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Effector Function of Diabetogenic CD4 Th1 T Cell Clones: A Central Role for TNF-α

Joseph Cantor and Kathryn Haskins

Effector function of T cells in autoimmune diabetes has been widely studied with mixed populations of lymphoid T cells stimulated ex vivo, but this approach does not permit evaluation of the contribution by a single T cell clone in the inflammatory site during pathogenesis. We have investigated cytokine production both in vitro and in vivo in a panel of diabetogenic CD4 Th1 T cell clones derived from the NOD mouse. SuperArray analysis showed a common pattern of mRNA expression for inflammatory cytokines and receptors in vitro after TCR stimulation. Ex vivo intracellular cytokine staining demonstrated that two important inflammatory cytokines, IFN-γ and TNF-α, were being made by these T cells recovered from the pancreas 6 days following adoptive transfer. TNF-α produced in the pancreas by pathogenic T cell clones and recruited macrophages was not the membrane-bound form. Secreted TNF-α can lead to production of multiple inflammatory chemokines, as were observed in the pathogenic clones by intracellular cytokine staining. Our results not only define the nature of an inflammatory cytokine response critical to development of diabetes, but also suggest its role in the regulation of other events during pathogenesis induced by CD4 T cells. Similar analyses in other models demonstrated that disease induced by CD4 T cell clones closely resembles spontaneous autoimmune diabetes in which both CD4 and CD8 T cells are required. Thus, cloned T cells in effect amplify effector function of T cells which otherwise may be difficult to detect without ex vivo stimulation.

A utoreactive T cells are key mediators of many autoimmune diseases, including type 1 diabetes (T1D). A variety of studies have documented the involvement of both CD4 and CD8 T cells in progression of disease (1), but as it has been demonstrated in both rodent and human spontaneous autoimmune diabetes that the strongest genetic linkage to disease susceptibility is MHC class II, we have focused on MHC class II-restricted CD4 T cell effectors. The importance of CD4 T cells has been well documented in the NOD mouse model of T1D, and efforts to determine the mechanisms whereby CD4 T cells contribute to pathogenesis have focused on several important aspects of T cell function, particularly cytokine production and, more recently, chemokine expression. For example, a recent study reported a variety of proinflammatory cytokines and chemokines expressed at the mRNA level in pancreatic tissue after adoptive transfers of CD4 Th1 T cells isolated from a NOD TCR-transgenic (TCR-Tg) mouse (2). The specific cell sources of these cytokines in the pancreas, however, were not identified. The complexity of effector responses in vivo is difficult to determine and conventional quantitative assays determining averages of cell populations may be misleading. Recent advances in technology have made possible the investigation of CD4 effector function at the level of the single T cell and elegant studies by Peixoto et al. (3) have demonstrated that there is great heterogeneity in the effector T cell response. These studies are complemented by those of Swain and colleagues (4) showing that this heterogeneity of effector T cells persists and is also found in memory T cells.

Because there is functional heterogeneity in effector T cells, how a particular T cell contributes to the pathological effector response is a critical question. The interest in this issue is illustrated by the considerable efforts being made to analyze at the single-cell level the activity of effector T cells through RNA amplification and gene expression analysis. Effector T cell activity is especially important in the case of T cell-mediated diseases, such as autoimmune diabetes, where T cells present high priority therapeutic targets. It has been particularly difficult to characterize the behavior of distinct T cells in the actual sites where autoimmune inflammation persists. This difficulty can be resolved through the use of T cell clones that provide a defined T cell population displaying a uniform functional phenotype. In addition to being powerful tools for the systematic analysis of T cell effector function in vitro, well-characterized T cell clones have the added advantage of being readily available in quantities that allow for testing in vivo.

To investigate in detail the effector function of pathogenic T cells in T1D, we have used islet Ag-specific, CD4 Th1 T cell clones that are highly diabetogenic in young NOD recipients (Table I). These T cell clones were derived from peripheral lymphoid organs of diabetic NOD mice and their diabetogenic properties have been described in detail (1, 5–7). Based on the hypothesis that patterns of cytokine and chemokine production determine their pathogenicity, we provide in this study a comprehensive analysis of the proinflammatory mediators produced by this panel of T cell clones. We have used gene array analysis to assess the expression of mRNA for a large group of inflammatory cytokines and chemokines. Expression of most of these factors has been confirmed at the protein level through ELISA and intracellular staining. Most significantly, however, we present new data on how CD4 Th1 T cells function directly in the inflammatory site through ex vivo...
analysis of cells recovered from the pancreas after adoptive transfer, and how the proinflammatory cytokine TNF-α may be at the center of the pathogenic T cell response.

Materials and Methods

Mice

NOD and NOD.scid breeding mice were initially acquired from The Jackson Laboratory or the Barbara Davis Center for Childhood Diabetes (Denver, CO), and were housed in specific pathogen-free conditions at the University of Colorado Health Sciences Center (UCHSC) for Laboratory Animal Care (CLAC). NOD.scid mice were housed in sterile isolation cages. Mice in NOD.scid litters (6–10 days old) were used as recipients in adoptive transfer experiments. Breeding mice and experimental animals were monitored for development of disease by urine glucose. The 6.9 TCR-Tg mouse was produced using TCR genes from a diabetogenic T cell clone, BDC-6.9 (8). All procedures used were in accordance with Institutional Animal Care and Use Committee guidelines and approved by the UCHSC Animal Care and Use Committee.

Culture and expansion of T cell clones

The T cell clones listed in Table I were established from spleen and lymph nodes of diabetic NOD mice (5, 6), and were restimulated every 2 wk with a β cell granule membrane fraction obtained from β cell tumors as a source of Ag (7), irradiated NOD spleen cells as APCs, and EL-4 supernant as a source of IL-2 in complete medium (CM). CM is DMEM supplemented with 44 mM sodium bicarbonate, 0.55 mM L-arginine, 0.27 mM L-asparagine, 1.5 mM L-glutamine, 1 mM sodium pyruvate, 50 mg/L gentamicin sulfate, 50 mM 2-ME, 10 mM HEPES, and 10% FCS. Cell numbers were expanded for transfer experiments by subcuturing 3–6 × 10^6 T cells 4 days after restimulation in a 5-fold volume of CM and additional IL-2. T cells were harvested, washed three times, and resuspended in HBSS for injection into young (≤10 days of age) NOD.scid recipients.

SuperArray analysis

At the end of the 2-wk cycle of restimulation with Ag and APCs, resting T cell clones were added to 6-well plates coated with 1 μg/ml anti-CD3 Ab at 1 × 10^5 cells/well and cultured overnight at 37°C. After removing supernatant, RNA was extracted in the plate by addition of TRIzol (Invitrogen Life Technologies). Cells were surface-stained in 50–100 μl of staining buffer (PBS, 0.5% BSA) containing rat anti-CD4 or isotype control Ab at optimized concentrations. After incubation on ice for 30–45 min, followed by three washes, cells were fixed in 2% formaldehyde for 10 min in the dark. Cells were washed once more before resuspending in permeabilization buffer (staining buffer plus 0.5% saponin), containing an isotype or specific Ab mix for intracellular cytokines. Polyclonal intracellular staining Abs used were obtained from R&D Systems and included polyclonal goat IgG anti-TCA-3, anti-RANTES, anti-MIP-1α, anti-MIP-1β, anti-MIP-1y, anti-C10, and anti-SLC as primary Abs, followed by FITC-rabbit anti-goat secondary Ab (Vector Laboratories). Monoclonal digoxigenin-MTAC-2 anti-lymphocytotin and Cy5-anti-digoxigenin. secondary Abs were kindly provided by B. Dorner (R. Koch Institute, Berlin, Germany) (9). Monoclonal anti-cytokine Abs used were: FITC-rat anti-IFN-γ (XMG1.2; BD Biosciences) and allophycocyanin-rat anti-TNF-α (MP6-XT22; BD Biosciences). After incubation with primary Ab, cells were washed three times in permeabilization buffer, incubated with the secondary Ab for another 30–45 min, washed again in permeabilization buffer, and resuspended into staining buffer. Stained cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences).

Adoptive transfer of diabetes

For disease transfer experiments, expanded cell cultures were harvested after 4 days with cell dissociation buffer and washed three times in HBSS. T cells (1 × 10^5) were injected i.p. into age-matched 6- to 10-day-old NOD.scid recipient mice. In some experiments, adoptive transfers were performed with spleen cells from diabetic NOD or TCR-Tg donors. In these instances, spleen cell suspensions were washed and injected (1 × 10^7 cells) into young NOD.scid recipients. Onset of diabetes was monitored by urine glucose screening; positive urine glucose readings were followed by blood glucose measurement. Blood glucose concentration >15 mM for more than 1 day was considered diagnostic of overt diabetes.

Recovery of diabetogenic T cells from pancreas

Several days after adoptive transfer with T cell clones (6–7 days unless otherwise indicated), or at onset of diabetes (4–6 wk) following diabetic spleen cell transfers, recipient NOD.scid mice were sacrificed, spleens and pancreata were removed and placed into cold Solution A (PBS, 5% FBS, 1% glucose, 1× GolgiPlug (BD Biosciences)). One milliliter of prewarmed Solution B (PBS, 15% FBS, 1× GolgiPlug, 5 mg/ml collagenase (Sigma-Aldrich)) was added to each pancreas, which was then placed in a 37°C

<table>
<thead>
<tr>
<th>Clone</th>
<th>Th</th>
<th>TCR</th>
<th>Islet Ag Reactivity</th>
<th>Diabetic in NOD (&lt;14 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDC-2.5</td>
<td>1</td>
<td>Vβ4Vα1</td>
<td>All mouse strains tested</td>
<td>+</td>
</tr>
<tr>
<td>BDC-3.10.3</td>
<td>1</td>
<td>Vβ4Vα (ND)</td>
<td>All mouse strains tested</td>
<td>+</td>
</tr>
<tr>
<td>BDC-6.3</td>
<td>1</td>
<td>Vβ4Vα3.1</td>
<td>All mouse strains tested</td>
<td>+</td>
</tr>
<tr>
<td>BDC-6.9</td>
<td>1</td>
<td>Vβ4Vα13.1</td>
<td>NOD, SWR</td>
<td>+</td>
</tr>
<tr>
<td>BDC-9.3</td>
<td>1</td>
<td>Vβ4Vα13.1</td>
<td>NOD, SWR</td>
<td>+</td>
</tr>
<tr>
<td>BDC-10.1</td>
<td>1</td>
<td>Vβ15Vα13</td>
<td>All mouse strains tested</td>
<td>+</td>
</tr>
<tr>
<td>BDC-2.4</td>
<td>1</td>
<td>ND</td>
<td>Nonreactive to islet cells</td>
<td>−</td>
</tr>
<tr>
<td>2.5 Tg/TZ-X</td>
<td>2</td>
<td>Vβ4Vα1</td>
<td>Same as BDC-2.5</td>
<td>+</td>
</tr>
</tbody>
</table>

*All mouse strains tested = all inbred mouse strains tested (NOD, NOR, BALB/c, CBA, C57BL/6, C57L/J, SWR, SI.L).
water bath for 20–40 min with periodic vortexing, until a cloudy suspension was observed. Digestion was stopped with the addition of 3–4 ml of cold CM/GolgiPlugs. Single-cell suspensions of the digested pancreata and spleens were made using glass homogenizers, with one wash of cold CM/GolgiPlugs. Cell suspensions were flash-spun to remove tissue debris, and the liquid was removed and spun at 300 × g for 10 min at 4°C to pellet. Cells were resuspended in 1 ml of CM/GolgiPlugs and incubated at 37°C for 3–5 h, with or without PMA (100 ng/ml) and ionomycin (1 μg/ml).

**Ex vivo analysis of cytokines and chemokines**

After ex vivo culture, cells were harvested using Cell Dissociation Buffer (Invitrogen Life Technologies) and washed with PBS/GolgiPlugs. After centrifugation, cell pellets were resuspended in staining buffer/GolgiPlugs and aliquoted to wells of a 96-well round-bottom plate: 2 wells for each set of Ab combinations, one for specific Ab mix, and one for isotype control mix. Surface and intracellular cytokine staining was performed as described in the protocol for intracellular cytokine staining, with the modification of adding GolgiPlugs to all reagents until fixation and preincubating in 25 μl/well staining buffer containing 2 μg/ml FcBlock (BD Biosciences) for 5 min on ice before addition of surface-staining Abs.

**Results**

**Diabetogenic CD4 Th1 T cell clones express mRNA for multiple inflammatory cytokines after TCR stimulation in vitro**

Screening of the inflammatory cytokines and chemokines produced by a panel of defined pathogenic CD4+ T cell clones after TCR stimulation was conducted by a limited gene array analysis of inflammatory cytokines (SuperArray Bioscience). To obtain a profile on each T cell clone, analysis was performed two or three times on RNA isolated from the clones, with repeat assays conducted on different RNA preparations. After resting T cells were stimulated overnight with anti-CD3, the cells were lysed and RNA was extracted using TRIzol reagent. As a non-Th1 T cell control, we used a Th2 T cell clone, 2.5 Tg/T2-X, which exhibits a typical Th2 cytokine profile and does not produce IFN-γ, but is nevertheless pathogenic under certain conditions (10). Using the SuperArray kit, RNA was reverse-transcribed and biotinylated cDNA was hybridized with the gene array membrane overnight. Expression of cytokine genes was detected using streptavidin-alkaline phosphatase and a chemiluminescent substrate.

Results from the SuperArray analysis, as illustrated in the array from one T cell clone, BDC-6.9, indicated that in addition to the more well-established Th1 T cell cytokines such as IL-2, IFN-γ, and TNF-α, the T cell clones produce the proinflammatory cytokines LT-α, MIF, and IL-17B. (Fig. 1). These pathogenic CD4+ T cell clones also express mRNA for cytokines that are typically anti-inflammatory, such as IL-11 and TGF-β, a finding that points to the complexity of the cytokine response. The results from gene array analysis are shown only for one T cell clone, but similar analyses on other pathogenic T cell clones in the panel indicated a common pattern of cytokine production, with little variation observed between clones.

**Expression of IFN-γ and TNF-α in pathogenic Th1 T cell clones at the protein level after TCR stimulation**

IFN-γ and TNF-α are perhaps the most important proinflammatory Th1 cytokines, in part because of their role in the activation of macrophages (11, 12). As both cytokines are critical to the development of diabetes (13), we compared IFN-γ and TNF-α protein expression by intracellular cytokine staining in expanded cell cultures, either with no additional stimulation or following stimulation with anti-CD3. The “unstimulated” condition reflects the activated state of the expanded T cells at the time of injection into adoptive transfer recipients whereas anti-CD3 stimulation simulates encounter with Ag after adoptive transfer and trafficking to the target organ. Thus, intracellular staining performed in this way represents in vitro the protein expression of these two critical

**FIGURE 1.** Analysis of inflammatory cytokine gene expression in pathogenic Th1 clones. After stimulation of resting T cells for 24 h with plate-bound anti-CD3 Ab, RNA was extracted and analyzed for purity and integrity. RNA (5 μg) for each clone was reverse-transcribed in a complex mixture of gene-specific primers using the SuperArray TrueRT kit to generate biotinylated cDNA probes representing mRNA expression levels for these inflammatory cytokines. After hybridization and nonradioactive detection on film, spots were identified using the gene array map. A representative array is shown and was obtained from the Th1 T cell clone BDC-6.9.

**FIGURE 2.** Cytokine production by four diabetogenic CD4 Th1 T cell clones. A, Intracellular staining for IFN-γ and TNF-α after CD3 stimulation. Th1 clones were expanded in IL-2 for 3 days and then stimulated for 24 h with anti-CD3 in the presence of brefeldin A for the final 4 h. T cells (1 × 10^6) were stained for intracellular cytokines with specific Abs. The difference between specific and nonspecific (isotype control) staining is expressed as change in mean fluorescence intensity (Δ M.F.I.). B, IFN-γ response to islet cell Ag over a 12-wk time course. IFN-γ response to Ag/APC was measured by specific ELISA of supernatants from the T cell clones each week for 12 wk. Resting T cells were cultured for 48 h in the presence of freshly isolated NOD peritoneal cells as APC and 5000 fresh NOD islet cells as Ag.
proinflammatory cytokines, both before T cell transfer and after exposure to their Ag in the pancreas. As indicated in Fig. 2A, the production of IFN-γ and TNF-α is robust after TCR stimulation and is consistent among the pathogenic clones in the panel; intracellular staining of these cytokines was not observed in the absence of anti-CD3 stimulation (data not shown).

To test the consistency of cytokine production by the Th1 T cell clones, we measured by ELISA the secretion of IFN-γ over time by several clones in response to islet-cell Ag and fresh APCs. Fig. 2B shows that while the magnitude of the T cell cytokine response varied from week to week upon stimulation by freshly isolated islet cells, the pattern of response is similar among the pathogenic T cell clones.

Expression of chemokines by diabetogenic CD4 Th1 T cell clones

Fig. 1 also shows that the diabetogenic T cell clones make several chemokines, and as with other cytokines, the pattern was similar between clones. As would be predicted from other studies in which chemokine/chemokine mRNAs were detected in the pancreas after using 2.5 TCR-Tg T cells in adoptive transfers (2), chemokine mRNAs detected by gene array analysis of the T cell clones included RANTES, MIP-1α, and MIP-1β, all of which are ligands for the chemokine receptor CCR5. Again, several factors were detected that have not been well characterized in T cells, including TCA-3, C10, SLC, MCP-3, and MIP-1γ. Table II provides a summary of the proinflammatory cytokines and chemokines made by diabetogenic T cell clones (six Th1 T cell clones and one Th2 clone). These data confirm that Th1 T cell clones are very different from Th2 T cells not only in their production of cytokines, but also in the chemokines they make.

To analyze chemokine production by the diabetogenic T cell clones at the protein level, intracellular cytokine staining was conducted on several clones, again either without additional stimulation of expanded subcultures or after incubation with anti-CD3. Fig. 3 demonstrates that chemokines could not be detected from expanded cultures of the T cell clones under “unstimulated” conditions, but were readily detectable after anti-CD3 activation. The single exception was SLC, which was not observed by intracellular cytokine staining under either condition.

The proinflammatory cytokines IFN-γ and TNF-α are produced by pathogenic CD4+ Th1 clones in the pancreas during progression to disease

In these studies on pathogenic CD4 T cell effector function, our ultimate goal was to investigate the activity of CD4+ Th1 T cells at the single-cell level in the target organ. We have developed a method whereby large numbers of defined T cells can be retrieved from the pancreas following adoptive transfer of pathogenic T cell clones into NOD.scid recipients. The cells are then analyzed for

Table II. Summary of cytokine and chemokine mRNA expression induced by anti-CD3 Ab stimulation of a panel of diabetogenic CD4 T cell clones

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>BDC-2.5 (Th1)</th>
<th>BDC-5.10.3 (Th1)</th>
<th>BDC-6.3 (Th1)</th>
<th>BDC-6.9 (Th1)</th>
<th>BDC-9.3 (Th1)</th>
<th>BDC-10.1 (Th1)</th>
<th>Tg/T2-X (Th2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>TNF-α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>IL-2</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>IL-17B</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>LT-α</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>RANTES</td>
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<td>+/-</td>
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<td>+/−</td>
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<td>C10</td>
<td>+</td>
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cells were analyzed by intracellular flow cytometry for IFN-γ and TNF-α production by CD4⁺ T cells.

FIGURE 4. Production of IFN-γ and TNF-α by BDC-2.5 ex vivo from pancreas after adoptive transfer. T cells (1 × 10⁷) from expansion subcultures were injected i.p. into 6-day-old NOD.scid recipients. Six days after transfer, pancreatic single-cell suspensions were incubated in the presence of brefeldin A, with or without PMA stimulation for 3 h. After harvesting, cells were analyzed by intracellular flow cytometry for IFN-γ and TNF-α production by CD4⁺ T cells.

Inflammatory cytokines are not produced in the pancreas by a nonpathogenic clone

To determine whether the inflammatory cytokines produced in the pancreas by diabetogenic T1 clones were a specific response resulting from their pathogenicity, or whether they were due simply to homeostatic proliferation induced by T cell deficiency, we performed adoptive transfers with a nonpathogenic CD4⁺ Th1 clone, BDC-2.4. The BDC-2.4 clone makes Th1 cytokines upon stimulation with anti-CD3, but it does not react to islet cells as Ag nor does it induce disease in young NOD recipients. Adoptive transfers into NOD.scid littermates were conducted with clone BDC-2.4 at the same time as transfers with a pathogenic clone, BDC-2.5, and cytokine production of the two clones ex vivo was compared 6 days later. As is apparent in Fig. 5 from the anti-CD4 staining, the nonpathogenic BDC-2.4 clone does not migrate to the pancreas; however, 2, 4, or 6 days after transfer there was little or no production of IFN-γ or TNF-α by BDC-2.4, whereas high levels of these cytokines were produced by BDC-2.5. This difference is apparent despite similar numbers of T cells in the pancreas, suggesting that the production of IFN-γ and TNF-α in the inflammatory site is indeed linked to T cell pathogenicity.

TNF-α produced in the pancreas by diabetogenic T cell clones is not in the membrane-bound form

TNF-α is produced as a membrane-bound protein, but can be cleaved by TNF-α-converting enzyme to generate secreted cytokine (14). Both forms are functional, but differ in their ability to bind TNFR1 or TNFR2 (15), and in their biological activity (16). Although the relative roles of membrane and soluble TNF-α in the induction of autoimmune inflammation have not been clearly defined, recent reports have suggested that secreted TNF-α is needed for optimal development of the inflammatory lesion (16). Because the form of TNF-α found in the pancreatic infiltrates of autoimmune diabetes in the NOD mouse has not been described previously, we conducted an experiment in which surface staining for the membrane-bound form of TNF-α was compared with total intracellular cytokine staining (representing both forms). Fig. 6A shows that in vitro, the Th1 T cell clone BDC-2.5 makes little or no TNF-α in the absence of anti-CD3 stimulation; after stimulation, however, there is a substantial amount of TNF-α staining both on the surface of these cells and intracellularly. In T cells recovered from the pancreas after adoptive transfer, there is negligible surface staining, even after a PMA/ionomycin stimulus (Fig. 6B), but intracellular staining of TNF-α is high with or without additional stimulation. These data indicate that although both forms of TNF-α are produced by the clones after PMA stimulation in vitro, the form detected in T cells recovered from the pancreas is not membrane bound, and is therefore likely to be the secreted form of this cytokine.

Macrophages recruited to the pancreas by pathogenic T cell clones also produce TNF-α

Because we observed that large numbers of F4/80⁺CD11b⁺ macrophages were present in the pancreas following adoptive transfer of the T cell clones, we wanted to know whether macrophage

FIGURE 5. A nonpathogenic Th1 T cell clone produces little or no cytokine in the pancreas. BDC-2.4 is a CD4⁺ Th1 T cell clone that produces IFN-γ in response to anti-CD3, but not Ag/MHC; it is not specific for islet Ag and is not pathogenic. BDC-2.4 was analyzed for cytokine production ex vivo in comparison to a transfer with the pathogenic clone BDC-2.5. At different time points following clone transfer, pancreatic tissue was harvested and cells were analyzed by intracellular staining for production of IFN-γ and TNF-α without further stimulation.
recruitment and cytokine production were contributing to the effector function of these pathogenic Th1 T cells. The results illustrated in Fig. 7A indicate that significant macrophage recruitment occurs only after transfer of a pathogenic Th1 T cell clone; the numbers of macrophages recruited to the pancreas by the non-pathogenic T cell clone BDC-2.4 were very low. Fig. 7B shows that the macrophages recruited into the pancreas and recovered after T cell transfer are making substantial levels of TNF-α and that intracellular staining of this cytokine can be detected without ex vivo stimulation of the cells. These results suggest that the recruitment and stimulation of macrophages in the inflammatory site are a manifestation of pathogenic CD4 Th1 T cell effector function.

Effector function of CD4 T cells is detectable at low levels and only after stimulation ex vivo in spontaneous models of disease in the NOD mouse

To determine whether ex vivo analysis of the T cell clones is representative of CD4 T cell activity in spontaneous disease, we conducted ex vivo analyses of T cells isolated from the pancreas of a diabetic NOD or TCR-Tg mouse and compared them to the results obtained from an adoptive transfer into NOD.scid mice of either diabetic NOD spleen cells or a pathogenic T cell clone BDC-2.5. Thus, Fig. 8 compares ex vivo staining of TNF-α in T cells from pancreas in four disease models: 1) a diabetic NOD mouse, 2) a monoclonal TCR-Tg mouse expressing the TCR from the diabetogenic Th1 T cell clone, BDC-6.9, 3) a NOD.scid recipient of a diabetic spleen cell transfer, and 4) a NOD.scid recipient of a pathogenic clone, BDC-2.5. Although large numbers of T cells can be retrieved in the first three models, the cytokine-producing T cells that can be detected are very low in number unless the T cells receive additional stimulation ex vivo with PMA/ionomycin. The only exception to this rule is the fourth disease model of pathogenic T cell clone transfer in which substantial numbers of CD4 T cells can be shown to be making significant levels of cytokine directly upon recovery from the pancreas, without further stimulation. Two important points can be made from this experiment. The first point is that it is difficult to demonstrate significant numbers of cells producing cytokines in an effector response at any given time in spontaneous models of disease or spleen cell transfers. The second point is that the cytokine production of pathogenic T cell clones in the pancreas after transfer appears to accurately represent the activity of infiltrating T cells in spontaneous disease or spleen cell transfers, and that by using a defined T cell population to induce disease, an amplified picture of events can be obtained.
data illustrate the very considerable advantage of using defined T cell populations in adoptive transfers where no other T cells are present, i.e., sufficient numbers of cells can be recovered from the inflammatory site to allow for analysis of the activity of a single T cell clone. These experiments readily allow for the unambiguous assignment of effector function to a single T cell clone after it has migrated to the pancreas, using methods that are considerably less demanding than other single-cell isolation approaches. Our diabetogenic CD4 T cell clone lines were all isolated from different mice except in the case of two clones, BDC-6.3 and BDC-6.9, that are from the same line (and mouse) and have different TCRs. Yet, all of these clones are strikingly similar in ability to cause disease in young recipients and in their cytokine profiles. Such a panel thus demonstrates that the properties of CD4 effector function do not vary randomly from clone to clone, but in fact are consistent among pathogenic CD4 T cells.

A criticism often leveled at studies using T cell clones is that cell lines carried in culture are likely to be different from primary T cells isolated directly from the animal and despite the difficulty in demonstrating contributions of single T cell types, investigations with primary T cells are frequently considered to be more relevant. Our experiments have demonstrated that diabetogenic CD4 T cell lines with a defined and stable phenotype are highly representative of events occurring in the pancreatic lesion, whether in spontaneous disease in a wild-type or a TCR transgenic NOD mouse, or in adoptive transfers with spleen cells obtained from a diabetic NOD mouse. In all of these cases, T cells recovered from the pancreas display an activated cytokine-producing phenotype when stimulated ex vivo with PMA/ionomycin. However, in the absence of additional stimulus, the T cells mediating inflammation are few in number and difficult to detect. What the use of diabetogenic T cell clones provides is an amplified snapshot of these events: sufficient numbers of defined T cells can be obtained that are producing high levels of inflammatory cytokines directly upon recovery and without the need for additional stimulation ex vivo. This approach to analyzing effector function should therefore be applicable to a wide variety of questions about conditions that regulate T cell activity, issues such as migration, stage of disease, and the presence of other cell types.

One such question is whether the effector function of pathogenic CD4 Th1 T cells is manifested through other cells recruited to the inflammatory site to allow for analysis of the activity of a single T cell clone.
lesion. Having observed that large numbers of macrophages appear in the pancreas after adoptive transfer, we wanted to know whether their recruitment was a result of adoptive transfer with the pathogenic clone and if the macrophages were activated to make inflammatory cytokine. Our results comparing pathogenic Th1 T cells to nonpathogenic Th1 T cells showed a marked difference in the macrophages recruited. Numbers of CD11b cells in the pancreas after clone transfer were very low when the clone was non-pathogenic whereas adoptive transfer of a diabeticogenic T cell clone resulted in large numbers of TNF-producing macrophages, suggesting that these cells play a part in augmenting the inflammatory environment. It is well accepted that during inflammation, macrophages are recruited into the disease site and contribute to pathogenesis through production of cytokines and other mediators. Our results demonstrate that macrophage recruitment and activation occurs as a direct consequence of pathogenic CD4 T cells migrating into the site, suggesting that this is a manifestation of pathogenic T cell effector function. This observation also correlates well with the finding that the diabeticogenic T cell clones produce several chemokines important in the migration and stimulation of macrophages. The pathogenic potential of a T cell clone considered in terms of its ability to stimulate macrophage cytototoxic damage could thus be important for understanding the pathogenesis of autoimmune diabetes.

Finally, our analysis of pathogenic T cell clone activity, in vitro and in vivo, has confirmed and extended findings of others pointing to the importance of the cytokine TNF-α in autoimmune inflammation. In T1D, however, the role of TNF-α has been controversial, with apparently contradictory data as to whether it promotes or inhibits disease progression, depending on the stage of disease (or age of the animal). Our results, obtained with defined CD4 T cells, adoptively transferred and then retrieved from the inflammatory site, provide direct evidence that TNF-α has a central role in the effector function of pathogenic CD4 T cells involved in disease, as a factor secreted both by Th1 T cells and by the macrophages they recruit and activate. Fig. 9 poses a model of how TNF-α may function as a result of CD4 infiltration into the pancreas, contributing directly to cytotoxicity of β cells, up-regulating adhesion molecules in tissue, and increasing chemokine expression which leads to recruitment of other inflammatory cell types. These three tenets of the model are supported by studies showing the importance to development of disease of TNFR expression on islets (17), interaction of adhesion molecules (18–22), and production of chemokines (23). Our data indicating a central role for TNF-α produced by infiltrating T cells and macrophages is, for example, in good agreement with the finding that the presence of TNFR1 on islets was necessary for destruction mediated by 2.5 TCR-Tg T cells to take place (17). The importance of clearly delineating how TNF-α contributes to disease progression is underscored by the many efforts underway to identify therapeutic agents that target this molecule. As highlighted by our studies, a critical goal in this search is to determine which specific cell types are producing which forms of TNF-α at what point in the disease process.

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Disclosures

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References