Virally Induced Inflammation Triggers Fratricide of Fas-Ligand–Expressing β-Cells

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E xpression of Fas-ligand (Fas-L) on the surface of cells has been shown to provide immune privilege by inducing apoptosis of “invading” lymphocytes expressing Fas. However, accelerated diabetes has been reported in transgenic mice expressing Fas-L in islets (RIP-Fas-L) as a result of Fas-dependent fratricide of β-cells after transfer of diabetogenic clones. Here we studied whether Fas-L could protect islets from autoaggressive CD8 lymphocytes in a transgenic model of virally induced diabetes (RIP-LCMV-NP transgenic mice), in which the autoimmune response is directed to a viral nucleoprotein (NP) expressed as a transgene in β-cells. Indeed, disease incidence after viral (lymphocytic choriomeningitis virus [LCMV]) infection was reduced by ~30%, which was associated with a decrease of autoaggressive CD8 NP-specific lymphocytes in islets and pancreatic draining lymph nodes. However, surprisingly, a high degree (50%) of diabetes was seen in mice that expressed only Fas-L but not the viral transgene (NP) in β-cells after infection with LCMV. This was due to induction of Fas on β-cells after LCMV infection of the pancreas, resulting in Fas/Fas-L–mediated fratricide. Thus, although Fas-L can lend some immune privilege to islet cells, local virus-induced inflammation will induce Fas on β-cells, leading to their mutual destruction if Fas-L is present. Expression of Fas-L therefore might not be protective in situations in which viral inflammation can be expected, resulting in Fas induction on the targeted cell itself. Diabetes 53:591–596, 2004

Tissue-specific expression of Fas-ligand (Fas-L) can provide immune privilege by inducing apoptosis of “invading” lymphocytes expressing Fas. However, accelerated diabetes has been reported in transgenic mice expressing Fas-L in islets (RIP-Fas-L) as a result of Fas-dependent fratricide of β-cells after transfer of diabetogenic clones. Here we studied whether Fas-L could protect islets from autoaggressive CD8 lymphocytes in a transgenic model of virally induced diabetes (RIP-LCMV-NP transgenic mice), in which the autoimmune response is directed to a viral nucleoprotein (NP) expressed as a transgene in β-cells. Indeed, disease incidence after viral (lymphocytic choriomeningitis virus [LCMV]) infection was reduced by ~30%, which was associated with a decrease of autoaggressive CD8 NP-specific lymphocytes in islets and pancreatic draining lymph nodes. However, surprisingly, a high degree (50%) of diabetes was seen in mice that expressed only Fas-L but not the viral transgene (NP) in β-cells after infection with LCMV. This was due to induction of Fas on β-cells after LCMV infection of the pancreas, resulting in Fas/Fas-L–mediated fratricide. Thus, although Fas-L can lend some immune privilege to islet cells, local virus-induced inflammation will induce Fas on β-cells, leading to their mutual destruction if Fas-L is present. Expression of Fas-L therefore might not be protective in situations in which viral inflammation can be expected, resulting in Fas induction on the targeted cell itself. Diabetes 53:591–596, 2004
which both are required for disease (19). Indeed, cytotoxic killing of β-cells is a major requirement for diabetes development along with local inflammation of the pancreas and islets. As early as 2 days after LCMV infection, “preconditioning” of the pancreas is occurring, reflected by upregulation of major histocompatibility complex class II molecules on activated antigen-presenting cells (22). This LCMV-induced inflammation is transient (day 2 through 6 postinfection) and is alone insufficient to cause diabetes in nontransgenic mice (23,24). However, in RIP-LCMV mice this activation of APCs and secretion of interferon and other inflammatory molecules is important to “entice” autoaggressive (LCMV-NP specific) lymphocytes to the pancreas and islets, where they will continue to attack β-cells that express the NP antigen until all of them have been destroyed.

For the present study, RIP-LCMV mice (Lk/KdIAg7) were crossed to RIP-Fas-L transgenic mice (Dk/KdIA87). We present two interesting observations that clearly illustrate the dual and opposing mechanisms that local Fas-L expression can elicit. First, virally induced disease in RIP-NP mice is significantly delayed by Fas-L expression in β-cells mediated by reduction of autoaggressive CD8 cells locally in islets and pancreatic draining lymph nodes. However, LCMV infection can induce diabetes in Fas-L expressor mice in complete absence of the LCMV-NP transgene. This occurs in conjunction with Fas upregulation on β-cells that enables Fas-L transgenic β-cells to kill their neighbors/brothers (fratricide).

RESEARCH DESIGN AND METHODS

Mice breeding scheme and origin. Generation of H-2d RIP-LCMV-NP transgenic mice used for this study has been described previously (19). RIP-Fas-L mice expressing Fas-L under the control of the rat insulin promoter had been generated previously (4). NOD (Dk/KdIA87) RIP-Fas-L mice were intercrossed with the H-2d (Dk/KdIA87) RIP-LCMV-NP line, resulting in double-transgenic mice. These mice were used for all studies here as littermates. Mice genotyping. The presence of transgenic RIP-LCMV-NP or Fas-L sequences was determined by performing two independent standard PCR reactions with genomic DNA obtained from mouse tails as described (4,19).

Virus. Virus used was LCMV strain Armstrong (arm), clone 33b. LCMV was plaque purified three times on Vero cells, and stocks were prepared by a single passage on BHK-21 cells. Mice were infected with a single intraperitoneal dose of 105 pfu LCMV-arm unless indicated otherwise (20).

Viraltiters. LCMV viral titers of organ homogenates were determined by infection of Vero cells as described elsewhere (18). Briefly, organ homogenates were diluted serially and cultured with Vero cells for 5 days. Viral titers were determined from the number of counted plaques (20).

Blood glucose measurements. Blood samples were obtained from the retro-orbital plexus, and plasma glucose concentration was determined using Accuchek III (Roche, Indianapolis, IN). Mice with blood glucose values >300 mg/dl were considered diabetic (24).

Islet cell enrichment. Islets were isolated as previously described (25). Briefly, the pancreas was removed, cut into little pieces, and digested with collagenase P (Roche). Islets were purified on histopaque-1077 density gradients (Sigma, St. Louis, MO). The obtained islet-enriched fraction and the remaining portion of the pancreas containing mainly acinar cells were either immediately homogenized in tri-reagent (Molecular Research Center) for subsequent isolation of total RNA or islets were further digested with trypsin (Sigma) to obtain single β-cell suspensions (26).

RNAse protection assay. Total RNA was isolated either from whole pancreas homogenates or from islet or acinar cell–enriched fractions using tri-reagent (Molecular Research Center). RNA was extracted with chloroform followed by isopropanol precipitation and washing with ethanol. Twenty micrograms of total pancreatic RNA was used for hybridization with a 32P-UTP–labeled multitemplate set containing specific probes for Fas, Fas-L, tumor necrosis factor-α, interferon (IFN-γ), and lymphotoxin-β provided by a commercial kit (Riboquant, mCK-3b and mAPO-3; Pharmingen, La Jolla, CA). The RNAse protection assay was carried out according to the manufacturer’s guidelines. The resulting analytical acrylamide gel was scanned using a Storm-860 phospho-imaging system (Molecular Dynamics), and the intensity of bands corresponding to protected mRNA was quantified using the ImageQuant image analysis software (Molecular Dynamics) using L22 as a reference gene (23).

Immunohistochemistry. Organs were harvested at week 6 after LCMV infection unless indicated otherwise, immersed in Tissue-Tek OCT (Bayer, Elkhardt, IN), and quick frozen on dry ice. Six- to 10-μm tissue sections were cut using a cryomicrotome and stained-coated “superfrost plus” slides (Fisher Scientific, Pittsburgh, PA). Sections were fixed with 90% ethanol at −20°C, and after washing in PBS an avidin-biotin blocking step was included (Vector Laboratories, Burlingame, CA). Primary and biotinylated secondary antibodies (Vector Laboratories) were reacted with the sections for 30 min each, and color reaction was obtained by sequential incubation with avidin-peroxidase conjugate (Vector Laboratories) and diaminobenzidine-hydrogen peroxide. Primary antibodies were rat anti-mouse CD8b (ly3), rat anti-mouse CD8a (ly2), and anti-LCMV-NP Alexa 1.3 antibody (19,27).

Cytotoxicityassays. LCMV-specific cytotoxic T-cell (CTL) activity in spleen was analyzed in a 5-h in vitro 51Cr-release assay (19). All samples were run in triplicate. Primary CTL activity was tested by harvesting spleens at day 7 after intraperitoneal infection with 106 pfu LCMV-arm. Splenocytes were co-incubated with major histocompatibility complex–matched (Balb/c H-2d) and mismatched (MC57 H-2d) target cells that had been loaded with 51Cr. Target cells were either LCMV-infected, uninfected but coated with the immune dominant LCMV major histocompatibility complex class I peptide NP396-404 ([RPAASGIVM], or uninfected and uncoated. For determination of secondary CTL activity, spleens were harvested 6 weeks after LCMV infection and splenocytes were cultured for 8 days on LCMV-infected, irradiated peritoneal exudate cells before testing CTL activity in a 51Cr release assay. For precursor frequency analysis, spleen cells were serially diluted and cultured in 96-well flat bottom plates in the presence of T-cell growth factor (primarily containing interleukin-2), irradiated LCMV-infected peritoneal exudate cells and spleen feeder cells. After 8–10 days, cultures were assayed for CTL activity on LCMV-infected and uninfected target cells. Precursor frequencies were calculated as described (28).

Flow cytometry. Spleen and pancreatic draining lymph node cells were harvested at week 6 after LCMV infection. Single-cell suspensions were stimulated with 100 μg/ml interleukin-2 and 2 μg/ml Brefeldin A (Sigma) for 5–6 h at 37°C. Cells were stained for cell surface markers using monoclonal antibodies (mAbs) against CD8 and CD4, permeabilized and fixed with paraformaldehyde/saponin, and stained for intracellular cytokines using fluorochrome isothiocyanate–conjugated anti-mouse tumor necrosis factor-α mAb and PE-conjugated anti-mouse IFN-γ mAb (Pharmingen). Cells were acquired and analyzed on a FACSort or FACS-Calibur flow cytometer (Becton Dickinson) using cell quest software (Becton Dickinson) (29).

RESULTS

Expression of Fas-L by β-cells leads to elimination of autoaggressive CD8 lymphocytes and lower diabetes incidence in RIP-LCMV mice. Littermates from F1 mating of RIP-LCMV-NP (H-2d) × RIP-Fas-L (NOD) transgenic were infected with LCMV. As shown in Fig. 1, LCMV infection resulted in 80% diabetes in RIP-NP+ Fas-L controls within 2–8 weeks. In contrast, type 1 diabetes incidence was significantly reduced in littermates that expressed Fas-L in addition to NP antigen in β-cells. Thus, expression of Fas-L has a clear protective effect in the RIP-LCMV diabetes model. Importantly, systemic numbers of cytotoxic CD8 cells were equivalent when comparing Fas-L− versus Fas-L+ NP expressors (Table 1). Comparable numbers of CTL precursors and primary killing activity was detected in spleens. In contrast, locally in the pancreatic draining lymph node, we detected a significant reduction of NP pCTL (Table 1) and, accordingly, a lesser degree of CD8 infiltration in Fas-L− NP+ protected compared with Fas-L+ NP diabetic mice (Fig. 2A versus B). This reduction of NP CTLS was not only site specific but also antigen specific, because CD8 lymphocytes producing IFN-γ in response to LCMV NP but not glycoprotein peptides were reduced (Table 1). Thus, local expression of Fas-L by...
105 pfu LCMV intraperitoneally, and CTL activities were determined as described (29). Significance as described by us previously (30). Lytic precursor CTLs were assessed by intracellular cytokine staining (ICCS) as described in our previous publications (24). For detecting NP-specific CTL precursors were assessed by limiting dilution as explained in detail in our previous publications (24).

**TABLE 1**

<table>
<thead>
<tr>
<th>Spleen</th>
<th>Fas-L+ /NP+</th>
<th>Fas-L+ /NP+</th>
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<tbody>
<tr>
<td>CTL, day 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCMV</td>
<td>43 ± 7</td>
<td>39 ± 11</td>
</tr>
<tr>
<td>pCTL, day 60</td>
<td>12 ± 2</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>PDLN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICCS (IFN-γ), day 45</td>
<td>1/5,211 ± 18</td>
<td>1/4,850 ± 15</td>
</tr>
<tr>
<td>GP33</td>
<td>1.7 ± 0.2</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>NP396</td>
<td>1.12 ± 0.5*</td>
<td>0.19 ± 0.11*</td>
</tr>
<tr>
<td>pCTL, day 60</td>
<td>1/6,430*</td>
<td>1/15,000*</td>
</tr>
</tbody>
</table>

Data are means ± SE. Groups of three mice were infected with 1 × 10⁵ pfu LCMV intraperitoneally, and CTL activities were determined at the times indicated. Lytic CD8 activities were determined from splenocytes on day 7 after LCMV infection by conventional Cr51 release assays using MC57 (H-2b) target cells coated with the immune dominant LCMV NP peptide (NP396: FYFPQNGQFL) or infected with LCMV 48 h before at a multiplicity of infection 1:1. Percentage of Cr51 release after a standard 5-h assay is shown ± SE. CTL precursors were assessed by limiting dilution as explained in detail in our previous publications (24). For detecting NP-specific precursors in limiting dilution assays (pCTL), target cells (MC57) were infected with vaccinia virus recombinants expressing LCMV-NP. On day 45 or 60 after LCMV infection pancreatic draining lymph node sections were harvested as described previously. Numbers of IFN-γ-producing CD8 lymphocytes specific for H-2Db restricted GP33 or NP396 peptides were assessed by intracellular cytokine staining (ICCS) as described by us previously (30). Lytic precursor CTLs were determined as described (29). *Significant differences are indicated (P < 0.05). Note that only in PDLN from Fas-L+ expressing NP+ mice CTLs directed to LCMV-NP are significantly reduced, indicating that the Fas-L-mediated effect on autoaggressive CD8 lymphocytes is antigen specific and locally restricted. PDLN, pancreatic draining lymph nodes.

β-cells can exert some protective capacity by eliminating autoaggressive CD8 lymphocytes in an islet antigen-specific manner. The antigen specificity of the process argues for the fact that interaction of Fas-expressing LCMV-NP-specific CD8 lymphocytes directly with Fas-L+ expressing NP+ β-cells leads to their demise.

**Viral (LCMV) infection of the pancreas induces Fas on β-cells, leading to fratricide by Fas-L+ expressing transgenic islet cells.** To our surprise, we observed almost 50% diabetes development in Fas-L+, NP-nonexpressing littersmates 6–10 months after LCMV infection (Fig. 1). This was not a spontaneous disease comparable to that found in the original Fas-L+ NOD lines, because the mice under observation were intercrosses between the original NOD and nondoniabetes-prone Balb/c mice, and spontaneous disease without LCMV infection was never seen in these mice (data not shown). It is interesting that the diabetes in the Fas-L+ NP mice was not associated with marked CD8 (Fig. 2C) and other lymphocytic infiltration (not shown) as compared with more extensive CD8 infiltration in NP+ littersmates (Fig. 2A and B) or in LCMV-infected RIP-NP Balb/c mice (Fig. 2D). This observation indicated that β-cell fratricide had occurred as a consequence of virally induced inflammation of the pancreas, because LCMV infects the pancreas and the islets directly as shown by immunohistochemical stains of pancreas sections with anti-NP antibody (clone 1.1.3.; Fig. 2E) (19,27). Because earlier studies had demonstrated expression of Fas by β-cells after transfer of diabetogenic clones, we hypothesized that LCMV-induced inflammation of the pancreas would lead to Fas induction by β-cells.

Indeed, we could detect Fas expression on both RNA and protein level by RNase protection assay (RPA) and flow cytometry of isolated islets, respectively. First, Fig. 3A shows that Fas gene expression, as detected by RPA, is seen on islet cells early after LCMV infection (day 4) immediately after viral antigen is first expressed (day 2 after infection) and interferons, chemokines, and other
inflammatory mediators are induced. Because Fas upregulation is induced by IFN-γ and can be blocked by neutralizing antibodies (30), we next treated LCMV-infected mice with neutralizing antibodies against IFN-γ and analyzed Fas gene expression by RPA. As expected, Fas gene expression at day 4 after LCMV infection was significantly lower in the pancreatic draining lymph node of mice that received four doses of anti–IFN-γ mAb at days 0, 1, 2, and 3 after infection than in mice that received an isotype-matched control antibody (Fig. 3B). However, no change in Fas gene expression was observed in the pancreas (Fig. 3B).

DISCUSSION

We demonstrate in this study a dual effect of Fas-L expression on β-cells. On one hand, Fas-L provides immune privilege by eliminating autoaggressive CD8 lymphocytes. This finding would encourage the use of Fas-L to protect β-cells and other cell types/organs from autoimmune attacks and fits well with the known role of Fas-mediated apoptosis in CD4 populations. On the other hand, viral infection of the pancreas but not the islets can be identified through their high autofluorescence detectable in the FL1 channel (Fig. 4A). Increased Fas expression was first noted at day 5 after LCMV infection, and it was >10-fold higher than on β-cells isolated from uninfected mice at day 7 after infection (Fig. 4B).
scatter (FSC) pattern (R1; left), low propidium iodine incorporation (R2; middle), and high autofluorescence in channel FL1 (R3; right). B: Fas expression was analyzed in β-cells (gated in R3) at several days after infection with 10^5 pfu LCMV. Data shown are average (±SE) of the mean fluorescence intensity (MFI) (n = 3–5 mice/group).

FIG. 4. Expression of Fas on the cell surface of isolated β-cells. A: Pancreatic islet cell preparations were stained with anti-Fas antibody and incubated with propidium iodine (PI). Viable β-cells were identified by flow cytometry on the basis of their side scatter (SSC) versus forward scatter (FSC) pattern (R1; left), low propidium iodine incorporation (R2; middle), and high autofluorescence in channel FL1 (R3; right). B: Fas expression was analyzed in β-cells (gated in R3) at several days after infection with 10^5 pfu LCMV. Data shown are average (±SE) of the mean fluorescence intensity (MFI) (n = 3–5 mice/group).

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