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Tc17 CD8⁺ T Cells Potentiate Th1-Mediated Autoimmune Diabetes in a Mouse Model

Amit Saxena,*⁺,‡ Sabine Desbois,*⁺,‡ Nadège Carrić,*⁺,‡ Myriam Lawand,*⁺,‡ Lennart T. Mars,*⁺,‡ and Roland S. Liblau*⁺,⁺,‡

An increase in IL-17–producing CD8⁺ T (Tc17) cells has been reported in the peripheral blood of children with recent onset type 1 diabetes (T1D), but their contribution to disease pathogenesis is still unknown. To directly study the pathogenic potential of β cell-specific Tc17 cells, we used an experimental model of T1D based on the expression of the neo-self Ag hemagglutinin (HA) in the β cells of the pancreas. When transferred alone, the IL-17–producing HA-specific CD8⁺ T cells homed to the pancreatic lymph nodes without causing any pancreatic infiltration or tissue destruction. When transferred together with small numbers of diabetogenic HA-specific CD4⁺ T cells, a strikingly different phenotype developed. Under these conditions, Tc17 cells sustained disease progression, driving the destruction of β-islet cells, causing hyperglycemia and ultimately death. Disease progression did not correlate with functional or numerical alterations among the HA-specific CD4⁺ T cells. Rather, the transferred CD8⁺ T cells accumulated in the pancreatic islets and a considerable fraction converted, under the control of IL-12, to an IFN-γ-producing phenotype. Our data indicate that Tc17 cells are not diabetogenic but can potentiate a Th1-mediated disease. Plasticity of the Tc17 lineage is associated with transition to overt disease in this experimental model of T1D. The Journal of Immunology, 2012, 189: 3140–3149.

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease in which the insulin-producing β cells within the pancreatic islets are selectively destroyed (1–3). Disease predisposition is most strongly conferred by alleles of the MHC class II locus (2, 4), which influence the structure of the MHC peptide-binding pockets (1, 5, 6), thereby affecting the manner by which CD4⁺ T cells recognize specific peptides. CD4⁺ T cells specific for β cell-derived self Ags drive spontaneous T1D in the NOD mouse model (7). In addition to CD4⁺ T cells, a large body of evidence supports a major role for MHC class I-restricted autoreactive CD8⁺ T cells in T1D pathogenesis. Genetic susceptibility is linked to inheritance of selected HLA-A and HLA-B alleles, either independently or in conjunction with MHC class II alleles (8, 9). Moreover, histopathological analysis of postmortem pancreatic organs of T1D patients revealed a direct correlation between the demise of β cells and the frequency of CD8⁺ T cells (10). This demise can result from a direct cytotoxic effect mediated by the infiltrating CD8⁺ T cells (3, 11). MHC class I expression can be induced on β cells during inflammation and renders them susceptible to CD8⁺ T cell-mediated killing (12, 13). In human T1D, several β cell autoantigens have been identified as targets for CD8⁺ T cells (3, 14, 15). In the NOD mouse, CD8⁺ T cells specific for proinsulin or islet-specific glucose-6-phosphatase catalytic subunit-related protein have been identified and can transfer T1D (16, 17). Islet-specific glucose-6-phosphatase catalytic subunit-related protein-specific CD8⁺ T cells are detectable during the earliest stage of islet infiltration, and the transition from insulitis to overt diabetes correlates with avidity maturation of this CD8⁺ T cell response (18). Collectively, these data illustrate the deleterious role of CD8⁺ T cells in T1D and indicate that the pathogenic CD8⁺ T cell response is composite and evolves over time.

Recent evidence indicates that CD8⁺ T cells express functions that extend beyond Ag-specific cell cytotoxicity. Notably, a subset of CD8⁺ T cells has recently been identified that is characterized by the expression of the transcription factor retinoic acid-related orphan receptor (ROR)γt and the production of IL-17 (19, 20). These cells have been termed Tc17 cells, despite being remarkably noncytotoxic with little expression of granzyme B, perforin, and Fas ligand (19, 21–23). The commitment of naive CD8⁺ T cells to the Tc17 lineage is driven by signals similar to those described for Th17 differentiation. Indeed, TGF-β and IL-6 drive naive CD8⁺ T cells to differentiate into IL-17–producing CD8⁺ T cells in a STAT3– and RORγt-dependent manner (19, 23, 24). The Tc17 cells also express the IL-23 receptor, the chemokine receptor CCR6, and produce IL-21, IL-22, and TNF-α (23, 25, 26). Furthermore, type I cytokines antagonize the development of both Tc17 (21) and Th17 cells (27, 28).

Tc17 cells have been identified during various inflammatory conditions that range from infection to cancer and autoimmunity (20, 22, 25, 26, 29–32). Notably, in patients, CD8⁺ T cells producing IL-17 can be identified in chronic inflammatory lesions such as psoriatic lesions (32) and multiple sclerosis plaques (30). Animal models corroborate the implication of IL-17–producing CD8⁺ T cells in a wide variety of immune conditions (22, 33–36). Adoptive transfer experiments of Tc17 cells have suggested that their ability to clear influenza virus and to protect against tumor
are related to the plasticity of the Tc17 lineage (22, 37). Indeed, the acquisition of a cytotoxic and IFN-γ-producing phenotype partly explains their capacity to provide immunity (22, 24, 37).

Tc17 cells might be implicated in the pathophysiology of T1D, as the proportion of circulating CD8+ T cells secreting IL-17 in children with new-onset T1D is increased (38) and their pathogenicity is suggested in mice (25). However, whether β cell-specific Tc17 can contribute to T1D pathogenesis remains undetermined. In this study we set out to directly study the pathogenic contribution of Tc17 in vivo using an experimental model of T1D. We demonstrate that β cell-specific Tc17 cells home to the pancreatic lymph nodes, but they fail to infiltrate the pancreas and to induce diabetes. Tissue damage to the pancreas is only initiated after transfer of Tc17 cells together with a subdiabetogenic dose of Th1 cells. We propose that CD4+ T cells initiate inflammation in the pancreas, thereby favoring the recruitment of the transferred β cell-specific CD8+ T cells. Furthermore, progression to diabetes is associated with a conversion of the noncytotoxic Tc17 cells into an IFN-γ-producing phenotype, which accumulate in the inflamed islets.

Materials and Methods

**Mice**

RIP-HA mice express the influenza virus hemagglutinin (HA) on pancreatic β-islet cells under the control of rat insulin promoter (39). CL4-TcR transgenic mice express a TcR specific for the influenza virus HA532-540 peptide/Ka complex on >95% of CD8+ T cells (40). Additionally, 6.5-TcR transgenic mice express a TcR specific for the influenza virus HAI0−119 peptide on ~30% of CD4+ T cells (41). CL4-TcR and 6.5 TcR transgenic mice were backcrossed at least 10 times onto the BALB/c background and crossed with Rag2−/− or IFN-γ−/− mice. All animal experiments were performed under specific pathogen-free conditions in accordance with the European Union guidelines and received approval from the local ethics committee.

**Cytokine measurement**

Using FACS, we fractionated CD8+ or CD4+ T cells into CD62L+CD44+ naive cells and CD62L−CD44+ memory cells (purity, >98%). Cytokine production was assessed following stimulation of 106 purified cells with beads coated with anti-CD3/anti-CD28 mAbs for 72 h by ELISA (R&D Systems, Minneapolis, MN).

**In vitro T cell differentiation**

To generate HA-specific Tc17 cells, we purified CD8+ T cells from spleen and lymph nodes of Rag2−/− CL4-TcR transgenic mice using MACS (Miltenyi Biotec) and stimulated them with 1 μg HA532-540 peptide and irradiated syngeneic spleen cells in DMEM supplemented with 10% FCS in the presence of neutralizing anti–IFN-γ (50 μg/ml, XMG1.2) and anti-IL-4 (10 μg/ml, 11B11) mAbs and the indicated cytokines (TGF-β, 3 ng/ml; IL-6, 20 ng/ml; IL-23, 3 ng/ml). For day 6, living cells were collected by Ficoll density separation and used in adoptive transfer experiments or for intracellular cytokine staining. HA-specific Tc17 cells (and Th1 cells; see Supplemental Fig. 1) were generated from Rag2−/− CL4-TcR and Rag2−/− 6.5-TcR mice, respectively, as previously described (42, 43). All cytokines were purchased from R&D Systems.

**Intracellular cytokine staining by flow cytometry**

Differentiated HA-specific CD8+ or CD4+ T cells or a single-cell suspension from spleen, pancreas-draining lymph nodes (PLNs), and pancreas were stimulated for 4 h with PMA (0.5 μg/ml; Sigma-Aldrich), ionomycin (1 μg/ml; Sigma-Aldrich), and GolgiPlug (BD Biosciences). After FcγR blockade with the 2.4G2 mAb, cells were stained with fluorescently labeled anti-Thy1.2 (53.2.1), anti-Vβ8.2 (F23.1), anti-CD8 (53.6.2), and anti-CD4 (RM4-5) mAbs and mAbs against IFN-γ (XMG1.2), IL-17 (TC11-18H10), granzyme B (16G6), and TNF-α (MP6-XT22) or IgGl PE (R3-34) and IgGl allophycocyanin (A110-1) isotype controls using a Cytofix/Cytoperm Plus kit (BD Biosciences). Labeled cells were analyzed with an LSRII flow cytomtert (BD Biosciences) using the BD FACSDiv software.

**Cell surface detection of IL-17 secretion**

Cultured Thy1.2 Tc17 cells and polyclonal Thy1.1 Tc1 cells were harvested by Ficoll density separation and washed in cold buffer. Cells were labeled either individually or in combination with a mouse IL-17 catch reagent for 5 min on ice. Prewarmed (37°C) medium was added and cells were incubated for 45 min at 37°C. Cells were then washed with cold buffer, and a biotinylated mouse anti–IL-17 detection Ab was added for 10 min on ice. Cells were washed again in cold buffer, stained with a PE-labeled anti-biotin Ab (Milenyi Biotec), and analyzed by flow cytometry.

**In vitro cytolotoxicity assay**

To generate target cells, BALB/c spleen cells were pulsed with either 1 μg HA532-540 (specific) or CW3 (control) peptide (2 h at 37°C), washed, and labeled with CFSE (Molecular Probes, Eugene, OR) for 10 min at a final concentration of 5 μM for HA-loaded cells or 0.5 μM for CW3-loaded cells. HA-pulsed and CW3-pulsed target cells at a 1:1 ratio were cocultured for 4 h at 37°C with different concentrations of effector Tc1 or Tc17 cells. Specific cytolysis was determined by FACS analysis using the following formula:

\[
\text{Number of CW3-pulsed target cells without T cells} - \text{Number of HA-pulsed target cells without T cells} \\
\times 100
\]

Adoptive transfer of T1D

After 6 d culture, live Tc17 or Tc1 cells were isolated using a Ficoll gradient and directly used for adoptive transfer experiments. Th1 cells from Rag2−/− 6.5-TcR mice were isolated similarly after 9 d culture (43). Syngenic nonirradiated 6- to 8-wk-old RIP-HA recipients received 5 × 106 HA-specific Tc1 or Tc17 cells and 106 HA-specific Th1 cells i.v., either alone or in combination in 0.1 ml PBS. Glycosuria was assessed daily for 30 d using test strips (Glukostech; Roche Diagnostics, Mannheim, Germany).

**Purification of mononuclear cells from pancreas**

Diabetic mice were sacrificed and perfused with PBS to limit contamination by blood cells. Thereafter, pancreata were injected with 3 ml HBSS containing 1.5 mg/ml collagenase P (Roche, Indianapolis, IN), transferred into tubes containing another 3 ml collagenase P solution, and incubated for 10 min at 37°C. Tissue digestion was stopped by three washes with HBSS containing 5% FCS. The digested pancreatic samples were then loaded on a Percoll gradient to collect the mononuclear cells at the interface.

**Small interfering RNA transfection**

Small interfering RNA (siRNA) targeting IL-12Rβ2 (ID nos. s68204 and s68205) and control siRNA (ID no. 4459405) were designed and synthesized by Ambion (Carlsbad, CA). A reverse transfection protocol was used (as per the manufacturer’s protocol). Briefly, siRNA was gently mixed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) and incubated for 1 h at room temperature to form transfection complexes. In vitro-differentiated Tc17 cells (0.5 × 106 cells/ml) were gently mixed with transfection complexes (50 nM final concentration) and coincubated for 6 h in Opti-MEM I medium without serum at 37°C. After 6 h, cells were washed and fresh Opti-MEM I medium was added. Twenty-four hours later, cells were washed and living cells were collected by Ficoll density separation and used in adoptive transfer experiments.

**Immunohistochemistry**

For immunohistochemistry, pancreata were snap-frozen and cryosections (5 μm thick) were stained using purified anti-CD3 (CD3-12), anti-B220 (RA3-682), anti-CD11c (N418), and anti-F4/80 (BM8) mAbs. A peroxidase-based ABC detection system (DakoCytomation, Glostrup, Denmark) using diaminobenzidine as the chromogenic substrate was used for detection.

**Statistical analysis**

The paired or unpaired t tests were used to compare the frequency of cytokine-positive T cell subsets. The log-rank test was used to compare occurrence of diabetes. A P value <0.05 was considered statistically significant. Statistical analyses were performed using the GraphPad Prism 4 program (p < 0.05, ***p < 0.01, ****p < 0.001).

**Results**

Ag-experienced CD8+ T cells can secrete IL-17

To test whether the memory CD8+ T cell compartment contains cells prone to produce IL-17, we first assessed ex vivo the proportion of circulating CD8+ T cells secreting IL-17 in chil-
duction of IL-17 by CD8+ and CD4+ T cells isolated from healthy unmanipulated BALB/c mice. Intracellular cytokine staining identified a distinct IL-17–producing CD8 subset at steady-state, as well as a larger IFN-γ–producing subset (Fig. 1A). To confirm this observation, we measured IL-17 release by CD8+ and CD4+ T cells isolated from unmanipulated BALB/c mice. To this end, we purified naive or in vivo-activated CD8 and CD4 T cells by FACS based on their CD62L and CD44 expression. Purified subpopulations were stimulated with anti-CD3/anti-CD28 mAb-coated beads and we measured IL-17A production by ELISA (Fig. 1B). We observed a clear production of IL-17A by memory CD8+ T cells, although 2- to 3-fold lower than their CD4+ counterparts. These data indicate that normal individuals harbor memory CD8 T cells able to release Th17-associated cytokines upon TcR triggering.

**TGF-β and IL-6 induce IL-17–producing CD8+ cells with reduced cytotoxicity**

Th17 differentiation from naive CD4+ T cells in mice requires the combination of IL-6 and TGF-β, as well as neutralization of IL-4 and IFN-γ (28, 44). We asked whether the same differentiating conditions would also be applicable to the CD8+ T cell compartment. We purified HA-specific CD8+ T cells from Rag2−/− mice. We purified HA-specific CD8+ T cells from Rag2−/− CL4-TcR transgenic mice, which exhibit a naïve phenotype, and cultured them for 6 d with APCs and the cognate HA512–520 peptide in presence of neutralizing anti–IFN-γ and anti–IL-4 mAbs and different cytokines (Fig. 2A). Intracellular cytokine staining on differentiated cells revealed that culture in presence of TGF-β and IL-6, with or without IL-23, induced IL-17 production by CD8+ T cells. These Tc17 cells produced little granzyme B as compared with the IL-2– and IL-12–driven Tc1 cells (Fig. 2B).

> **FIGURE 1.** Ag-experienced CD8+ T cells produce IL-17. (A) Splenocytes from unmanipulated BALB/c mice were stained for intracellular IL-17 and IFN-γ or with isotype control mAbs 4 h after PMA/ionomycin stimulation. The data are gated on CD8 (left) and CD4 (right) T cells. (B) CD8+ or CD4+ T cells from unmanipulated BALB/c mice were fractionated into CD62L+CD44− naïve cells and CD62L−CD44+ memory cells by FACS (purity, >98%). Purified populations (75 × 10^3 cells/well) were stimulated for 72 h with beads coated with anti-CD3 and anti-CD28 mAbs. IL-17A (left) and IFN-γ (right) were measured by ELISA. Data shown are from two independent experiments involving a total of four mice.

Consistent with this finding, in vitro-differentiated Tc17 cells were unable to kill Ag-loaded target cells, whereas in vitro-differentiated Tc1 cells were highly cytotoxic (Fig. 2C). Importantly, using an IL-17 secretion assay that captures the IL-17 being released at the cell surface, we established that 95–99% of Tc17 cells generated under IL-6 and TGF-β differentiating conditions secreted IL-17 (Fig. 2D). In contrast, none of the independently cultured Tc1 cells was positive using this assay (Fig. 2D). The specificity of the IL-17 signal was assessed by performing experiments using a mixture of genetically marked Tc1 and Tc17 cells. Although Tc1 exhibited an increased background staining in these conditions, only the Tc17 cells were strongly positive for IL-17 (Fig. 2D). Therefore, the culture of naive CD8 T cells in the presence of IL-6 and TGF-β generates nontoxic Tc17 cells that homogeneously produce IL-17.

**IL-17–producing CD8+ T cells do not infiltrate the pancreas**

In autoimmunity, the deleterious potential of autoreactive CD4+ T cells depends critically on their effector subset differentiation. Therefore, we wondered whether a similar rule would apply to CD8+ T cells and assessed whether Tc17 cells, expressing a TcR specific for a β-islet–derived neo-self Ag, would exhibit diabetogenic properties. To this end, we injected 5 × 10^6 HA-specific Tc1 or Tc17 cells into recipient mice expressing HA specifically in β-islet cells (the RIP-HA transgenic mice). HA-specific Tc1 cells induced rapid and lethal T1D in all RIP-HA recipients (Fig. 3). This severe autoimmune diabetes was not observed upon transfer of Tc17 cells, generated using TGF-β and IL-6 with or without IL-23, because none of the mice developed glycosuria and all mice survived (Fig. 3). Moreover, no histological signs of inflammation were observed in the pancreas at days 3 and 6 following Tc17 transfer in RIP-HA mice (data not shown). Thus, HA-specific Tc1 cells are highly pathogenic, whereas Tc17 cells fail to cause tissue infiltration in RIP-HA recipients. Because migration of autoreactive T cells to the pancreas is largely dependent on the expression of receptors for chemokines (45, 46), we assessed expression of a large panel of these receptors on diabetogenic Tc1 and Th1 cells and on nondiabetogenic Tc17 cells. We did not observe any clear differences in the expression of CCR4, CCR5, CXCR3, CXCR4, or CXCR5. Interestingly, however, Tc1 and Th1 cells harbored low levels of CCR7, whereas Tc17 cells maintained strong CCR7 expression (Supplemental Fig. 2). These data are compatible with retention of Tc17 cells in secondary lymphoid organs. Furthermore, CCR6 was clearly expressed on Tc17 cells, as previously shown for Th17 cells.

**IL-17–producing CD8+ T cells can synergize with Th1 cells to induce T1D**

To assess whether Tc17 cells can contribute to pathogenesis once inflammatory tissue damage has been initiated in the pancreas, we transferred a subdiabetogenic dose of HA-specific Th1 cells (10^6 cells) with or without Tc17 cells (5 × 10^6 cells) in RIP-HA mice. This subdiabetogenic dose of Th1 cells induced diabetes at a low frequency (Fig. 4A), permitting us to test whether the cotransfer of Tc17 cells would aggravate disease. Indeed, whereas mice injected with Tc17 cells only remained free of disease, a significant
increase in diabetes prevalence was observed upon cotransfer of Th1 (10^6) and Tc17 (5 × 10^6) cells (Fig. 4A). Disease onset in mice that received both Th1 and Tc17 occurred 5–6 d earlier than in mice that only received Th1 cells (Fig. 4A). To determine whether the exacerbation of T1D was associated with IFN-γ production, we transferred Tc17 cells from IFN-γ knockout mice in combination with Th1 cells. We observed a similar exacerbation of T1D, indicating that IFN-γ production by Tc17 is dispensable for diabetes potentiation (Fig. 4B). This suggests that the in vitro-differentiated noncytotoxic Tc17 cells can contribute, in an IFN-γ–independent manner, to the pathogenic process of autoimmune diabetes once inflammatory tissue damage has been initiated.

**IL-17–producing CD8+ T cells retain their phenotype in lymphoid organs**

It has been suggested that the Tc17 phenotype is not stable in vivo and that transferred Tc17 cells may lose their signature in Ag-bearing hosts and turn into IFN-γ–producing Tc1 cells (24). To investigate whether a similar change occurs in our model of T1D, we transferred Thy1.2 HA-specific Tc17 cells and Thy1.2 HA-specific Th1 cells, either alone or in combination, in Thy1.1 congenic RIP-HA recipients and analyzed the donor T cells 6 d after their adoptive transfer. No significant increase in the number of Thy1.2 congenic T cells was observed in the spleen and PLNs of cotransferred mice as compared with mice receiving Tc17 or Th1 cells alone (Fig. 5A, 5B). Importantly, when transferred alone, Tc17 cells retained their IL-17+IFN-γ phenotype at day 6 d after transfer, both in spleen and PLNs as assessed by intracellular cytokine staining (Fig. 5C, 5D). In the PLNs, however, following cotransfer of Th1 cells, the Tc17 cells exhibited a decrease in the proportion of IL-17–producing cells and a concomitant increase in the proportion of IFN-γ+ cells (Fig. 5B). The transferred Th1 cells maintained their original IFN-γ–producing phenotype in both spleen and PLNs, regardless of the presence or absence of Tc17 cells.

**IL-17–producing CD8+ T cells acquire a type 1 phenotype within the pancreas**

Given the potential of Tc17 to aggravate Th1-mediated pancreatic inflammation and T1D development, we asked whether Tc17 cells would alter quantitatively or qualitatively the inflammatory infiltration of the pancreas. Mononuclear cells were purified from the pancreas at day 6 after transfer and analyzed by FACS. Consistent with the histological data, transfer of Tc17 cells alone into RIP-HA recipient mice did not elicit mononuclear cell infiltration. Transfer of Tc17 cells in combination with low doses of Th1 cells non-significantly increased the number of pancreas-infiltrating B cells,

**FIGURE 2.** TGF-β and IL-6 differentiate naive CD8+ T cells into Tc17 cells. Naïve HA-specific CD8+ T cells from Rag2−/− CL4-TcR transgenic mice were cultured for 6 d with APCs and cognate Ag in presence of neutralizing anti–IFN-γ and anti–IL-4 Abs and the specified cytokine or combination of cytokines. At day 6 intracellular staining was performed. The analysis gated on CD8+ T cells shows (A) IL-17 and IFN-γ staining, and (B) granzyme B and TNF-α staining. The data shown are representative of five independent experiments. (C) In vitro assessment of the Ag-specific lysis of target cells by in vitro-differentiated Tc17 cells or by conventional cytotoxic Tc1 cells. Data are from one representative of three independent experiments providing similar results. (D) Surface detection of IL-17 secretion by FACS was performed on polyclonal Thy1.1+ Tc1 cells and Thy1.2+ CL4-TcR Tc17 cells either alone or mixed in the same cell suspension. Data are from one of three independent experiments giving consistent results.
macrophages, and dendritic cells as compared with the group receiving Th1 cells alone (Fig. 6). There was no clear difference in the distribution of infiltrating immune cell subsets between the Th1 and Th1 plus Tc17 groups. However, at day 6, the transfer of low-dose diabetogenic Th1 cells into RIP-HA mice caused a pronounced peri-insulitis with little destruction of the β-islet cells, whereas cotransfer of Th1 and Tc17 cells led to destruction of the structural integrity of the islets (Supplemental Fig. 3).

To assess the phenotype of the pathogenic pancreas-infiltrating T cells, we analyzed by FACS the transferred Thy1.2+ T cells in Thy1.1-RIP-HA recipient mice at the onset of diabetes (Fig. 7A). Ex vivo analysis of pancreas-infiltrating T cells after 4 h PMA/ionomycin stimulation revealed that Th1 cells maintained their cytokine production phenotype (Fig. 7B). In contrast, the vast majority of the Tc17 cells had lost their original IL-17 signature and converted into an IFN-γ-producing phenotype (Fig. 7B, 7C), sharply differing from what was observed in the lymphoid organs (p = 0.0003 for comparison of the percentage of IL-17+CD8 T cells between PLNs and pancreas; p = 0.0009 for comparison of the percentage of IFN-γ+CD8 T cells between PLNs and pancreas; paired Student t test). These data indicate that in the inflamed pancreas the transferred HA-specific Tc17 cells switched their functional properties.

**FIGURE 3.** Tc17 cells are nonpathogenic upon adoptive transfer into RIP-HA mice. HA-specific Tc17 or Th1 cells (5 × 10^6) were adoptively transferred into immunocompetent RIP-HA mice and recipients were followed for glycosuria for up to 30 d. Data are pooled from three independent experiments. Diabetes development differed very significantly between Th1-injected and Tc17-injected mice (p < 0.0001 by log-rank test).

**FIGURE 4.** β cell-specific Tc17 cells aggravate autoimmune T1D. The pathogenic potential of β cell-specific Tc17 (5 × 10^6) cells was assessed in a suboptimal model of autoimmune diabetes induced by transfer of 10^6 HA-specific Th1 cells into RIP-HA recipient mice. (A) The prevalence of diabetes in the Th1/Tc17 cotransfer group was higher than in the Th1 alone group (***p < 0.0001 by log-rank test). Pooled data from five independent experiments are shown. In two additional experiments the Th1 cells induced rapid diabetes in all the recipients and, therefore, these experiments were excluded from the analysis. (B) Similar experiments were performed using HA-specific Tc17 cells originating from IFN-γ-deficient CL4-TcR transgenic donor mice. Data from two independent experiments are shown.

**FIGURE 5.** Tc17 cells retain their IL-17–producing phenotype in lymphoid organs after adoptive transfer into congenic Thy1.1 RIP-HA mice. HA-specific Tc17 (Tc17) or 10^6 HA-specific Th1 (Th1) Thy1.2+ cells were transferred alone or in combination into Thy1.1 congenic RIP-HA mice. At day 6, the numbers of transferred Tc17 (filled bars) and Th1 (open bars) T cells were assessed in spleen (A) and PLNs (B) by FACS. The percentage of IL-17+IFN-γ* (top panel) and IFN-γ*IL-17* (bottom panel) T cells was assessed by intracellular cytokine staining on gated Thy1.2+CD8* (filled bars) or Thy1.2+CD4* (open bars) from spleen (C) and PLNs (D). Data are pooled from five independent experiments; the number of mice analyzed individually is specified on the figure. *p < 0.05, **p < 0.01.
HA512–520 peptides in IFA. Enumeration and ex vivo FACS detectable.

ND, Not dependent experiments, each involving a pool of three mice per group. No and CD11c (N418) for dendritic cells. Data shown are from three independent experiments, each involving a pool of three mice per group. No significant differences were observed between the group that received Th1 cells alone and the group that received both Th1 and Tc17 cells.

To assess their respective phenotypes, we analyzed the donor Thy1.2+ CD8+ T cells by intracellular cytokine staining. The congenic Tc17 cells demonstrated a similar intracellular IFN-γ and IL-17 profile irrespective of whether the Tc17 cells had been transferred alone or in the presence of Th1 cells (Fig. 8). These data suggest that the concomitant activation of HA-specific Tc17 and Th1 cells in the draining lymph nodes is not sufficient to elicit IFN-γ production by Tc17 cells.

**Inhibition of IL-12 receptor expression on HA-specific Tc17 cells prevents their conversion to a type 1 phenotype and reduces their pathogenicity**

Because Tc17 cells expressed the IL-12 receptor on their cell surface (Fig. 9A), we assessed the effect of IL-12 on the Tc17 cells both in vitro and in vivo. In vitro addition of IL-12 significantly converted Tc17 into IFN-γ-producing CD8+ T cells (Fig. 9B). Next, we tested whether this IL-12–mediated conversion is implicated in the exacerbation of diabetes observed when Tc17 cells are cotransferred with low-dose diabetogenic Th1 cells into RIP-HA mice. To this end, we used a siRNA approach to knock down the expression of IL-12Rβ2 on differentiated Tc17 cells before their adoptive transfer into RIP-HA mice. We established an efficient protocol permitting a robust inhibition of IL-12Rβ2 surface expression (91 ± 2.9% at 72 h). We then transfected the IL-12Rβ2 siRNA-treated or control siRNA-treated Tc17 cells in combination with Th1 cells in Thy1.1 RIP-HA mice. Intracellular cytokine staining of pancreatic lymph node cells 6 d after transfer revealed that treatment with IL-12Rβ2 siRNA caused a significant reduction in the proportion of Tc17 cells that converted to IFN-γ+IL-17− CD8+ T cells as compared with Tc17 cells treated with control siRNA (Fig. 9C). We further compared Tc17 cells treated with IL-12Rβ2 siRNA with Tc17 cells treated with control siRNA for their ability to aggravate the clinical disease induced by the cotransfer of low-dose Th1 cells in Thy1.1 RIP-HA mice. Mice that received Th1 cells alone with control siRNA-treated Tc17 cells developed severe diabetes with high prevalence (80%, n = 10). Strikingly, invalidating the expression of IL-12 receptor expression by...
and reproducibly generates noncytotoxic IL-17–producing CD8+ T cells when starting with unfractionated CD8+ T cells, which is a naturally arising subset of CD8+ T cells. Naive CD8+ T cells differentiated in vitro in the presence of IL-23 (25). As noted above, the presence of memory T cells in the total CD8+ T cell cultures might explain this difference.

Cytotoxic CD8+ T cells are pivotal effectors in T1D that are involved in disease induction and progression (3, 11). Our data indicate that noncytotoxic Tc17 cells are unlikely to individually drive disease initiation. We show that after transfer, the noncytotoxic Tc17 cells accumulate in the PLNs but fail to infiltrate the pancreas. In contrast, the transfer of CD8+ Tc1 T cells or CD4+ Th1 T cells readily induces severe diabetes in the RIP-HA model (43, 49). This differential migration to the pancreas is associated with differences in chemokine receptor expression on nonpathogenic Tc17 cells versus diabetogenic Tc1 cells. Indeed, Tc17 cells express higher levels of CCR7 than do Tc1 cells. CCR7 is the major chemokine receptor involved in T cell homing to secondary lymphoid organs (50). Therefore, it is tempting to suggest that CCR7 expression by Tc17 cells promotes their retention into the PLNs and, as a result, prevents the induction of the autoimmune T1D.

We demonstrate that despite their incapacity to induce diabetes, noncytotoxic Tc17 cells can become detrimental once inflammation in the pancreas was initiated. When subclinical disease was induced with low doses of diabetogenic HA-specific CD4+ T cells, the transfer of Tc17 cells sustained disease progression, leading to the destruction of the β-islet cells and resulting in hyperglycemia and ultimately death. Under these conditions, the HA-specific CD8+ T cells infiltrated the pancreatic islets, indicating that local inflammation permits the recruitment of Tc17 cells into the pancreas. This change in Tc17 cell behavior is likely to drive the aggravation of diabetes, as no functional or numerical alterations were observed among the diabetogenic Th1 CD4+ T cells in the PLNs and within the infamed β-islet cells. However, most transferred CD8+ T cells had converted to an IFN-γ-producing phenotype, losing even the signature IL-17A expression. As >95% of the HA-specific CD8+ T cells cultured in the presence of IL-6 and TGF-β secrete IL-17A, it seems unlikely that the IFN-γ–producing CD8+ T cells present in the pancreas are derived from contaminating CD8+ T cells that had not committed to the Tc17 lineage. Consequently, our data demonstrate that noncytotoxic Tc17 cells retain a remarkable level of plasticity, permitting their redifferentiation toward IFN-γ–producing Tc1 cells during chronic inflammation in the pancreas. Although IFN-γ is a marker of Tc17 redifferentiation, this cytokine was not involved in the aggravation of autoimmune diabetes. Indeed, generating Tc17 cells from IFN-γ–deficient mice did not abrogate their pathogenic potential in the Th1 cotransfer model system. It is therefore likely that the in vivo–converted Tc17 cells promoted diabetes exacerbation through secretion of proinflammatory cytokines other than

![FIGURE 8. HA-specific Th1 cells are not sufficient to promote the conversion of HA-specific Tc17 cells in vivo. Thy1.2+ HA-specific Tc17 cells (5 x 10^6) were transferred either alone (Tc17) or in combination with 1 x 10^6 Thy1.2+ HA-specific Th1 cells (Th1+Tc17) into Thy1.1 congenic BALB/c mice followed by subcutaneous immunization with IFA and HA peptides. Six days after immunization, the draining lymph nodes were collected and single-cell suspensions were analyzed by FACS. The percentages of IL-17+ (filled bars), IFN-γ+ (open bars), and IL-17+IFN-γ+ (checked bars) cells were assessed by intracellular cytokine staining on gated Thy1.2+CD8+ T cells. The data are the means ± SEM of three experiments.](http://www.jimmunol.org/)

Discussion

In the present study, we assessed whether autoreactive CD8+ T cells producing IL-17 can contribute to the destruction of pancreatic β cells in an animal model of T1D. We demonstrate that Tc17 cells can aggravate autoimmune diabetes induced by Th1 cells, even though they fail to induce disease alone. Our data therefore extend the synergy between diabetogenic CD4+ and CD8+ T cells to the Tc17 subset, which is increased in the blood of patients with recent-onset T1D (38).

Our data indicate that Tc17 cells reside within the Ag-experienced CD62L<sup>lo</sup>CD44<sup>hi</sup> T cell pool of unmanipulated BALB/c mice. This further demonstrates that Tc17 cells are a naturally arising subset of CD8+ T cells. Naive CD8+ T cells isolated from Rag2<sup>−/−</sup> TcR transgenic mice could be differentiated into IL-17–producing cells upon Ag stimulation in the presence of IL-6 and TGF-β and concomitant neutralization of IL-4 and IFN-γ. This protocol has been applied in other studies and reproducibly generates noncytotoxic IL-17–producing CD8+ T cells (22, 23). However, when starting with total CD8+ T cells from TcR transgenic mice on a conventional Rag2<sup>−/−</sup> background, we generated IL-17–producing CD8+ T cells that also secrete IFN-γ and express granzyme B (data not shown). This is consistent with previous reports of the induction of cytotoxic IFN-γ<sup>+</sup> Tc17 cells when starting with unfractionated CD8+ T cells, which is attributed to the presence of memory T cells (22, 25).

IL-6 and TGF-β are sufficient to drive naive CD8+ T cells to the Tc17 lineage. This commitment is supported by the addition of IL-23 that augments RORγt expression and IL-17 production by Tc17 cells (21). Similar to their CD4+ counterparts (47), this could indicate a role for IL-23 in the terminal differentiation of CD8+ T cells. However, the impact of IL-23 might be less stringent for CD8+ T cells. In the absence of IL-23, the differentiation of CD4+ T cells generates Th17 cells that produce IL-10 and inhibit CNS inflammation (48). In contrast, when differentiating naive CD8+ T cells with TGF-β and IL-6, the ensuing Tc17 cells produce little IL-10 protein (Ref. 24 and A. Saxena and R. Liblau, unpublished observations) or transcripts (22). Furthermore, our cotransfer of Tc17 cells with diabetogenic CD4+ Th1 cells did not reveal any evidence for an in vivo immunoregulatory capacity of the IL-6– and TGF-β–differentiated Tc17 cells. In our hands, the addition of IL-23 in vitro did not improve the differentiation of naive CD8+ T cells into Tc17 cells. Furthermore, the adoptive transfer into RIP-HA mice of Tc17 cells cultured in the presence of IL-23 resulted in their accumulation in the PLNs without insulin or diabetes, thereby reproducing the phenotype of Tc17 cells cultured in the absence of IL-23. As such, our observations with noncytotoxic Tc17 cells differ from those obtained with cytotoxic Tc17 cells that become pathogenic when total CD8+ T cells are differentiated in vitro in the presence of IL-23 (25). As noted above, the presence of memory T cells in the total CD8+ T cell cultures might explain this difference.
IFN-γ or through direct cytotoxicity on β-islet cells. Similar observations were made regarding the antiviral activity of converted Tc17 cells in a model of vaccinia virus infection (33). Additionally, production of IFN-γ by the cotransferred Th1 cells can compensate for the lack of IFN-γ production by the IFN-γ−/− CD8+ T cells.

Multiple mechanisms may explain the conversion of noncytotoxic Tc17 cells toward the Tc1 lineage. It has been reported that a second round of Ag stimulation in vitro generates IL-17–producing CD8+ T cells that coexpress IFN-γ, possess lytic granules, and are functionally cytotoxic (33). We confirmed these observations, following in vitro Ag restimulation of differentiated noncytotoxic Tc17 cells, even in the continued presence of IL-6 and TGF-β stimulation (data not shown). Although these observations may suggest instability of the Tc17 lineage, we found that transferring the noncytotoxic Tc17 cells into RIP-HA mice resulted in the persistence of the Tc17 program even after Ag encounter in the PLNs. These data indicate that secondary restimulation by cognate Ag in vivo is insufficient to convert Tc17 cells, implying that additional factors are required to promote the acquisition of a cytotoxic IFN-γ–expressing phenotype. These factors are not directly derived from CD4+ T cells, as the concomitant activation of Th1 cells fails to convert the Tc17 cells both in vitro and in vivo. IL-12 has been implicated in the conversion of Tc17 cells into Tc1 CD8+ T cells (33, 51), indicating that cytokines that endow type 1 differentiation are potentially implicated. The expression of the IL-12Rβ2 on our Tc17 cells is in support of this scenario. To test this experimentally, we used an siRNA knockdown approach and revealed that IL-12 is implicated in vivo in instructing the functional changes on the injected Tc17 population. This phenotype is reminiscent of in vitro–differentiated Th17 cells (52, 53) that retain sensitivity to inflammatory stimuli, permitting the sequential differentiation into IFN-γ–producing Th17 cells after exposure to IL-12. Th1 fate-reporter mice have revealed that prior Th17 differentiation is essential in the generation of pathogenic IFN-γ+CD4+ T cells in vivo during experimental autoimmune encephalomyelitis (54). Retaining lineage plasticity is therefore an intrinsic feature of type 17 cells permitting continued adaptation to their local inflammatory environment. This flexibility augments the pathogenic potential of Tc17 cells during organ-specific autoimmune responses, including T1D.

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**FIGURE 9.** IL-12Rβ2 expression on HA-specific Tc17 contributes to their conversion in vivo and to the exacerbation of T1D in RIP-HA recipient mice. (A) IL-12Rβ2 expression was assessed by FACS on HA-specific Tc17 (empty curve). Staining with an isotype control mAb served as a control (gray curve). (B) HA-specific Tc17 cells were cultured with or without recombinant IL-12 (1 μg/ml) in vitro. After 6 d, the percentage of IL-17+ (filled bars), IFN-γ+ (open bars), and IL-17+IFN-γ+ (checked bars) cells was assessed by intracellular cytokine staining on gated live Thy1.2+CD8+ T cells. The data represent the means ± SEM of four independent experiments. A paired Student t test was used. (C and D) Thy1.2+ HA-specific Tc17 cells were treated in vitro with IL-12Rβ2 or control siRNA. siRNA-treated Tc17 cells (5 × 10⁶) were transferred 24 h later in combination with 10⁶ Thy1.2+ HA-specific Th1 cells into Thy1.1 RIP-HA mice. (C) Six days after transfer, the percentage of IL-17+ (black bars), IFN-γ+ (white bars), and IL-17+IFN-γ+ (checked bars) cells was assessed by intracellular cytokine staining on gated Thy1.2+CD8+ PLN cells. Data present means ± SEM of two independent experiments. An unpaired Student t test was used for statistical comparison. (D) The prevalence of diabetes in the control siRNA-treated Tc17 group was significantly higher than in the IL-12Rβ2-siRNA treated Tc17 group (p < 0.016 by log-rank test). Pooled data from two independent experiments are shown. *p < 0.05, **p < 0.01.
Disclosures
The authors have no financial conflicts of interest.

References