Different Diabetogenic Potential of Autoaggressive CD8+ Clones Associated with IFN-γ-Inducible Protein 10 (CXC Chemokine Ligand 10) Production but Not Cytokine Expression, Cytolytic Activity, or Homing Characteristics

Mette Ejrnaes, Nicoline Videbaek, Urs Christen, Anne Cooke, Birgitte K. Michelsen and Matthias von Herrath

*J Immunol* 2005; 174:2746-2755; doi: 10.4049/jimmunol.174.5.2746

http://www.jimmunol.org/content/174/5/2746

---

**References**  This article cites 85 articles, 47 of which you can access for free at: [http://www.jimmunol.org/content/174/5/2746.full#ref-list-1](http://www.jimmunol.org/content/174/5/2746.full#ref-list-1)

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions**  Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Different Diabetogenic Potential of Autoaggressive CD8+ Clones Associated with IFN-γ-Inducible Protein 10 (CXC Chemokine Ligand 10) Production but Not Cytokine Expression, Cytolytic Activity, or Homing Characteristics

Mette Ejrnaes,* Nicoline Videbaek,† Urs Christen,* Anne Cooke,‡ Birgitte K. Michelsen,† and Matthias von Herrath2*

Type 1 diabetes mellitus is an autoimmune disease characterized by T cell-mediated destruction of the insulin-producing β cells in the islets of Langerhans. From studies in animal models, CD8+ T cells recognizing autoantigens such as islet-specific glucose-6-phosphatase catalytic subunit-related protein, insulin, or glutamic acid decarboxylase (GAD) are believed to play important roles in both the early and late phases of β cell destruction. In this study, we investigated the factors governing the diabetogenic potential of autoaggressive CD8+ clones isolated from spleens of NOD mice that had been immunized with GAD6515–23 or insulin B-chain15–23 peptides. Although these two clones were identical in most phenotypic and functional aspects, for example cytokine production and killing of autologous β cells, they differed in the expression of IFN-γ-inducible protein-10, which was only produced at high levels by the insulin-specific clone, but not by the GAD65-specific clone, and other autoantigen-specific non-pathogenic CD8 T cell clones. Interestingly, upon i.p. injection into neonatal mice, only the insulin B-chain15–23-reactive CD8+ T clone accelerated diabetes in all recipients after 4 wk, although both insulin- and GAD-reactive clones homed to pancreas and pancreatic lymph nodes with similar kinetics. Diabetes was associated with increased pancreatic T cell infiltration and, in particular, recruitment of macrophages. Thus, secretion of IFN-γ-inducible protein-10 by autoaggressive CD8+ lymphocytes might determine their diabetogenic capacity by affecting recruitment of cells to the insulitic lesion. The Journal of Immunology, 2005, 174: 2746–2755.

The Journal of Immunology

Received for publication November 19, 2003. Accepted for publication December 16, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*Address correspondence and reprint requests to Dr. Matthias von Herrath, La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA 92121. E-mail address: matthias@liai.org

†Hagedorn Research Institute, Gentofte, Denmark; ‡Department of Pathology, University of Cambridge, Cambridge, United Kingdom

© 2005 by The American Association of Immunologists, Inc.
using β2-microglobulin knockout mice leads to inhibition of T1D (33–36), underlining the role of CD8+ T cells in disease progression. Additionally, cytokines such as IL-1, IFN-γ, or TNF-α secreted by infiltrating T cells and macrophages may lead to β cell destruction either directly or indirectly (37–41). One of the cytotoxic mechanisms used by MHC class I-restricted T lymphocytes is the release of perforin and/or granzymes to induce apoptosis in the target cell (42). Lysis of β cells that causes diabetes can occur via the perforin pathway, because perforin-positive CD8+ T cells have been found in pancreas of animals developing T1D (43) and perforin knockout mice have a reduced incidence and delayed onset of diabetes (44). However, studies with the NOD model have also implicated the Fas pathway as an important contributor to the demise of β cells (45, 46).

As a rationale for the present study, we reasoned that it would be important to better understand the phenotypic and functional features of autoaggressive CD8+ lymphocytes in correlation to their ability to cause diabetes. We were fortunate in obtaining two clones (GAD65515–524 and InsB15–23) that exhibited clearly different pathogenic potentials, although they shared most phenotypic and functional properties, in particular cytokine production, homing to islets, and the ability to lyse autologous β cells in vitro. Because only the InsB-specific clone was capable of causing profound insulitis and diabetes, the ability to attract other cells to the insulitic lesion was a major distinction between the two clones. Interestingly, this correlated with secretion of the chemokine IFN-γ-inducible protein-10 (IP-10) by the InsB-, but not the GAD-specific clone. These findings indicate that IP-10 might be one of the major factors involved in orchestrating the autoaggressive response in the islets.

Materials and Methods

Mice

NOD/Bom and NOD.scid mice were bred under specific pathogen-free conditions at M & B. Experiments were conducted at the animal facility at the Hagedorn Research Institute. To measure glucose levels in the mice, blood samples were obtained from the tail vein and measured on an AccuTrend glucose sensor (Roche Diagnostics). Mice were defined as diabetic using the major factors involved in orchestrating the autoaggressive response in the islets.

Peptides

Murine InsB15–23 peptide sequence: LYLVCGERG (Bethyl Laboratories); murine proinsulin I23–34; rGAD65515–524; WVPFPSRLVL; rGAD67515–524: WYIPQSLRGV; influenza virus nucleoprotein: TYQTRALVRT (both from Schafer-N). Peptide dose in all in vitro assays was 10 μg/ml.

Cell cultures

Spleen cells and P815 cells were cultured in RPMI 1640 (Invitrogen Life Technologies, and source for in vitro studies in this work) supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Invitrogen Life Technologies, respectively), and 5 × 10–3 M 2-ME (Bio-Rad). All cells were grown at 37°C, 5% CO2.

Generation of T cell clones

Immunization. NOD female mice (5 wk) were immunized s.c. with 50 μg/mouse InsB15–23, proinsulin I23–34, GAD65515–524, or GAD67515–524 peptide (Bethyl Laboratories) emulsified 1:1 with IFA (Sigma-Aldrich). NOD mice receiving GAD65 or GAD67 peptide were primed with a mixture of GAD peptide (50 μg) together with a Th peptide (OVA 323–339, 50 μg) in 1:1 emulsion with IFA. After 2 wk, mice were boosted with an equal amount of peptide in adjuvant. Splenocytes were prepared from the immunized mice (day 10 postimmunization) by mincing the spleens, washing twice with 1 × HBSS without Ca2+ or Mg2+ ( Invitrogen Life Technologies).

Stimulation of cells in vitro. Cell cultures were stimulated with 1 × 106 syngeneic APCs irradiated with 2900 rad, InsB15–23, proinsulin I23–34, GAD65515–524, or GAD67515–524 peptide at 10 μg/ml (Bethyl Laboratories), 20 U/ml IL-2 (BD Pharmingen), and 10 ng/ml IL-7 ( Peprotech). Cultures were grown with 100 ng/ml anti-CD4+ and 50 ng/ml anti-MHC class II Abs (L3T4 and OK-6; BD Pharmingen) to avoid overgrowth by CD4+ T cells.

Limited dilution cloning. Spleen cells from peptide-immunized mice were Ag stimulated and limited diluted in 96-well, flat-bottom plates. Cells were diluted to 0.25 cells/well and stimulated twice per month. Proliferating cells were further expanded. One clone proliferating to InsB15–23 and one proliferating to proinsulin I23–34 were isolated: clones A1 and P4.10, respectively. Additionally, two GAD-reactive clones proliferating to GAD65515–524, clone R1, and GAD67515–524, clone C2, were isolated (47). Finally, a lymphocytic choriomeningitis virus (LCMV) nuclear protein (NP)-specific clone (NP199–204) recognizing the LCMV nucleoprotein NP199–204 was generated and maintained in long-term cultures, as previously described (48). Shortly, the NP199–204-specific clone was isolated from LCMV-infected recipients and stimulated frequently with irradiated LCMV-infected syngeneic macrophages and 50 U/ml human IL-2 (Sigma-Aldrich).

Flow cytometry

To stain for cell surface markers, we used mouse mAbs (BD Pharmingen) to CD4 (PE-labeled), CD8+ (FITC-labeled), CD3 (PE-labeled), K562 (FITC-labeled), D9 (FITC-labeled), CD25 (FITC-labeled), and CD95 and CD95L (FITC-labeled). Vβ TCR screening panel kit containing FITC-conjugated Abs that recognize mouse Vβ 2, 3, 4, 5, 1, and 5.2, 6, 7, 8.1, and 8.2; and 8.3, 9, 10, 11, 12, 13, 14, and 17, respectively, was used in accordance with the manufacturer’s instructions.

Islet cytotoxic assays

To determine the amount of MHC-restricted CTL lysis, a standard in vitro 51Cr release assay was used. Target cells used were freshly isolated islets or P815 cells. Briefly, islets were isolated from NOD (Kd, D9) or B6 (Kb, D8) pancreas using a collagenase digestion method (41). Briefly, pancreas was shaken by hand for 10 min in 1 mg/ml collagenase type 4 (Valent Pharmaceuticals). Pancreas was transferred to new collagenase containing vials (10 mg/ml) and shaken for 5 min. This procedure was repeated until pancreas was completely digested. The islet-containing supernatant was washed three times with HBSS, 0.5% FBS. Finally, islets were handpicked under the microscope. The freshly isolated islets were labeled with 100 μCi of 51Cr (DuPont Pharmaceuticals) for 1 h at 37°C and washed. Islets were trypsinized into single cells by incubating the islets with 1 ml of trypsin-EDTA (Invitrogen Life Technologies) for 2 min and resuspended in RPMI 1640 with 10% FBS. E:T ratios for the islet killing assay were 50:1 and 25:1. Additionally, P815 target cells were labeled with 100 μCi of 51Cr (DuPont Pharmaceuticals), as described above. A total of 1 × 106 cells P815 cells was either incubated without or with the InsB15–23, GAD65515–524, or influenza virus NP peptide (10 μg/ml for all purified peptides). Release of 51Cr to the supernatant was measured in a gamma counter. Percent-specific 51Cr release was determined by the formula: (cpm experimental release – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release) × 100. Medium or 10% lysis buffer Triton X-100 (Sigma-Aldrich) was added to wells containing target cells to determine spontaneous and maximum release, respectively.

Proliferation

The 200-μl cultures containing 7 × 104 insulin- or GAD-specific CD8 T cells (quadruplicates) ≥ peptides (10 μg/ml), and 1 × 105 syngeneic splenic APCs were grown for 120 h in round-bottom 96-well plates. To verify MHC class I restriction Abs against MHC class I (Kb and D8) (10 μg/ml) were added together with peptide. Proliferation was assessed by measuring the amount of [3H]thymidine (Amersham Biosciences) incorporation after a 16-h pulse (0.5 μCi/well), and results were expressed as mean cpm.

Determination of T cell cytokine secretion

From the above cultures, supernatants were harvested after 24 and 48 h. Capture ELISA was used to assess IL-2, IL-4, IL-10, IFN-γ, and TNF-α levels. Cytokine analysis was conducted by use of kits and performed as proposed by BD Pharmingen, which supplied the capture and detection Abs.

Homing of the clones in vivo

To detect homing of the InsB15–23 and GAD65515–524, clones, cells were labeled for 10 min with CFSE (Molecular Probes). A total of 1 × 107 CFSE-labeled insulin- or GAD-specific T cells was injected i.p. into...
NOD.scid mice. Two days after the injection, mice were sacrificed, and spleen, pancreas, mesenteric, and pancreatic lymph nodes were removed and analyzed for the presence of CFSE-labeled cells by flow cytometric analysis or microscopy.

**Histological and immunochemoanalysis of tissues**

Tissues taken for histological analysis were fixed in 10% zinc Formalin and stained with H&E. Immunochemoanalysis studies were conducted on 6- to 10-μm freshly frozen cryostome sections for immunostaining of islets to detect presence of CD8 T cells and macrophages. Primary Abs were applied for 1 h. These consisted of rat anti-mouse CD8 (clone 53-6.7) and anti-F4/80 (clone A3-1) (BD Pharmingen). After washing in PBS, the secondary Ab, biotinylated goat anti-mouse IgG (Vector Laboratories), was applied for 1 h. Color reaction was developed with sequential treatment using avidin-HRP conjugate (Boehringer Mannheim) and diaminobenzidine-hydrogen peroxide.

**Clonal RNA analysis/RNase protection assays**

Chemokine expression was determined in the InsB15–23 and proinsulin II23–34 clones (A1 and P4.10), the GAD65515–524 (R1), and GAD67751–524 (C2). These four clones were all derived from the NOD mouse model. Additionally, a LCMV-NP396–404-specific clone derived from the rat insulins promoter (RIP)-LCMV model was tested (48, 49). The specific clones were homogenized in 2 ml of Tri-reagent (Molecular Research Center) day 5 postpeptide stimulation. Total RNA was extracted with chloroform, followed by isopropanol precipitation and washing with ethanol. Twenty micrograms of total T cell RNA were used for hybridization with a 32P-UTP-labeled multitemplate set containing specific probes for RANTES, IP-10, IFN-γ, and TNF-α provided by a commercial kit (Riboquant, mCK-3b; BD Pharmingen). Two different probes were used. The RNase protection assay was conducted according to the manufacturer’s guidelines. The resulting analytical acrylamide gel was scanned using a STORM-860 Phosphor-Imaging System (Amersham Biosciences), and the intensity of bands corresponding to protected mRNAs was quantified using the ImageQuant image analysis software (Amersham Biosciences).

**Adoptive transfer of diabetes**

Litters of 2- to 8-day-old NOD or NOD.scid mice were randomized and injected i.p. with PBS, 1 × 10^7 CD8 T spleen cells purified from 4-wk-old NOD mice, or 10 × 10^6 InsB15–23 or GAD65515–524-specific T cell clones, and the development of diabetes was monitored. Mice were considered diabetic when blood glucose levels in two consecutive measurements exceeded 15 mmol.

**Results**

**Ag specificity of insulin- and GAD-specific CD8^+ clones**

To investigate the properties that govern diabetogenicity of autoreactive CD8 T lymphocytes, we generated an insulin-specific CD8 clone by immunizing NOD mice with InsB15–23 peptide. Afterward, spleen cells from peptide-immunized mice were restimulated twice per month with InsB peptide. After 2 mo of culture, cells were limited diluted, and one proliferating clone was further characterized: the InsB15–23 clone (see Materials and Methods). Using immunization of NOD mice with GAD65515–524 peptide, a GAD65515–524-specific clone was generated using the same approach (50). We first tested and compared the ability of both clones to proliferate in response to cognate and irrelevant peptides. As
shown in Fig. 1a, insulin and GAD clones proliferated when APCs presented insulin or GAD 9mer peptides, but not influenza NP or GAD67 peptides. Both clones recognized Ag in a K\\textsuperscript{\textalpha}-restricted manner, because peptide-specific proliferation could be blocked almost completely with Abs against MHC class I K\\textsuperscript{\textbeta}, but not D\\textsuperscript{\textbeta} (Fig. 1a, middle and lower panel). These results demonstrated that both clones were CD8\\textsuperscript{a} and MHC class I K\\textsuperscript{\textalpha}-restricted peptide restricted.

**Autologous target cells and b cells are specifically lysed by insulin- and GAD-specific CD8\\textsuperscript{a} clones**

Next, the cytotoxic activity of both clones toward APCs presenting relevant or irrelevant peptides was tested in a standard 51Cr release assay. As shown in Fig. 1b, insulin-specific T cells lysed 51Cr-labeled P815 (K\\textsuperscript{\textbeta}) cells presenting InsB\\textsubscript{15–23} peptide (48-65%). In a comparable fashion, GAD68-specific CD8 cells lysed P815 cells presenting GAD65\\textsubscript{515–524} peptide (40-65%). Additionally, both clones were highly cytolytic (42-50%) even at low E:T ratios (2.5:1) (Fig. 1b). P815 cells alone or incubated with the control influenza NP peptide were not lysed to significant degrees (0-4%), underlining the peptide specificity of both autoantigen-specific clones (data not shown). Most importantly, we tested whether both clones were capable of lysing autologous b cells in vitro. As shown in Fig. 1c, comparable killing of autologous NOD b cells by both clones was observed after 5 h of incubation in an E:T-dependent manner. Furthermore, MHC class I-mismatched islets from B6 mice (H-2b) were lysed at 0-5% only, underlining that the effector cells lysed NOD islets in a MHC class I K\\textsuperscript{\textalpha}-restricted manner.

Additionally, expression of cell surface molecules and TCR usage were determined by flow cytometry. Both clones expressed activation markers. Insulin-specific T cells expressed CD8, TCR\textalpha, V\\textbeta13, K\\textsuperscript{\textalpha}, D\\textsuperscript{\textbeta}, CD44, CD69, and CD25, markers characteristic for an activated CD8\\textsuperscript{a} T cell, but no Fas (CD95) or Fas ligand (CD95L) (Table I). GAD6T5 cells expressed similar cell surface molecules. However, they differed in terms of V\\textbeta usage. The insulin-specific CD8\\textsuperscript{a} clone expressed V\\textbeta13, whereas the GAD65-specific clone expressed V\\textbeta8.1 (Table I). Thus, both clones exhibited similar lytic activity toward fibroblasts and b cells in vitro and showed a comparable activation profile.

Insulin- and GAD-specific clones secrete similar Tc1 cytokine, but not chemokine profiles

To further characterize the two clones, cytokine and chemokine production was investigated by ELISA (Table II) and RNase protection assays (Fig. 2). Phenotypically, upon Ag stimulation, all clones generated cytokines characteristic of Tc1 cells: IFN-\gamma (Table II and Fig. 2a) and TNF-\alpha (Table II and Fig. 2a), but no IL-4 and IL-10 (Table II). Because IFN-\gamma is known to stimulate MHC class I expression, up-regulation of MHC class I on b cells leading to increased Ag presentation would be a possible effect of IFN-\gamma released from the clones, making b cells more susceptible to CTL lysis (see Fig. 1c) (41). Additionally, as shown in Fig. 2b, top panel, lanes 1 and 2, both clones expressed the chemokine RANTES. However, IP-10 was only expressed at high levels by the InsB (Fig. 2, a, lane 1, and b, lower panel), but not the GAD65-specific T cell clone (Fig. 2, a, lane 2, and b, lower panel). Given the otherwise similar characteristics of the two clones, we reasoned that this difference could potentially be important in determining in vivo diabetogenicity. We therefore expanded the investigation of chemokine expression to include several other autoantigen-specific CD8 T cell clones. We included additional clones derived from the NOD mouse model, as well as clones derived from the RIP-LCMV mouse model, to compare the role of autoantigen-specific T cell chemokine expression in two different models of T1D. The significance of Ag-specific cytotoxic CD8 T cells had been demonstrated in both models. All clones were highly Ag specific and lysed target cells in a peptide-specific manner (Fig. 1b and data not shown). We evaluated the expression of RANTES and IP-10 in a proinsulin II\\textsubscript{25–34}-specific T cell clone (P4.10) (Fig. 2, a, lane 3, and b), a clone reactive to GAD67\\textsubscript{515–24} (C2) (Fig. 2, a, lane 4, and b), both derived from NOD. As shown in Fig. 2b, illustrating quantification of Fig. 2a the proinsulin II (P4.10)- and GAD67 (C2)-reactive clones both expressed high levels of RANTES, but only low levels of IP-10. The proinsulin- and GAD-specific clones also expressed Tc1-like cytokines as TNF-\alpha and IFN-\gamma (Fig. 2a, lanes 3 and 4). The cytokine phenotype was further confirmed by ELISA (data not shown). Furthermore, we evaluated one autoantigen-specific CD8 T cell clone recognizing the LCMV nucleoprotein CD8 epitope 396–404 (NP396-404) derived from the RIP-LCMV model and shown to be diabetogenic in vivo (49). We observed that this clone expressed TNF-\alpha and IFN-\gamma (Fig. 2a, lane 5), which was also confirmed by ELISA (data not shown). Importantly, as illustrated in Fig. 2, this clone expressed high levels of IP-10. Thus, because IP-10 is known to act as a major chemotactant of lymphocytes and macrophages (51–54), it was conceivable that distinct in vivo effects could be associated with its production.

**Increased diabetogenic potential of the InsB\\textsubscript{15–23} but not the GAD65\\textsubscript{515–524} CD8\\textsuperscript{a} clone**

To dissect the in vivo diabetogenic effects of the clones, InsB (●) or GAD65-specific (▲) T cells (1 × 10\\textsuperscript{7}) were injected into neonatal NOD (n = 10 mice/clone) recipients, and the development of diabetes was monitored (Fig. 3). NOD mice receiving purified CD8\\textsuperscript{a} T cell (n = 5, □) from naive NOD mice served as controls to exclude the possibility that a high number of CD8\\textsuperscript{a} T cells had a nonspecific effect. Furthermore, neonatal NOD mice injected with PBS (n = 10, filled line) served as controls to monitor spontaneous development of diabetes. As shown in Fig. 3, no acceleration of diabetes was observed in mice receiving purified naive CD8\\textsuperscript{a} T cells or PBS. Based on the exquisite killing capacity of

---

**Table I. Cell surface markers expressed by insB- and GAD-specific CD8\\textsuperscript{a} clones**

<table>
<thead>
<tr>
<th>Cell Surface Molecules and TCR Usage</th>
<th>GAD65\textsubscript{515–524} clone</th>
<th>Ins\textsubscript{\textbeta}\textsubscript{15–23} clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TCR\textalpha</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V\textbeta8.1</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>V\textbeta13</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MHC class I K\textalpha, D\textbeta</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD95</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD95L</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD25</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>CD44</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>CD69</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

---

**Table II. Cytokine profiles of insB- and GAD-specific CD8\\textsuperscript{a} clones**

<table>
<thead>
<tr>
<th>Cytokine Production (pg/ml)</th>
<th>GAD65\textsubscript{515–524} clone</th>
<th>Ins\textsubscript{\textbeta}\textsubscript{15–23} clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>68</td>
<td>78</td>
</tr>
<tr>
<td>IFN-\gamma</td>
<td>14,811</td>
<td>25,000</td>
</tr>
<tr>
<td>TNF-\alpha</td>
<td>47</td>
<td>105</td>
</tr>
<tr>
<td>IL-4 and IL-10</td>
<td>0/0</td>
<td>0/0</td>
</tr>
</tbody>
</table>
FIGURE 2. Strong difference in IP-10 production by InsB- and GAD-specific CTL clones. RANTES and IP-10 expression by InsB15–23, GAD65515–24, GAD67515–24, proinsulin H35–44, and NP396–404-specific CD8+ clones was analyzed after stimulation with irradiated APCs and GAD65, GAD67, InsB, or proinsulin peptide, respectively. The LCMV-NP396–404-specific clone was stimulated with irradiated LCMV-infected macrophages. RNase protection analysis was performed. Two different probes specific for RANTES, IP-10 (left marker), TNF-α, RANTES, IFN-γ, and IP-10 (right marker) were used. Relative signal intensity was determined using a Storm PhosphorImager with ImageQuant image analysis software (Amersham Biosciences). a, RNase protection analysis data; b, the average signal intensity of RANTES and IP-10 for the five clones.

the GAD65 clone in vitro (Fig. 1, b and c), it was surprising that GAD65-specific T cells did not have any in vivo diabetogenic effect. The GAD65 T cell recipients remained normoglycemic until 18 wk of age, and at 30 wk of age onset of T1D was 30% as compared with 50 and 60% in the control groups. The relatively low overall incidence of diabetes at 30 wk of age can be explained by the fact that both male and female recipients were used (65% of the mice were female and 35% male mice). In the group of mice receiving the insulin-specific CD8+ clone, a dramatic increase in blood glucose was observed. All mice had blood glucose levels >22 mmol 4 wk posttransfer. Acceleration of diabetes onset in all (10 of 10) mice 4–5 wk after receiving the insulin-specific clone was not gender specific, but equally distributed between males and females. Moreover, to verify that induction of disease was independent of endogenous lymphocytes, diabetes incidence was investigated in NOD.scid recipients using the same experimental approach as above. One hundred percent of NOD.scid mice receiving insulin-specific T cells developed diabetes compared with 0% of recipients receiving the GAD65 clone (data not shown).

Thus, the InsB-specific CD8+ clone induced rapid diabetes in male and female NOD and NOD.scid recipients, whereas the GAD65 clone did not. Because the clones were both specific for autoantigens thought to play a role in the development of diabetes and exhibited similar Tc1 characteristics, it was surprising that the in vivo effects were strikingly different. In additional experiments, diabetes could not be induced in mice receiving two consecutive injections of 1 × 10⁷ GAD65515–324-specific T cells (data not shown). These results indicated that T cells specific for the autoantigen InsB15–23 had diabetogenic potential, whereas GAD65515–324-specific T cells were not pathogenic, even upon multiple injections. This observation might be linked to the difference in IP-10 expression because diabetes was not accelerated in mice receiving the proinsulin II- and GAD67-specific clones, both low IP-10 expressors (data not shown). The correlation between IP-10 expression and diabetogenic potential was further underlined with the finding of high IP-10 expression in one Ag-specific CD8 T clone from the RIP-LCMV diabetes model. Interestingly, it was previously shown that this clone was found in the pancreas and had diabetogenic potential upon adoptive transfer into RIP-NP × RIP-B7.1 recipients (48). The clone recognizes the LCMV nucleoprotein CD8 epitope NP396–404. This strengthens the link between high IP-10 CD8 T cell expressers and their diabetogenic potential. Because we had determined in vitro that high IP-10 levels were only observed in CD8 T cells with diabetogenic potential, like the InsB clone, we further concentrated on analyzing the insulitic lesions in respect to cellularity and composition after injection of the InsB15–23 and GAD65515–324 clones to address potential differences in in vivo trafficking and recruitment of cells to the islets.

FIGURE 3. In vivo diabetogenic capability is much higher by the InsB than the GAD clone. Groups of 2- to 3-day-old NOD (n = 10 mice/clone) mice received 1 × 10⁷ InsB CD8+ T cells (■) or 1 × 10⁷ GAD65 CD8+ T cells (▲). Additionally, 10 NOD mice received PBS (thin line), and 5 NOD mice received purified CD8+ T cells from young NOD mice (□). Mice were bred under specific pathogen-free conditions at M & B. Mice were considered diabetic when blood glucose levels in two consecutive measurements exceeded 15 mmol/dL. Two additional experiments using NOD or NOD.scid mice as recipients of InsB or GAD T cells were repeated with similar results.
Increased islet infiltration dominated by CD8\(^+\) cells and macrophages following injection of the InsB- but not the GAD-specific CTL clone

Histological examination of pancreata from NOD.scid mice 4 wk after receiving insulin-specific T cells revealed strong infiltration of CD8\(^+\) T cells (Fig. 4b), whereas islets from recipients of GAD65 T cells (Fig. 4c) showed low degrees of peri-insulitis. As expected, because recipients were NOD.scid mice, no CD8 T cell infiltration was detected in saline-injected mice (Fig. 4a). Because the clones differed in the secretion of the chemokine IP-10, we wanted to investigate whether expression of this chemokine would correlate with an increased influx of macrophages. We observed that islets infiltrated with insulin-specific CD8\(^+\) T cells (Fig. 5a, left panel) also showed increased presence of macrophages (Fig. 5a, right panel). CD8 T cell and macrophage infiltration was significantly increased (8.6 score) in islets of NOD mice receiving the insulin-specific T cells (Fig. 5c, left and right panels, respectively). NOD mice receiving GAD65-specific T cells had T cell and macrophage infiltration comparable to control mice receiving saline (2.5 and 2.6 score) (Fig. 5, b and c, left and right panels, respectively).

Because the insulin and the GAD65 clone shared many functional similarities in vitro (except for the secretion of IP-10), these results indicated that IP-10 played a major role in increasing the insulitic lesion by recruiting more CD8 lymphocytes as well as macrophages. In vivo, increased accumulation of the InsB CD8\(^+\) clone is seen in islets already early after injection, although both clones reach islets and lymphoid organs.

We wished to investigate whether the InsB or GAD65 clones would enter the pancreas and islets after in vivo injection, and additionally assess quantitatively how many injected CD8\(^+\) cells reached the pancreas. This was achieved by injecting CFSE-labeled insulin (clone A1)- or GAD65-specific clones into neonatal NOD mice (2 days old). As shown in Fig. 6, more InsB (Fig. 6b) than GAD65 CD8\(^+\) CTL (Fig. 6c) were found in the islets on day 2 postinjection using FACS analysis to detect CFSE-positive cells. This was also reflected in histological examination, in which higher numbers of CD8\(^+\) InsB clones (Figs. 4b and 5a) were present in islets compared with GAD65 clone-injected mice (Figs. 4c and 5b). Further proliferation of the injected clones was not detected after they entered the pancreas, which could be explained by the fact that the clones were peptide stimulated 5 days before injection and therefore effector cells at the time of injection, not likely to be prone for proliferation. In addition, CFSE-positive T cells were detected in pancreatic lymph nodes and spleens of the recipient mice 1 day postinjection (data not shown). Note that the FACS plot data were gated for the CFSE-stained clone. These results showed that both clones had the ability to enter the pancreas and islets. Additionally, we observed that the proinsulin- and GAD67-specific clones were found in the pancreas upon adoptive transfer into the NOD model (data not shown). Furthermore, we observed that in regular LCMV infection of RIP-LCMV mice,
NP<sub>306−405</sub>-specific CD8<sup>+</sup> T cells were found in the islet infiltrates of diabetic mice.<sup>4</sup>

In conclusion, both InsB and GAD65 clones could reach the islets, whereby slightly higher numbers of InsB cells were found early after injection, which could be caused by autologous attraction due to IP-10 production. However, only the IP-10-expressing InsB clone led to destructive insulitis, which was characterized by the presence of many more T cells and, in particular, macrophages.

Discussion

Although strong evidence now implicates MHC class I-restricted effector CD8<sup>+</sup> T cells recognizing islet Ags in early as well as late phases during the development of T1D, the precise relative contributions of their different effector functions, i.e., cytokine and chemokine secretion, cytolytic activity, and Fas-mediated killing, are still not clear. Activated CD8<sup>+</sup> T cells were shown to recognize MHC class I-presented self epitopes on β cells (7, 32, 41, 55, 56), and tissue damage induced by activated CTLs can lead to activation and amplification of additional CD4 or CD8 effector T cell populations with different autoantigenic specificities. In this way, CD8<sup>+</sup> T cell-mediated cytoxicity could contribute from the beginning to progression from insulinis to clinically manifest diabetes (57, 58). Various attempts have been made to tolerate or eliminate CD8<sup>+</sup> T cells, and some success in animal models highlights the importance that they might have in the pathogenic process. Elimination of CD8<sup>+</sup> T cells with in vivo depleting anti-CD8 mAbs inhibited diabetes in animal models of T1D (32). Similarly, β<sub>2</sub>-microglobulin knockout mice, which due to the deficiency of MHC class I lack functional CD8<sup>+</sup> T cells, were protected against development of diabetes (33–35). In elegant follow-up studies, evidence was provided that expression of MHC class I on β cells, but not other cells of the body, is the essential component important during T1D pathogenesis (55). The low precursor frequency of autoantigen-reactive CD8<sup>+</sup> T cells in the blood has been a barrier to study them, a situation that has recently been greatly improved through the usage of MHC class I tetramers in animal models to track such cells. Insulin-specific cells were detected early during pathogenesis of T1D in the NOD (8), which is followed by appearance of IGRP-specific CD8 lymphocytes, whose accumulation correlates well with progression of the disease (9). Thus, the role of islet-reactive CD8<sup>+</sup> T cells is of importance, and the numbers of publications on this subject are now rising (7, 8, 41, 44, 56, 57, 59). In this study, we evaluated the role of effector functions and antigenic specificity of autoantigen-reactive CD8<sup>+</sup> T cells in β cell lysis and subsequent development of diabetes. We gathered evidence indicating that the chemokine IP-10 plays a crucial role in orchestrating the autoaggressive response; in our model, IP-10 secretion appears to be more important than Ag specificity, because insulin and GAD clones lysed autologous β cells in vitro with comparable efficacy.

Given the relatively much higher expression levels of insulin compared with GAD65 by β cells (60, 61), one could have expected that insulin-specific CD8 clones would be more pathogenic than GAD65 CTL. This, indeed, was the case in our study. We observed that the insulin-specific clone (A1) induced diabetes in neonatal mice independent of gender and with high efficiency in both NOD and NOD.scid recipients (100%) and relatively short mean time (Fig. 3). In contrast, animals receiving the GAD65-specific clone caused only minor peri-insulitis (Fig. 4c), but no acceleration of diabetes (Fig. 3). Do these results indicate that insulin is a better target Ag than GAD65 for the autoaggressive response? Based on our in vitro data showing comparable killing of autologous NOD β cells by both clones, one has to be careful with this conclusion (Fig. 1c). Killing of β cells by CTL only occurs if a sufficient amount of MHC class I is expressed on their surface. β cells derived from NOD mice express intermediate levels of MHC class I already very early in life, presumably because autoreactivity is initiated early in this strain (40). In contrast, β cells from nonautoimmune prone mice are not susceptible to CTL lysis, unless IFN-γ and/or other factors lead to up-regulation of MHC class I (40, 41). In our experiments in this study, we used β cells from young NOD or B6 mice as autologous targets. Because both clones produce equal and substantial amounts of IFN-γ and β cells from 6-wk-old NODs already express MHC class I, the amount of MHC was most likely not a limiting factor in our in vitro studies (Fig. 1c). Thus, one can conclude that at least under in vitro conditions, NOD-derived β cells must have expressed a sufficient amount of GAD peptide in context with the proper MHC to enable the GAD clone to lyse them. Thus, islets from MHC class I-mismatched recipients were not lysed (Fig. 1c). If one considers that as few as one MHC-peptide complex (62) can be sufficient for CTL lysis of a target cell, this is conceivable, even if overall expression levels of insulin compared with GAD in β cells

---

are much higher. These considerations do not imply that GAD or insulin as essential autoantigens in T1D. Because the initial antigenic signal is not known in human or NOD T1D, both Ags might contribute to aggressive as well as regulatory (63, 64) autoreactive responses without being essential. Other specificities might be invoked through antigenic spreading and might play as important or more important roles. Indeed, GAD65-deficient (GAD−/−) mice, which have a targeted mutation of the GAD65 gene, backcrossed to the NOD model show homing of activated T cells to the pancreas and development of diabetes comparable to wild-type mice (GAD65+/−) (65). A similar situation applies to insulin, in which insulin-deficient mice exhibit accelerated T1D (66, 67). Thus, at any rate, insulin and GAD are not essential Ags for the autoregressive response, but might play important roles, if targeted by autoreactive regulatory cells (68).

Why does adoptive transfer of the IP-10-expressing InsB clone only cause T1D in young NOD mice? Although of unknown origin, an initiating release of autoantigen leading to the activation of the autoreactive T cells must occur. Prediabetic NOD mice have been shown to exhibit changes in islet function very early during pathogenesis. For example, 4-wk-old female NOD mice show signs of islet hyperactivity and higher basal insulin levels are detected compared with control strains (69, 70). Interestingly, activation of InsB CTL in vivo has been shown very early as well at ~2 wk of age, when the pancreatic islets undergo remodeling. Increased neonatal apoptosis (71) has been found to precede insulitis in NOD mice (72). In this case, although apoptosis is thought to cause tolerance in many instances (73), apoptotic cells might litis in NOD mice (72). In this case, although apoptosis is thought to cause tolerance in many instances (73), apoptotic cells might

Acknowledgments

We thank Diana Frye for her assistance with this manuscript preparation, and Dr. M. B. A. Oldstone for providing the LCMV-specific clone.

Disclosures

The authors have no financial conflict of interest.

References


