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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. The Journal of Immunology, 2005, 175: 7738–7745.
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 used in this work as 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 supernatant 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 monitored for development of disease by urine glucose. The 6.9 TCR-Tg mouse was used in this work as 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.

**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 × 10⁶ 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 anti-CD8 Ab at 100 ng/ml, anti-CD3 Ab for an additional 24 h, at which time cells were harvested using Cell Dissociation Buffer (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. Polyconal intracellular staining Abs used were obtained from R&D Systems and included polyclonal goat IgG anti-TCR-α, 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-lymphoactin 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-INF-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 then analyzed on a FACSCalibur flow cytometer (BD Biosciences).

**Adaptive transfer of diabetics**

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 × 10⁶) 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 × 10⁶ 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 2 days was considered diagnostic of overt diabetes.

**Recovery of diabetogenic T cell clones**

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 incubator with gentle shaking. At 15 min, pancreas was digested in collagenase and washed several times with cold HBSS until the supernatant was clear. pancreatic islets were dispersed with a 20-gauge needle and the islet cell suspension was then filtered through a 70-μm cell strainer (BD Biosciences). Islets were then washed, suspended in HBSS, and dispensed into 48-well plates at 4–8×10⁶ islets/well. Islets were plated for 5 days, after which time the supernatant was aspirated and replaced with fresh media. Islets were then cultured in the presence of IL-2 (10 U/ml) and IL-15 (100 ng/ml) for 7 days. Media was changed daily, and islets were cultured in fresh media with IL-2 and IL-15 for a total of 10 days. Islets were then harvested, washed in HBSS, and stained with rat anti-CD4 or anti-CD8 Ab at 100 ng/ml and anti-CD3 Ab at 1 μg/ml. For intracellular cytokine staining, cells were cultured in the presence of GolgiPlug (BD Biosciences) and brefeldin A (1 μg/ml) for 3 h at 37°C. Intracellular cytokine staining was performed as above.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Th</th>
<th>TCR</th>
<th>Islet Ag Reactivity</th>
<th>Diabetic in NOD</th>
<th>(≤14 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDC-2.5</td>
<td>1</td>
<td>Vβ4Vαl</td>
<td>All mouse strains tested*</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>BDC-5.10.3</td>
<td>1</td>
<td>Vβ4Vα (ND)</td>
<td>All mouse strains tested</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>BDC-6.3</td>
<td>1</td>
<td>Vb4Vα3.1</td>
<td>All mouse strains tested</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>BDC-6.9</td>
<td>1</td>
<td>Vβ4Vα13.1</td>
<td>NOD, SWR</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>BDC-9.3</td>
<td>1</td>
<td>Vβ4Vα13.1</td>
<td>NOD, SWR</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>BDC-10.1</td>
<td>1</td>
<td>Vβ15Vα13</td>
<td>All mouse strains tested</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>BDC-2.4</td>
<td>1</td>
<td>ND</td>
<td>Nonreactive to islet cells</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>2.5 Tg/T2-X</td>
<td>2</td>
<td>Vβ4Vαl</td>
<td>Same as BDC-2.5</td>
<td>+</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, SJL).
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 in vitro culture of Th1 T cells isolated from NOD mice on day 12 posttransfer. Resting T cells from NOD mice were stimulated with anti-CD3 for 24 h. Cytokine production was measured by specific ELISA of supernatants from the stimulated T cell clones. 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 activation 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
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 chemokines, and as with other cytokines, the pattern was similar.

The proinflammatory cytokines IFN-γ and TNF-α are produced by pathogenic CD4+ 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

![FIGURE 3. In vitro intracellular chemokine staining of CD4+ Th1 T cell clones. T cell clones were harvested 3 days after expansion in IL-2 and cultured for an additional 24 h with or without additional stimulation with plate-bound anti-CD3 Ab or 5 h with PMA/ionomycin. GolgiPlug was added for the final 4 h; cells were harvested and fixed before intracellular staining with Abs specific for the indicated chemokines. The filled peaks represent specific staining while those that are unfilled show isotype control staining levels.](http://www.jimmunol.org/)

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>
</tr>
<tr>
<td>TNF-α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>IL-2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>IL-17B</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LT-α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mif</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>TCA-3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RANTES</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
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<tr>
<td>MIP-1α</td>
<td>+</td>
<td>+</td>
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<td>MIP-1γ</td>
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<td>MCP-3</td>
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<td>Lymphotactin</td>
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<td>C10</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>
intracellular cytokine production, with and without additional stimulation ex vivo. Fig. 4 shows that upon recovery from the pancreas and without any additional stimulus, both IFN-γ and TNF-α can be detected ex vivo in the pathogenic CD4⁺ Th1 clone BDC-2.5. This result identifies individual pathogenic CD4⁺ Th1 clones as major sources of these two critical inflammatory cytokines during the effector stage of an autoimmune attack in the pancreas and constitutes the first definitive demonstration of this cytokine response by diabetogenic T cells in the inflammatory site.

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

To determine whether the inflammatory cytokines produced in the pancreas by diabetogenic Th1 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 T cell 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
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 nonpathogenic 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, which is of particular relevance to this study, 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.
inherent differences between pathogenic and normal T cells. These
in the presence of anti-CD3 stimulation), suggests that there are
either in vitro or in vivo (but does produce inflammatory cytokines
nonpathogenic T cell clone BDC-2.4 does not respond to Ag/MHC
fers. The fact that pathogenic CD4 T cells produce inflammatory
cytokines and TNF-α, and TNF-α, suggests 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 clones are 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 diabe-
togenic 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
inflammation 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 dia-
abetic 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.

Discussion
We have described here studies on the effector function of patho-
genic CD4 Th1 T cells. Earlier studies addressing mechanisms of
CD4 T cell-mediated pathogenicity in the NOD mouse, using either
isolated T cell subsets or short-term T cell lines, have demon-
strated that mRNAs for a variety of cytokines and chemokines
are found in total pancreatic tissue after adoptive transfers (2). We
have confirmed and extended these findings, demonstrating that
mRNA for a large number of cytokines and chemokines, summa-
ized in Table I, are expressed specifically in diabetogenic T cells
after TCR stimulation in vitro. Moreover, we have shown by
ELISA or intracellular cytokine staining that most of these cyto-
kines and chemokines can be detected in the T cell clones at the
protein level. For the most part, detection of protein corresponded
very well with the gene array results, although in some cases ex-
pression at the protein level seemed to be stronger and more con-
sistent than was suggested by mRNA expression (e.g., for the che-
mokine RANTES). In a few instances (SLC, IL-17, MCP-3), we
either have not yet determined the conditions or have not obtained
reagents to confirm expression of a cytokine or chemokine at the
protein level.

In this report, we have also provided the first definitive demon-
stration that diabetogenic T cells are making the cytokines IFN-γ
and TNF-α upon recovery from the pancreas after adoptive trans-
fer. In addition, our data establish that production of these inflam-
matory cytokines is a hallmark of pathogenic CD4 clones and is
not observed if nonpathogenic CD4 Th1 T cells are used in trans-
fers. The fact that pathogenic CD4 T cells produce inflammatory
cytokines in response to islets in vitro and in vivo, and that the
nonpathogenic T cell clone BDC-2.4 does not respond to Ag/MHC
either in vitro or in vivo (but does produce inflammatory cytokines
in the presence of anti-CD3 stimulation), suggests that there are
inherent differences between pathogenic and normal T cells. These
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 dia-
betic 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, investiga-
tions 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 diabe-
togenic 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

![FIGURE 8. Ex vivo TNF-α response from CD4+ cells in spontaneous
vs adoptive transfer models. Single-cell suspensions prepared from pan-
creas of spontaneously diabetic mice are compared with those from mice
adoptively transferred with either spleen cells from a diabetic NOD or with
a diabetogenic T cell clone, BDC-2.5. After harvesting and 3–4 h of ex
vivo culture, cells were analyzed by intracellular flow cytometry for TNF-α
production by CD4+ T cells.]

![FIGURE 9. Central role of TNF-α. Upon stimulation by islet cell Ag,
pathogenic CD4 Th1 T cell clones migrate to the pancreas and secrete a
variety of proinflammatory cytokines and chemokines, including IFN-γ
and TNF-α. These cytokines and chemokines recruit and stimulate macro-
phages, which further contribute to the pool of TNF-α in the pancreatic
islets. TNF-α has multiple downstream effects: direct islet cell cytotoxicity,
up-regulation of adhesion molecules, and stimulation of chemokine
production.]}
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 nonpathogenic 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 cytophilic 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 which leads to recruitment of other inflammatory cell types, 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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