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Relative Diabetogenic Properties of Islet-Specific Tc1 and Tc2 Cells in Immunocompetent Hosts[1]

Csaba Vizler,* Nadège Bercovici,* Agnès Heurtier,* Nathalie Pardigon,[†] Karine Goude,* Karine Bailly,* Christophe Combadière,[‡] and Roland S. Liblau[2]*

CD8^{+} T cells are important effectors, as well as regulators, of organ-specific autoimmunity. Compared with Tc1-type CD8^{+} cells, Tc2 cells have impaired anti-viral and anti-tumor effector functions, although no data are yet available on their pathogenic role in autoimmunity. Our aim was to explore the role of autoreactive Tc1 and Tc2 cells in autoimmune diabetes. We set up an adoptive transfer model in which the recipients were transgenic mice expressing influenza virus hemagglutinin (HA) specifically in their pancreatic β islet cells (rat insulin promoter-HA mice) and islet-specific Tc1 and Tc2 cells were generated in vitro from HA-specific CD8^{+} cells of TCR transgenic mice (CL4-TCR mice). One million Tc1 cells, differentiated in vitro in the presence of IL-12, transferred diabetes in 100% of nonirradiated adult rat insulin promoter-HA recipients; the 50% diabetogenic dose was 5×10^5. Highly polarized Tc2 cells generated in the presence of IL-4, IL-10, and anti-IFN-γ mAb had a relatively low, but definite, diabetogenic potential. Thus, 5×10^6 Tc2 cells caused diabetes in 6 of 18 recipients, while the same dose of naive CD8^{+} cells did not cause diabetes. Looking for the cause of the different diabetogenic potential of Tc1 and Tc2 cells, we found that Tc2 cells are at least as cytotoxic as Tc1 cells but their accumulation in the pancreas is slower, a possible consequence of differential chemokine receptor expression. The diabetogenicity of autoreactive Tc2 cells, most likely caused by their cytotoxic activity, precludes their therapeutic use as regulators of autoimmunity. *The Journal of Immunology,* 2000, 165: 6314–6321.

C ytotoxic T cells were originally known for their capacity to secrete type 1 cytokines, such as IFN-γ or TNF-α (1). However, not long after the description of Th1 and Th2-like Th cells, an analogous functional division of the CD8^{+} T cell subset was also revealed (2–4), and the Tc1/Tc2 terminology was later introduced by Sad et al. (5). Two main types of Tc2 cells have been described, apparently depending on the conditions of in vitro T cell differentiation. In one of the early reports, CD8^{+} T cells stimulated with PMA and ionomycin in the presence of IL-4 were found to produce IL-4, IL-5, and IL-10; they lost cytotoxic activity and CD8 expression, and provided cognate help to B cells (6). Noncytotoxic CD8^{+} T cell clones, which expressed CD40 ligand and helped B cells, were also described by Cronin et al. (7). However, in other models, Tc2 cells of an apparently different type were described. These Tc2 cells exerted perforin-mediated cytotoxicity; expressed the CD8 α- and β-chains; secreted IL-4, IL-5, and IL-10; and provided bystander, but not cognate, help to B cells. The cytokine profile of Tc2 cells generated by stimulation with alloantigen, H-Y Ag, or specific peptide was found to be stable both in vitro (5) and in vivo (8). Functional in vivo assays demonstrated that Tc2 cells are able to trigger a delayed-type hy-persensitivity reaction and have strong graft-vs-lymphoma activity (9–11). Interestingly, upon adoptive transfer, in vitro-polarized influenza virus hemagglutinin (HA)[3]-specific Tc2 cells were found to be inefficient in clearing influenza virus infection (12). In another model, in vitro-generated tumor-reactive Tc2 cells were less efficient in eliminating lung tumors than Tc1 cells (13).

Tc2-like CD8^{+} cells have been detected in diverse pathological conditions in humans as well as in mice. CD8^{+} T cells producing type 2 cytokines were found in the respiratory tract, mediastinal lymph nodes, and spleen of mice infected with influenza virus (14–16). Furthermore, Tc2 cells seem to be involved in human pathology, because IL-4-producing CD8^{+} cells have been detected in patients with AIDS and Job-like syndrome and also in lepromatous leprosy (17–19). A functional role for IL-4- and IL-5-producing T cells has been suggested in experimental bronchial hyperreactivity; development of IL-5-producing CD8^{+} cells was detected in lymphocytic choriomeningitis virus (LCMV) TCR transgenic mice undergoing a Th2 immune response against OVA emulsified in incomplete Freund adjuvant immunized concomitantly with LCMV peptide via the airways (20). These Tc2 cells were found to trigger a lung eosinophilic cell infiltration upon challenge with the viral Ag.

However, no data are available on the role of Tc1 and Tc2 cells in autoimmunity. Like CD4^{+} cells, CD8^{+} cells are also known to have an important role in both the induction and the down-regulation phase of organ-specific autoimmune diseases, such as insulin-dependent diabetes mellitus (IDDM) and experimental allergic encephalomyelitis (EAE; Ref. 21–26). According to most of the experimental data available, autoreactive Th1 cells are effectors, whereas Th2 cells are regulators, of the autoimmune tissue damage

[3] Abbreviations used in this paper: HA, hemagglutinin; LCMV, lymphocytic choriomeningitis virus; IDDM, insulin-dependent diabetes mellitus; EAE, experimental allergic encephalomyelitis; RIP, rat insulin promoter; NOD, nonobese diabetic mouse; IP-10, IFN-inducible protein 10; MIP, macrophage inflammatory protein; SLC, secondary lymphoid tissue chemokine; CFSE, 5,6-carboxy-succinimidyl-fluorescein-ester.

in organ-specific autoimmunity (27, 28), so an analogous functional division of Tc1-Tc2 cells might also apply. Furthermore, CD8$^+$ cells were found to be mediators of oral tolerance to autoantigens (29). Whether these TGF-β-producing CD8$^+$ cells are related to the Tc2 cells described in other models has not been determined. However, investigation of the potential Ag-specific immunoregulatory activity of Tc2 cells is of theoretical, as well as practical, interest; if autoreactive Tc2 cells were found to be devoid of diabetogenic potential they could be tested as regulators of the destructive autoimmune response mounted by Tc1 and Th1 cells.

The aim of this study was to explore the role of autoreactive Tc1 and Tc2 cells in an organ-specific autoimmune disease, IDDM, employing an adoptive transfer model based on the use of HA-specific T cells from TCR transgenic mice (CL4-TCR mice) and recipients rat insulin promoter (RIP)-HA transgenic mice expressing HA in the pancreatic β cells (30, 31). This approach enabled us to generate high purity autoreactive Tc1 and Tc2 populations from naive CD8$^+$ cells, providing the means to compare directly the pathogenic potential of Tc1 and Tc2 effectors expressing the same TCR. Tc2 cells produced small amounts of IFN-γ and high but variable amounts of IL-4 and IL-10. We found that Tc1 and Tc2 cells had similar levels of cytotoxic activity. However, pancreas-specific homing and proliferative capacity of Tc2 cells was lower than that of Tc1 cells. Transferred to adult, nonirradiated RIP-HA recipients, Tc1 cells were highly diabetogenic, while Tc2 cells had a definite, but reduced, diabetogenic potential.

Materials and Methods

Mice and cell transfer experiments

The CL4-TCR transgenic mouse line (30) expresses an H-2K^d-restricted TCR (Vα10; Vβ8.2) against the influenza virus HA transmembrane peptide amino acids 512–520 (IYSTVASSL). RIP-HA mice, used as recipients in the adoptive transfer experiments, express HA specifically in the pancreatic β cells (31). The CL4-TCR and RIP-HA transgenic mice, both backcrossed 6–8 times with BALB/c mice, were bred in a conventional animal facility, while normal BALB/c mice were purchased from IFFA-Credo (St-Germain-sur-l'Arbresle, France).

In the adoptive transfer experiments, nonirradiated, 6- to 8-wk-old RIP-HA hemizygote recipients were injected i.v. with different doses of naive or preactivated CL4 CD8$^+$ T cells in 0.2 ml PBS. Glucosuria was tested daily using Multistix 8 SG test strips (Bayer Diagnostics, Puteaux, France). Mice that had glucosuria for 2 consecutive days were considered to be diabetic; control measurements confirmed that these mice had more than 3.5 g/L glucose in their blood. Severely diabetic mice were euthanized.

Cell culture

CL4 spleen and lymph node single-cell suspensions were incubated with anti-CD8α mAb (CT-CD8a; Caltag, Burlingame, CA) and positive selection of CD8$^+$ cells was performed by magnetic-activated cell sorting magnetic beads and separation columns (Miltenyi Biotech, Bergish Gladbach, Germany). The efficacy of the selection was controlled by flow cytometry; the resulting cell suspension typically contained more than 80% Vβ8$^+$, CD8$^+$, CD62L^{high}, CD25$^-$ lymphocytes and less than 2% contaminating CD4$^+$ cells.

To obtain Tc1 cells, 5×10^5 purified CL4 CD8$^+$ T cells were stimulated with 5×10^6 irradiated syngeneic spleen cells in 2 ml complete DMEM supplemented with 10% FCS (Life Technologies, Paisley, Scotland) containing 1 μM HA peptide, 1 ng/ml IL-2, and 20 ng/ml IL-12. On day 3, the cultures were split into four aliquots and fed with fresh medium containing IL-2. For the generation of Tc2 cells, 5×10^5 CL4 CD8$^+$ T cells were stimulated with 5×10^6 spleen cells in the presence of 1 μM HA peptide, 1 ng/ml IL-2, 80 ng/ml IL-4, 20 ng/ml IL-10, and 10 μg/ml anti-IFN-γ mAb. On day 3, the cultures were split into two to four aliquots and fed with fresh medium. On day 6, cells were harvested and living cells were collected by Ficoll density separation and washed with tissue culture medium at least three times.

The polarized Tc1 and Tc2 cells were then used in adoptive transfer experiments. Their cytokine production was assessed in parallel by restim-

ulating 10^6 cells with 5×10^6 irradiated BALB/c spleen cells in 2 ml complete DMEM in the presence of 1 μM HA peptide. Splenocytes and pancreatic lymph nodes were removed from RIP-HA mice 4 days after adoptive transfer of either 20×10^6 Tc1 or Tc2 cells. Single-cell suspensions were incubated (8×10^5 cells/well) in the presence or absence of 1 μM HA peptide. Supernatants were collected after 48 h incubation, aliquoted, and stored at $-20°$C until the cytokine determination by ELISA test was performed.

Cytokines, ELISA, and flow cytometry

Recombinant mouse IL-2, IL-4, and IL-10 were purchased from R&D Systems (Minneapolis, MN). Recombinant mouse IL-12 was a gift from Dr. Stanley Wolf, Genetics Institute (Cambridge, MA). The neutralizing anti-IFN-γ mAb (clone XMG1.2) used in the Tc2 culture was purchased from PharMingen (San Diego, CA).

Cytokines were determined by sandwich ELISA using Cytoset matched Ab pairs and recombinant cytokine standards (Biosource International, Camarillo, CA). IFN-γ: polyclonal rabbit anti-mouse IFN-γ capture Ab and biotinylated anti-IFN-γ mAb (clone DB-1); IL-10: anti-IL-10 capture mAb (clone JES5-SXC1) and biotinylated anti-IL-10 mAb (clone JES5-2A5); IL-4: anti-IL-4 capture mAb (clone BVD4-1D11) and biotinylated anti-IL-4 mAb (clone BVD4-24G2). The cytokine ELISA were performed according to the instructions of the supplier using Costar (Cambridge, MA) enxyme immunoassay plates (No. 3590) and Biosource streptavidin-HRP conjugate.

The anti-CD8 (clone CT-CD8a) and anti-CD4 (clone CT-CD4) mAbs were purchased from Caltag Laboratories (Burlingame, CA) while the anti-B220 (clone RA3-6B2), anti-Vβ8 (clone F23.1) and anti-CD25 (clone 7D4) mAbs were obtained from PharMingen. Intracellular IFN-γ was detected by FITC-labeled anti-IFN-γ mAb (clone XMG1.2) using FITC-labeled clone R3-34 as an isotype control mAb (PharMingen). Tc1 and Tc2 cells were previously stimulated for 5 h with 1 μg/ml PMA (Sigma, St. Louis, MO) and 1 μg/ml ionomycin (Sigma); after the first hour, 3 μM monensin (Sigma) was added. Fixation and membrane permeabilization were performed using 2% paraformaldehyde and 0.1% saponin, respectively.

FACS analysis was performed by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), and the data were analyzed using CellQuest software (Becton Dickinson).

Cytotoxicity assay

The cytotoxic effect of Tc1 and Tc2 cells on P815 mastocytoma cells (H-2^d) pulsed with 1 μM HA peptide was assessed as previously described (32). To assess the contribution of the perforin pathway in the cytotoxic activity, the cytotoxicity test was performed in the presence or absence of 4 mM EGTA and 3 mM MgCl$_2$.

Tracing of adoptively transferred CD8$^+$ cells

In some experiments, Tc1 or Tc2 cells labeled with an intracellular fluorescent dye, 5,6-carboxy-succinimidyl-fluorescein-ester (CFSE; Molecular Probes, Eugene, Oregon), were used for adoptive transfer experiments. For the labeling, the cell suspension (5×10^7 cells/ml) was incubated for 10 min with 5 mM CFSE at 37°C, then the reaction was stopped by adding cold medium and the cells were washed three times. One or 4 days after the cell transfer, the recipients were sacrificed. Spleen and pancreas single cell suspensions were prepared using a wire mesh and a syringe plunger and the percentage of the CFSE-labeled cells in the lymphocyte forward light scatter-side light scatter gate and the mean fluorescence level of positive cells were determined by FACS analysis.

RNase protection assay

Total RNA was prepared from 10^7 Tc1 or Tc2 cells, at day 6 after initiation of the culture under polarizing conditions, either before or after 12 h of in vitro restimulation with 10 μg/ml of plate-bound anti-CD3 mAb (clone 2C11; PharMingen) using the RNA Plus extraction kit (Quantum Biotechnologies, Montréal, Canada). Ribonuclease protection assays were performed with the RiboQuant kit (PharMingen) using the mCR-5 and the mCR-6 radiolabeled probe sets (PharMingen) according to the manufacturer's recommended protocol. The dried gel was placed on film and developed at $-70°$C. Quantitation of the radioactivity on the gel was performed using a high-resolution β-imager (Biospace, Paris, France) allowing us to obtain a direct measurement of numeric images obtained from actual counting of β-particles emitted. Data were normalized against the L32 and GAPDH housekeeping genes.

Intracellular [Ca^{2+}] measurements

Cells (10^7/ml) were incubated in HBSS with Ca^{2+} and Mg^{2+} (pH 7.4), containing 2.5 μM fura 2-AM (Molecular Probes) for 60 min at 37°C in the dark. Cells were washed twice with HBSS, and resuspended at 2×10^6/ml. Two milliliters were placed in a continuously stirred cuvette at 37°C in a fluorometer (Perkin-Elmer LS-5B, Bois d'Arcy, France). Fluorescence was monitored at $\lambda_{ex1} = 340$ nm, $\lambda_{ex2} = 380$ nm, and $\lambda_{em} = 510$ nm. Data were recorded every 200 ms. The following recombinant chemokines were tested at 50 nM: RANTES, macrophage inflammatory protein (MIP)-1β, eotaxin, secondary lymphoid tissue chemokine (SLC), I309, fractalkine, IL-8, and IFN-inducible protein 10 (IP-10) (all from PeproTech, Rocky Hill, NJ). Calcium concentrations were calculated using the following equation as described (33): $[Ca^{2+}]_i = 225 \times (R - R_{min})/(R_{max} - R) \times$ Sf380/Sb380. R_{max} and R_{min} were evaluated in 1 mM Ca^{2+} containing medium by lysing the cells with 0.5% Triton X-100 for R_{max} ($R_{max} = 9.4$), followed by the addition of 3 mM EGTA for R_{min} ($R_{min} = 1.5$).

Results

Generation of Tc1 and Tc2 cells

The extent of Tc1- or Tc2-type cell differentiation was controlled by restimulating the purified cells in vitro and measuring the production of prototypic type 1 (IFN-γ) and type 2 (IL-4 and IL-10) cytokines. Tc1 cells harvested after 6 days of culture in the presence of IL-12 and restimulated with APC and peptide were invariably found to produce large amounts of IFN-γ and no detectable IL-4 or IL-10 (Fig. 1). In our initial experiments, we found that IL-4, IL-10, and anti-IFN-γ mAb synergize in inducing Tc2-type differentiation. The presence of IL-4 alone in the culture resulted in a mixed cytokine profile, but the addition of IL-10 to the culture elevated the production of both IL-4 and IL-10, while anti-IFN-γ mAb decreased the production of IFN-γ (Fig. 1). Because the most complete Tc2-type polarization was obtained in the presence of IL-2, IL-4, IL-10, and anti-IFN-γ mAb, this protocol was chosen for the adoptive transfer experiments. The individual Tc2 cell populations used in the add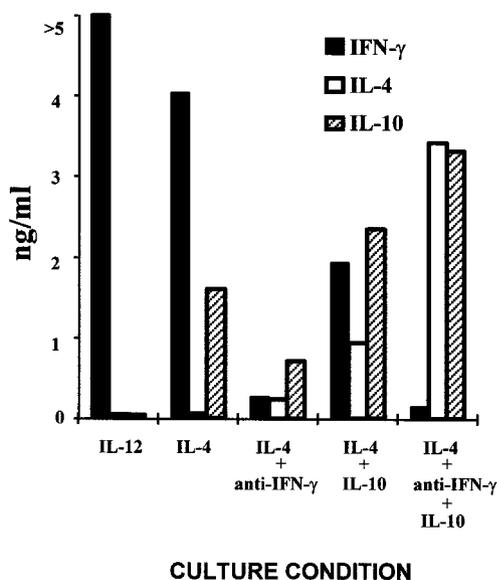itional experiments produced variable quantities of the three cytokines measured. However, the results of the cell transfer experiments shown in Fig. 5 were obtained using highly polarized Tc2 cells, which, according to our arbitrarily but a priori established criteria, produced at least a 2-fold higher level of both IL-4 and IL-10 than IFN-γ. Six of 14 independent Tc2 populations fell into this category; the supernatant of these cells contained 169 ± 61 pg/ml IFN-γ, 1435 ± 409 pg/ml IL-4, and 5868 ± 1943 pg/ml IL-10 (mean $\pm$ SEM).

Both Tc1 and Tc2 cell cultures resulted in substantial cell proliferation in vitro in response to Ag. However, the yield of Tc1 cell was about three times greater than that of Tc2 cells. After the 6-day primary culture in five independent experiments, the recovery of Tc1 cells was 16.9 ± 1.8-fold (mean $\pm$ SEM) the initial cell input, whereas it was only 6.6 ± 1.0-fold for Tc2 cells generated in parallel.

Both Tc1 and Tc2 cells collected on day 6 stained homogeneously for the transgenic Vβ8.2 TCR chain and contamination with CD4$^+$ cells was less than 1%. Cells were highly activated in both cases and showed only slight differences in the CD25 and CD62 ligand expression between the two cell subsets (Fig. 2 and data not shown). A varying percentage of the Tc1 cells, typically about 50%, and a minor fraction of Tc2 cells were found to be CD8^{low} (Fig. 2). The CD8^{low} and the CD8^{high} cells had identical side-scatter/forward-scatter characteristics and similar levels of TCR and CD25 expression (data not shown).

Because most of the Tc2 cell populations produced IFN-γ, we performed intracellular cytokine staining to assess the cellular source of this production. After 5 h of incubation in the presence of PMA, ionomycin, and monensin, over 85% of Tc1 cells were highly positive for IFN-γ. The majority of Tc2 cells were also positive; however, a heterogeneity was found in the level of IFN-γ and the mean fluorescence intensity of IFN-γ^{high} Tc2 cells was lower than that of corresponding Tc1 cells (Fig. 3). These results suggest that the IFN-γ present in the culture supernatant of most of our Tc2 cultures was not due to contamination with Tc1 cells, but

CULTURE CONDITION

FIGURE 1. IL-4, IL-10, and anti-IFN-γ mAb synergize in inducing Tc2 differentiation. Purified CD8$^+$ cells were stimulated with irradiated syngeneic spleen cells and HA peptide in the presence of 1 ng/ml IL-2 and 20 ng/ml IL-12 or IL-2 and the specified combinations of 80 ng/ml IL-4, 20 ng/ml IL-10, and 10 μg/ml anti-IFN-γ mAb. On day 6, living cells were purified by Ficoll density separation and restimulated with irradiated spleen cells and peptide for 48 h. The cytokine content of the culture supernatants was then tested by ELISA. The experiment was repeated, with similar results. The level of IFN-γ produced by the Tc1 cells was above the upper threshold of our ELISA test, 5 ng/ml.
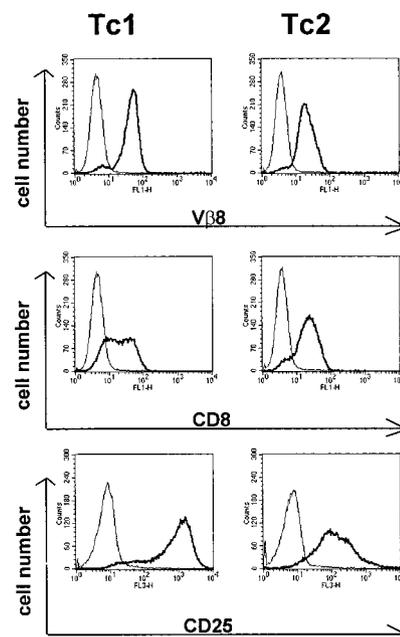

FIGURE 2. Cell surface phenotype of Tc1 and Tc2 cells. After 6 days of culture, Tc1 (*left*) and Tc2 (*right*) cells were purified by Ficoll density separation, labeled by anti-CD8, anti-Vβ8, or anti-CD25 mAb, and tested by flow cytometry; the thin lines represent the unstained control Tc1 or Tc2 cells. Four independent experiments were performed with similar results.
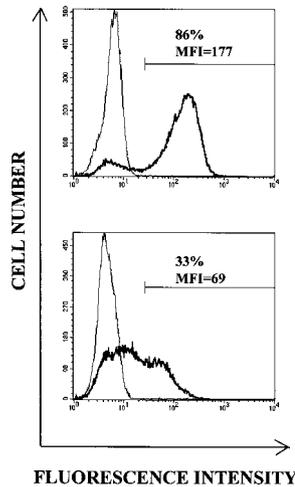
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FIGURE 3. Comparison of the intracellular IFN-γ content of Tc1 and Tc2 cells. Tc1 (*top*) and Tc2 (*bottom*) cells were stimulated for 5 h with PMA and ionomycin in the presence of monensin. Cells were then fixed by paraformaldehyde and permeabilized with saponin and stained. The thick lines represent cells stained by FITC-labeled anti-IFN-γ mAb and the thin lines represent cells stained by a FITC-labeled isotype control mAb. The percentage and mean fluorescence intensity of high positive cells is given. Three independent experiments were performed with similar results.

rather to a low/intermediate level of IFN-γ production by Tc2 cells.

Both Tc1 and Tc2 cells were found to be highly cytotoxic to Ag-pulsed target cells in vitro. In fact, Tc2 cells generated according to our protocol killed ^{51}Cr-labeled P815 cells at least as effectively as did Tc1 cells. The cytotoxic activity in both cases seemed to be entirely due to perforin-dependent lysis, because addition of EGTA abolished the cytotoxic activity of both cell subsets (Fig. 4).

Tc2 cells are diabetogenic in immunocompetent hosts, though less so than Tc1 cells

CL4-TCR Tc1 cells transferred into adult, nonirradiated, RIP-HA transgenic recipients induced diabetes reproducibly. The 50% diabetogenic dose was 5×10^5, and as few as 10^5 cells were sufficient to cause diabetes at a low frequency (Fig. 5). High cell doses (10^6 or more) invariably caused disease 4–5 days after the cell transfer, while, in the case of lower cell doses, a longer time (up to 3 wk) was necessary for the development of glucosuria (Fig. 5).
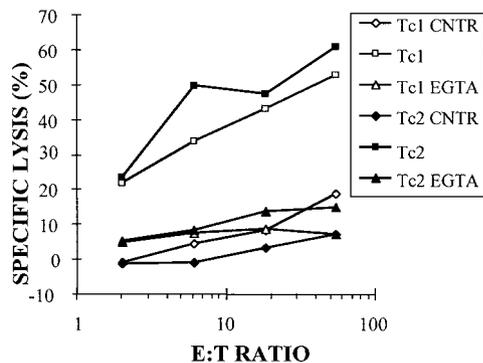

FIGURE 4. Both Tc1 and Tc2 cells kill ^{51}Cr-labeled P815 target cells efficiently. HA peptide-pulsed (squares) or nonpulsed (diamonds) target cells were incubated with Tc1 (empty symbols) or Tc2 (filled symbols) cells. Cytotoxicity assays were also performed in the presence of EGTA (triangles). The experiment was repeated three times, with similar results.
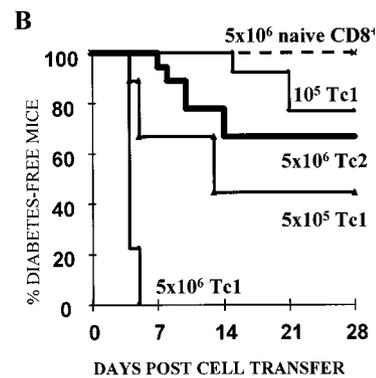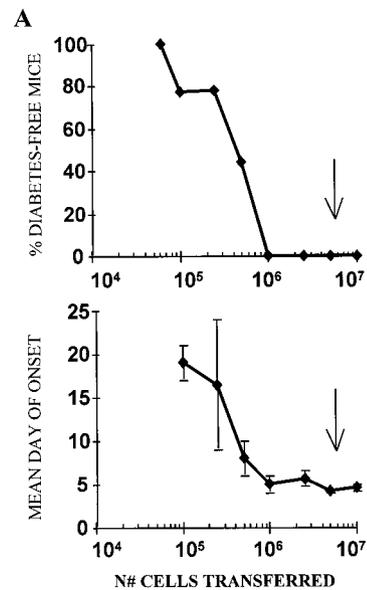

FIGURE 5. Tc1 and Tc2 cells cause diabetes in nonirradiated adult RIP-HA recipients. *A*, Graded doses of CL4 Tc1 cells were injected i.v. into RIP-HA recipients and glucosuria was controlled by test strips. Each data point represents 7–13 recipients, except for two cell doses, 1×10^6 and 2.5×10^6 cells, which represent three recipients each. The *upper panel* shows the percentage of diabetes-free animals and the *lower panel* shows the day of onset of glucosuria (mean ± SEM). The arrows indicate the cell dose chosen for the comparative cell transfer experiments using naive CD8$^+$ cells and Tc2 cells (5×10^6 cells, i.e., more than ten times the 50% diabetogenic dose of Tc1 cells). *B*, Comparison of diabetogenic potential of Tc1, Tc2, and naive CD8$^+$ cells. RIP-HA recipients were injected i.v. with the indicated doses of Tc1, Tc2, or purified naive CD8$^+$ cells, and glucosuria was controlled by test strips. The numbers of RIP-HA recipients were 7 for transfer of 5×10^6 naive CD8$^+$ T cells, 13 for 10^5 Tc1, 9 for 5×10^5 Tc1, 9 for 5×10^6 Tc1, and 18 for 5×10^6 Tc2.

Tc1 (10^7 cells) transferred into nontransgenic control recipients ($n = 5$) did not cause diabetes.

To assess the diabetogenic potential of Tc2 cells, a relatively high cell dose, 5×10^6 cells, was chosen. This number of Tc1 cells, which corresponds to 10 times the 50% diabetogenic dose, always transferred diabetes within 4–5 days (Fig. 5). Highly polarized Tc2 cells (5×10^6) were found to have a definite, but reduced, diabetogenic activity. Overall, 6 of 18 mice injected with Tc2 cells developed diabetes. Therefore, Tc1 cells were more than 10 times more diabetogenic than Tc2 cells (Fig. 5). Tc2 cells were nonetheless more diabetogenic than naive CD8$^+$ cells, because the same dose of purified naive CD8$^+$ cells, 5×10^6, did not transfer diabetes (Fig. 5). However, higher doses of naive cells were not

completely harmless, because 10^7 naive cells caused diabetes in 2 recipients of 11 (data not shown).

As previously described (8), the polarized phenotype of HA-specific Tc1 and Tc2 cell populations was stable in vivo. Splenocytes and pancreatic lymph node cells from RIP-HA mice adoptively transferred 4 days earlier with 20×10^6 Tc1 cells specifically produced large amounts of IFN-γ but no detectable IL-4 or IL-10 in response to HA peptide in vitro (Fig. 6). Conversely, splenocytes and pancreatic lymph node cells from RIP-HA mice adoptively transferred with 20×10^6 Tc2 cells produced both IL-4 and IL-10 as well as low levels of IFN-γ in response to HA peptide (Fig. 6). No cytokine production was detected using splenocytes or pancreatic lymph node cells from uninjected RIP-HA control mice. This indicates that Tc1 and Tc2

cells retain their cytokine profile in vivo and that a phenotype switch is not likely to explain induction of diabetes in Tc2 recipients.

Tc1 cells preferentially accumulate in the pancreas of RIP-HA recipients

To look for possible causes of the different diabetogenic potential of Tc1 and Tc2 cells we compared the homing and proliferation of Tc1 and Tc2 cells in the pancreas and the spleen. In these experiments, the transferred Tc1 or Tc2 cells had previously been labeled with CFSE and homing of the transferred cells was monitored by flow cytometry. Furthermore, a comparison of the mean fluorescence level of CFSE-positive cells enabled us to assess the difference between the number of divisions performed by Tc1 and Tc2 cells. The homing and proliferation of Tc1 and Tc2 cells in the spleen was comparable on both days 1 and 4 after the transfer (Fig. 7). In contrast, in the pancreas, there were 2.0 times more Tc1 than Tc2 cells on day 1, but by day 4 this difference had increased to 6 times more. Furthermore, the decreased CFSE content of Tc1 cells in the pancreas on day 4 indicated that they had divided on average twice more than Tc2 cells (Fig. 7).

Because different profiles of chemokine receptors could lead to distinct homing properties, we investigated whether Tc1 and Tc2 effector cells differed in the expression of these receptors either before or 12 h after in vitro restimulation with immobilized anti-CD3 mAb. Distinct chemokine receptors were expressed on Tc1 vs Tc2 as assessed by ribonuclease protection assays (Fig. 8A). Indeed, CCR1 and CCR4 were preferentially expressed in Tc2, whereas CCR2 and CCR5 expression was higher on Tc1 than on Tc2 cells but was reduced upon restimulation. Both cell types had
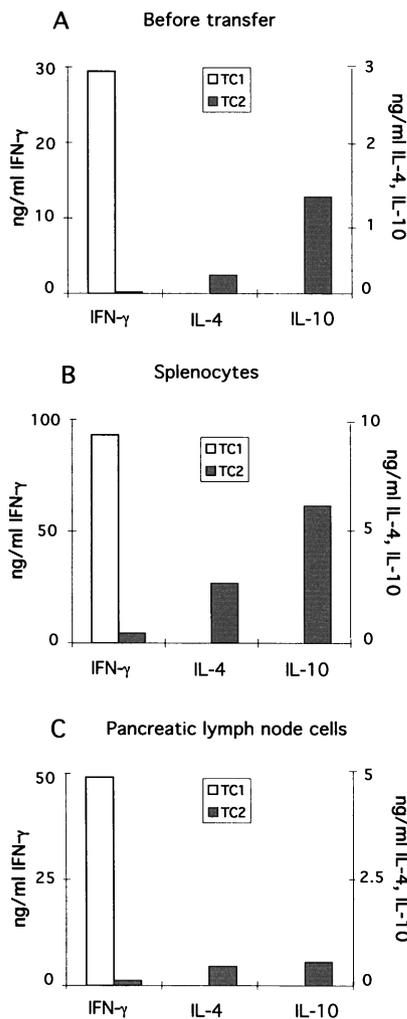

FIGURE 6. Cytokine production by Tc1 and Tc2 cells before and after adoptive transfer in RIP-HA mice. A, The cytokine profile of the Tc1 and Tc2 cells before transfer was established by analyzing cytokines released following stimulation of 6400 in vitro-differentiated Tc1 or Tc2 cells with HA-pulsed (or unpulsed) irradiated splenocytes. B and C, RIP-HA transgenic mice were adoptively transferred with 20×10^6 Tc1 or Tc2 generated according to the protocol described in *Materials and Methods*. Their splenocytes (*B*) and pancreatic lymph node cells (*C*), harvested 4 days post transfer, were restimulated in vitro (8×10^5/well) with or without HA peptide. Cytokines released in the supernatants at 48 h were measured by specific ELISAs. Note that IFN-γ results and IL-4 and IL-10 results are on different scales. No or minimal cytokine production was detected in the absence of the HA peptide (data not shown). Two independent experiments were performed with similar results.
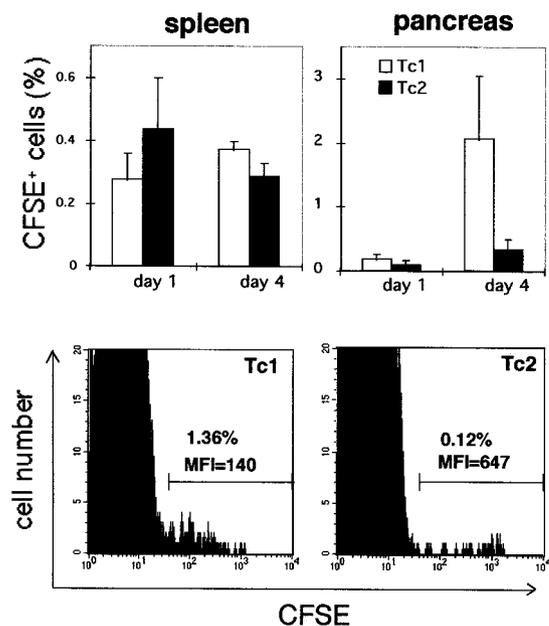

FIGURE 7. Different homing and proliferation of Tc1 and Tc2 cells in RIP-HA recipients. RIP-HA recipients injected with 5×10^6 CFSE-labeled Tc1 or Tc2 cells were sacrificed at days 1 or 4, and single-cell suspensions of their spleen and pancreas were analyzed by FACS. *Top panels*, Percentage of CFSE-labeled cells in the lymphocyte forward light scatter-side light scatter gate in the spleen and pancreas (mean ± SEM is plotted; $n = 4–5$). At day 4, the pancreas of recipients of Tc1 cells contained 6 times more CFSE-labeled cells than that of recipients of Tc2 cells (2.06% vs 0.33%; $p = 0.06$; two-tailed Mann-Whitney test). *Bottom panels,* Distribution of the CFSE$^+$ cells in the pancreatic cell suspensions of two representative recipients sacrificed at day 4.
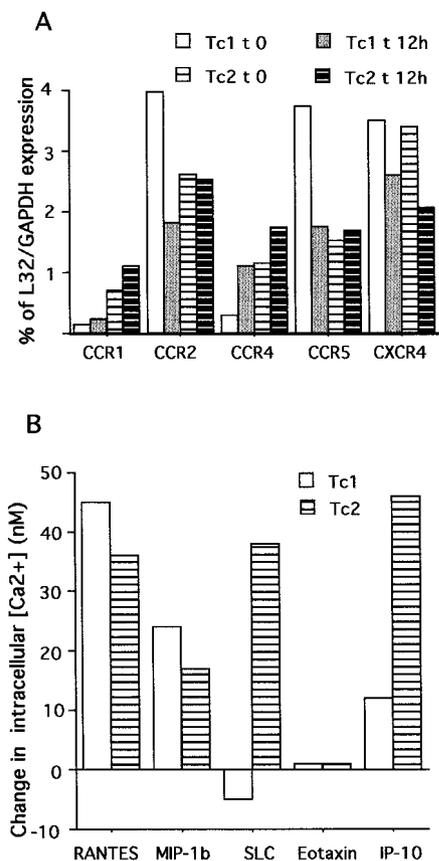
FIGURE 8. Tc1 and Tc2 cells differentially express certain chemokine receptors. *A*, RNA was extracted from Tc1 and Tc2 cells, 6 days after initiation of culture under polarizing conditions, either before (t 0) or after 12 h (t 12h) of restimulation with immobilized anti-CD3 mAb. RNase protection assay was performed using the RiboQuant template sets mCR-5 and mCR-6 as described in *Materials and Methods*. Quantitation was performed using a high-resolution β-imager and data were normalized against the L32 and GAPDH housekeeping genes. Two independent experiments were performed, with similar results. *B*, Fura-2-labeled Tc1 and Tc2 were placed at 37°C in a fluorometer. Following the addition of 50 nM of recombinant chemokine, increases in intracellular Ca^{2+} concentrations were recorded. Data are plotted as the difference of baseline intracellular Ca^{2+} concentration and the maximum response to a given chemokine. Two independent experiments were performed, with similar results.

undetectable CXCR2 and CCR3 mRNA expression. Measurements of calcium flux induced in Tc1 or Tc2 cells by incubation with 50 nM recombinant chemokines (IL-8, SLC, eotaxin, RANTES, MIP-1β, IP-10, fractalkine, and I309) confirmed the somewhat higher surface expression of functional CCR5 on Tc1 as compared with Tc2 cells, and the lack of CXCR2 and CCR3 on both cell types (Fig. 8*B*). Moreover, they revealed a selective response of Tc2 cells to SLC, indicative of surface expression of CCR7, and a greater response of Tc2, as compared with Tc1 cells, to IP-10, indicative of surface expression of CXCR3 (Fig. 8*B*). A lack of fractalkine (CX₃CR1), and I309 (CCR8) responsiveness by both cell types was also found (data not shown). It is likely that the distinct pattern of chemokine receptor expression by Tc1 and Tc2 cells contributes to their different rate of accumulation in the inflamed pancreas.

Discussion

We have demonstrated that in vitro-generated islet-specific Tc1 cells are highly diabetogenic, while Tc2 cells have a lower, but definite, diabetogenic potential. The different pathogenic potential

cannot be attributed to reduced cytotoxicity or CD8 expression of Tc2 cells but correlates with their reduced proliferation and homing to the pancreas. To our knowledge, this is the first report on the role of Tc1 and Tc2 cell subsets in autoimmunity.

In accordance with literature data, Tc1 and Tc2 cells generated according to our protocol were phenotypically similar. However, a surprising feature of our model is the partial loss of the CD8 molecule, which was more pronounced in Tc1 than in Tc2 cells. The contribution of an expanded minor CD4⁻CD8⁻ compartment to the recovered cell population is highly improbable, because our cultures start with highly purified, positively selected CD8⁺ cells and because the CD8^low Tc1/Tc2 cells still express the molecule at a low level. Therefore, a partial loss of the CD8 marker by the originally positive cells would seem the more plausible explanation. A similar loss of CD8 expression has been described by Erard et al. (6) in the case of CD8⁺ cells stimulated with PMA and ionomycin, or allogeneic thymoma cells, in the presence of IL-4. A loss of the CD8 molecule is also known to occur in the case of CD8⁺ cells stimulated by mitogens, and has also been described as a means to silence autoreactive CD8⁺ T cells (34, 35). However, it is interesting that CL4-TCR CD8⁺ cells stimulated with APC and peptide can also lose the CD8 molecule, especially in light of the fact that activation of CL4-TCR-expressing T cells by Ag-pulsed APC or by soluble monomeric MHC-peptide complexes was found to be CD8-dependent (30, 36). Because the decrease in the expression level of CD8 was less marked in Tc2 than in Tc1 cells, the reduced expression of this molecule cannot explain the lower diabetogenic potential of the Tc2 cells.

We found that Tc2 cells, though they are much more diabetogenic than naive CD8⁺ cells, do not induce diabetes as efficiently as Tc1 cells. Analogous adoptive transfer experiments performed with Th1 and Th2 cells gave consistent results: transferred islet-specific Th2 cells did not cause disease in immunocompetent recipients (37, 38). However, whether autoreactive Th2 cells were protective depended on the model. In the adoptively transferred diabetes model of Katz et al. (37) Th2 cells could not protect against the damage caused by Th1 cells. In EAE models, Th2 cells were either protective (39, 40), or harmless but inefficient in suppressing the disease induced by Th1 cells (41). However, Th2 cells caused diabetes in severely immunocompromised nonobese diabetic mouse (NOD).SCID mice (42). Furthermore, myelin basic protein-specific Th2 cells caused CNS inflammation in an analogous experimental setup (43).

In our system, the reduced diabetogenic potential of the Tc2 cells might be due to different factors. Differential cytokine production could contribute to this effect. Indeed, an important pathogenic role has been proposed for IFN-γ in IDDM based on experiments involving transgenic expression of IFN-γ in β islet cells, treatment of NOD mice with neutralizing anti-IFN-γ mAbs, or use of IFN-γ- or IFN-γR-deficient mice (44–48). Our preliminary results indicate that 2×10^6 Tc1 cells from IFN-γ-deficient CL4-TCR mice fail to transfer diabetes in RIP-HA mice (our unpublished results) suggesting that the reduced pathogenic potential of Tc2 cells could be due to their low level of IFN-γ production. Another possibility is that the IL-4 or IL-10 produced by the Tc2 cells might protect the recipients from the β cell damage. Nevertheless, the role of these two cytokines remains ambiguous. Local expression of IL-4 has been shown to prevent the autoimmune rejection of transplanted islet grafts (49), while IL-4 is not required for control of either autoimmune diabetes (50) or EAE (51). Interestingly, IL-10 has been found to have a dual role in the development of autoimmune tissue damage. Though administration of IL-10, considered to be a prototypic immunosuppressive cytokine,

decreased the incidence of diabetes in NOD mice (52), local expression of IL-10 in the pancreas of NOD mice led to CD4- and B cell-independent CD8$^+$ infiltration of the organ (53, 54).

Another potential explanation for the different pathogenicity of Tc1 and Tc2 cells could be their different homing and proliferative properties. By analogy, islet-specific diabetogenic Th1 and non-diabetogenic Th2 cells have been found to have different kinetics of migration to the pancreas (55). The migration pattern of mouse Th1 and Th2 is correlated with differential expression of chemokine receptors (56). In particular, CCR5 is preferentially expressed on Th1 cells, whereas its ligands (RANTES, MIP-1α, and MIP-1β) are expressed in the pancreas during development of destructive autoimmune insulitis in NOD mice (55, 57). It is tempting to speculate that the higher expression of CCR5 on Tc1 as compared with Tc2 cells, previously reported by Cerwenka et al. (12) and confirmed in this report, contributes to the preferential recruitment of Tc1 cells in the pancreas. Conversely, CCR7 expression on Tc2 cells could promote their homing in secondary lymphoid tissues, which constitutively express SLC (56, 58) and this interaction may prevent T cells from responding to agonist MHC-peptide complexes (59). However, the difference in homing capacity seems to be quantitative rather than qualitative (55). In our model, 1 day after the injection, slightly more CFSE-labeled Tc1 than Tc2 cells were found in the pancreas of the recipients while on day 4 the difference was more marked; six times more Tc1 than Tc2 cells were found in the pancreas of recipients. This increase might have been due to different homing, or to different rates of proliferation of the two cell subsets because the decreased amount of CFSE in Tc1 cells indicated a higher number of cell divisions. This reduced pancreas-specific homing/proliferation, combined with the different cytokine profile, might contribute to the compromised diabetogenic potential of Tc2 cells.

In contrast, the residual diabetogenic potential of our Tc2 populations might be explained by their retained cytotoxic activity. Fully in line with the observation of Cerwenka et al. (12), our Tc2 cells generated in vitro by stimulation with APCs and specific peptide were cytotoxic. Because EGTA abolished the cytotoxic activity of both Tc1 and Tc2 cells, the cytotoxicity in both cases seems to be preferentially mediated by perforin rather than by alternative pathways. Literature data on the Fas-L-dependent killing by in vitro cultured Tc1 and Tc2 cells are equivocal. Although cytotoxicity of both Tc1 and Tc2 cells was almost completely abolished in perforin$^{-/-}$ mice, Fas-L mediated killing was demonstrable by wild-type Tc1 or, in other models, by both Tc1 and Tc2 cells (9, 10, 60, 61). The interpretation of these data is currently difficult.

Perforin-dependent lysis seems to be a major mechanism of β cell damage in IDDM, because the incidence of spontaneous diabetes was drastically decreased in NOD mice lacking perforin (21). Furthermore, a lack of perforin blocked the development of diabetes, but not insulitis, in an adoptive transfer model based on the use of LCMV-GP transgenic mice (22). Being a necessary mechanism of tissue damage in diabetes, the cytotoxic activity of the Tc2 populations could explain their diabetogenic capacity.

In conclusion, although highly polarized Tc2 cells are more diabetogenic than naive CD8$^+$ cells, they are more than 10 times less diabetogenic than Tc1 cells. This difference between Tc1 and Tc2 cells may due to a combination of several factors, namely differences in cytokine production, proliferative capacity, and homing pattern. The diabetogenicity of autoreactive Tc2 cells, caused most likely by their cytotoxic activity, precludes their clinical use to down-regulate autoimmunity.

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