have lower expression levels of the dominant-negative receptor (39), which allows for some signaling of IFN-γ to upregulate MHC class I on β-cells (51) (H.E.T., T.W.H.K., unpublished observations). Alternatively, there might be an additional yet unidentified IFN-γ receptor present on β-cells that is not associated with the β-cell–death pathways but linked to MHC class I induction. Additionally, other cytokines, such as

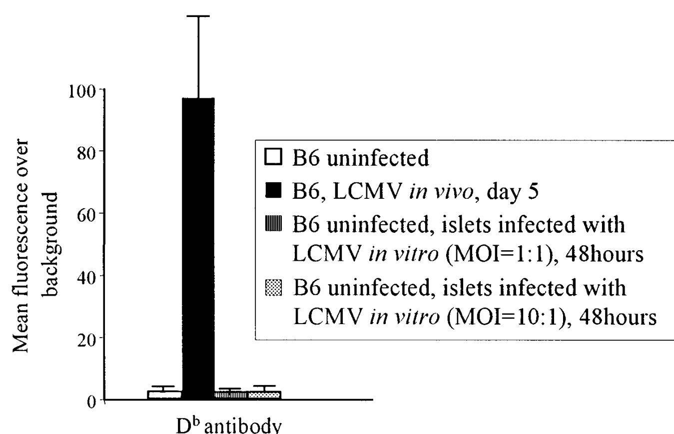

FIG. 2. LCMV infection of islet cells in vitro does not directly lead to upregulation of MHC class I. β-Cells were identified by flow cytometry based on their size and their autofluorescence after cultivation in low glucose medium, and dead cells were gated out after staining with propidium iodide. MHC in FL-2 was detected as described in RESEARCH DESIGN AND METHODS. LCMV infection was carried out in vitro by adding infectious plaque forming units (pfu) at an MOI of 1:1 or 10:1 as indicated and incubating with islet cells for 48 h in a humidified CO_2 incubator at 37°C. In parallel, MC57 (H-2^b) fibroblasts were infected in vitro with LCMV. For both cell types (fibroblasts and islets), LCMV infection was productive as assessed by antigen expression (staining with LCMV-NP antibody 1.1.3) (54) and, for fibroblasts, by recognition in CTL assays. However, LCMV infection in vitro did not lead to upregulation of MHC class I in either cell type (FACS data for β-cells displayed). The experiment was performed twice with standard errors on display.

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 by CTL 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$^{-/-}$-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 crossed with IFN-γ–deficient mice (23). From these studies, it can be concluded that MHC class I needs to be increased on β-cells predominantly through virally induced IFN-γ before the β-cells can be killed by perforin-mediated CTL lysis. Interest-

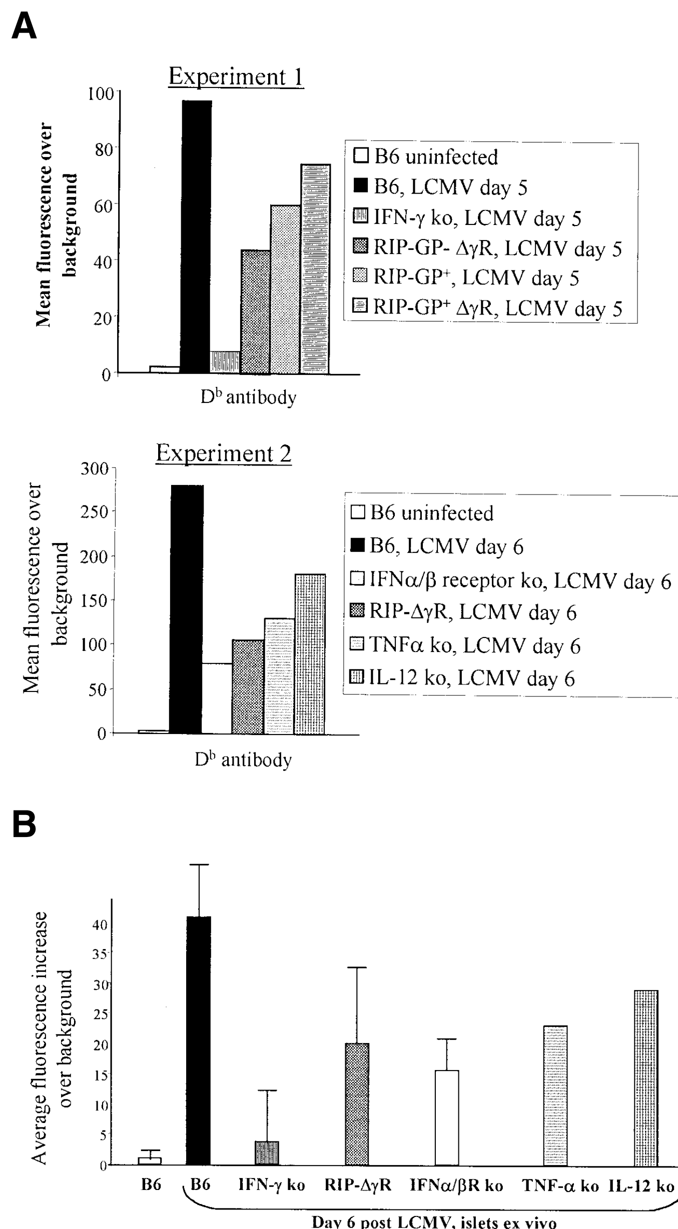

FIG. 3. MHC class I induction in cytokine and cytokine receptor deficient mice. Islet cell FACS staining for MHC class I was performed as described in the legend to Fig. 1. Cytokine and cytokine receptor knockout mice were obtained as described in RESEARCH DESIGN AND METHODS. The graph shows increase of MHC class I on islet cells after LCMV infection over background levels, which are very low to nondetectable when measured before LCMV infection (no visible difference among islets incubated with secondary antibody alone, secondary and primary antibody, and islets deficient for MHC class I [B2m deficient H-2^b mice] incubated with primary and secondary antibody). RIP-ΔγR receptor transgenic mice expressing a mutated dominant negative variation of the IFN-γ receptor in their β-cells were crossed to RIP-LCMV-glycoprotein transgenics, and $F_{3–4}$ back-crosses were used for all studies as indicated in RESEARCH DESIGN AND METHODS. *A*: Panel *A* shows two experiments comparing MHC class I on islet cells of RIP- glycoprotein$^+$ with RIP- glycoprotein$^+$ × RIP-ΔγR mice, RIP-glycoprotein negative (–) × RIP-ΔγR mice, TNF-α, IL-12 and IFNα/β receptor deficient mice 6 days after infection with 1 × 10^5 pfu LCMV i.p. and IFN-γ–deficient mice 5 days after infection with 1 × 10^5 pfu LCMV i.p. *B*: Panel *B* shows the relative increase of MHC class I to allow comparison between different experiments performed on islets isolated ex vivo 6 days post-LCMV infection.

TABLE 2
Uninfected islets ex vivo 7 days after LCMV (MHC^{hi})

		^{51}Cr release (%) from targets									
		B6/H-2^b GP^-		RIP-GP^+		RIP-ΔγR$^+$GP$^+$		IFN-γ KO		Perforin KO	
	Spo/tot	237/1,013		1005/6,547		246/1,670		881/2,365		132/660	
Effector cells		nil	Peptide	nil	Peptide	nil	Peptide	nil	Peptide	nil	Peptide
B6 LCMV day 7	50:1	4 ± 2	78 ± 22	35 ± 15	65 ± 19	28 ± 14	71 ± 26	0	11 ± 2	0	56 ± 10
	25:1	0	55			17 ± 3	34 ± 5	0	0		
	12:1	0	12			0	10	0	0		
IFN-γ KO LCMV day 7	50:1							2 ± 1	18 ± 8		
RIP-Δγ LCMV day 7	50:1					33 ± 14	70 ± 21				
Perforin KO LCMV day 7	50:1									13 ± 3	16 ± 4
IFN-γ + TNF-α + IL-1β		96 ± 4									

Effector lymphocytes and various knockout mice with 1×10^5 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-2^b restricted, see RESEARCH DESIGN AND METHODS) were added simultaneously to islet-cells just before the assay at 10^{-5} mol/l. In parallel to each islet-cell cytotoxicity assay, MC57 (H-2^b) fibroblasts were used as targets to ensure that effector splenocytes were showing equivalent LCMV CTL activities 7 days after LCMV infection (see Table 1).

ingly, cytokine (IFN-γ, TNF-α, and IL-1β)-mediated lysis of islet cells occurring over a 20-h period was always higher than direct 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).

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 lysis after maximal induction of MHC class I 7 days post-LCMV infection (Table 2) and, in accordance with these findings, type 1 diabetes did not occur in RIP-glycoprotein$^+$ transgenic IFN-γ–deficient mice (23). However, surprising results were obtained when we analyzed the double transgenic mice that expressed a dominant mutated nonfunctional IFN-γ receptor (RIP-ΔγR mice), in addition to the LCMV-glycoprotein on their β-cells, but not any other cells (as evidenced by FACS analysis) (see RESEARCH DESIGN AND METHODS). These mice were still capable of upregulating MHC class I on islets, similarly to other cytokine-deficient mice (Fig. 3), which was sufficient to render RIP-ΔγR/glycoprotein$^+$ islets susceptible to perforin-mediated lysis in vitro (Table 1). Surprisingly, such double transgenic mice did not develop type 1 diabetes to a significant degree (<10%, 1 of 12 mice F_3 or 0 of 5 mice F_4, Fig. 4) compared with a >50% type 1 diabetes incidence in RIP-glycoprotein single transgenic controls. From these experiments, it can be concluded that, although perforin-mediated CTL lysis of β-cells is apparently needed to potentiate islet destruction, as evidenced by the lack of diabetes in most perforin-deficient RIP-glycoprotein mice (Fig. 4) (26), complete loss of β-cells and consequentially type 1 diabetes occurs only through a mechanism that involves direct IFN-γ signaling to islets and not perforin-mediated lysis, because a mutated dominant negative IFN-γ receptor protects islets from death in vivo. The sources of IFN-γ are probably LCMV CTL and CD4 lymphocytes, which produce large amounts on activation (24,32). Thus, heterozygous expression of a dominant mutated form of the IFN receptor on β-cells is sufficient to allow for IFN-γ–induced class I upregulation but insufficient to allow for IFN-γ–mediated islet cell death. This observation suggests a different sensitivity of β-cells to IFN-γ for MHC upregulation versus β-cell killing and presumably redundant mechanisms (involving other cytokines) for MHC class I induction,

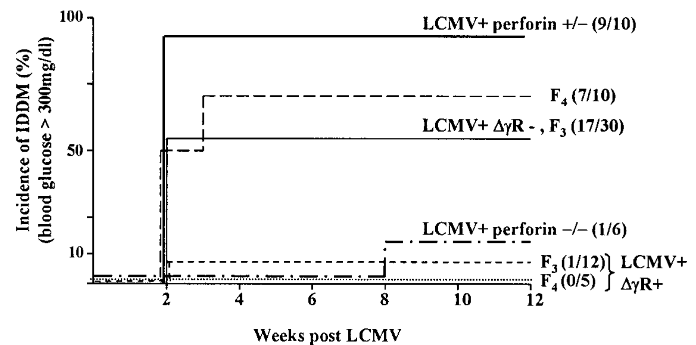

FIG. 4. Type 1 diabetes is drastically reduced in RIP-LCMV transgenic mice expressing a dominant mutated IFN-γ receptor selectively on their β-cells. RIP-ΔγR transgenic mice were back-crossed to RIP-LCMV-glycoprotein transgenics for 3 or 4 generations and littermates were infected with 1×10^5 pfu LCMV. Similarly, RIP-glycoprotein mice were crossed with perforin-deficient mice (Jackson Laboratories, B6), and the F1 were intercrossed resulting in perforin$^{+/-}$ or perforin$^{-/-}$ 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$^{+/-}$ or $^{+/+}$ (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_4 backcross single transgenic RIP-glycoprotein$^+$ mice have a slightly higher incidence than the F_3 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$^{+/-}$ and $^{-/-}$ RIP-glycoprotein mice as well as ΔγR expressing versus nonexpressing RIP-glycoprotein mice.

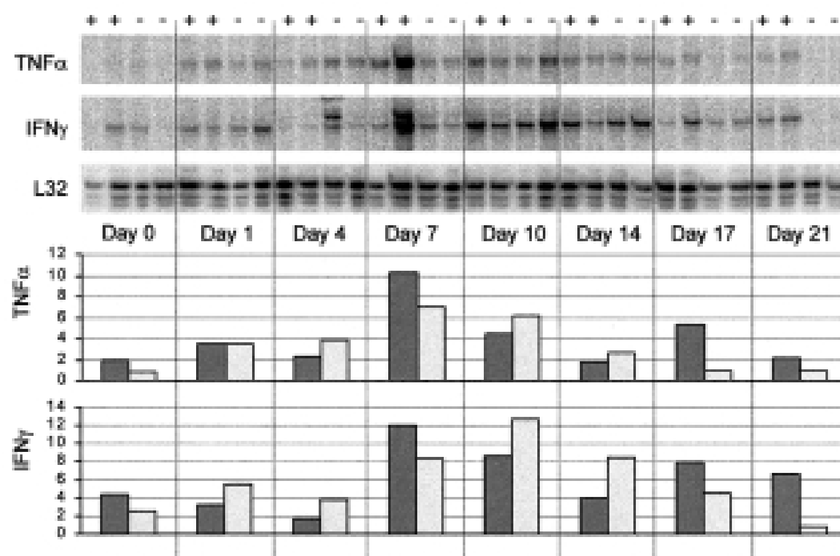

FIG. 5. Expression of IFN-γ and TNF-α comparing pancreata of nontransgenic H-2^b (B6) mice with transgenic RIP-glycoprotein mice after LCMV infection. RIP-glycoprotein (+) or C57bl/6 wild-type (–) mice were infected with LCMV and analyzed for pancreatic IFN-γ and TNF-α expression at several time points thereafter. RNAse protection analysis was performed as described in RESEARCH DESIGN AND METHODS and PAGE of protected cytokine mRNA isolated from two representative mice per group are shown. Relative signal intensity was determined using a Storm phospho-imager with ImageQuant image analysis software (Molecular Dynamics) and mean values obtained from three to five animals per group are displayed. The upper panel shows RNAse protection analysis data from two representative mice per group and the lower panel shows the average signal intensity for RIP-LCMV-glycoprotein$^+$ (■) compared with nontransgenic littermates (□).

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 nontransgenic littermates. In contrast, TNF-α mRNA levels dropped at 21 days postinfection to levels found at 2 days postinfection in both nontransgenic 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 insulitis 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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