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Isolation of Self Antigen-Reactive Cells from Inflamed Islets of Nonobese Diabetic Mice Using CD4^{high} Expression as a Marker[1]

Kristina Lejon and C. Garrison Fathman[2]

The low precursor frequency of Ag-reactive CD4$^+$ T cells has been a barrier to the study of CD4$^+$ T cell responses to conventional Ags as well as CD4$^+$ T cell responses to autoantigens recognized during the course of an autoimmune disease. We have recently reported that all "conventional Ag" reactive CD4$^+$ T cells are contained within the subpopulation expressing high levels of the CD4 molecule, termed CD4^{high}. We have identified a CD4^{high} population in the islets of Langerhans of prediabetic nonobese diabetic (NOD) mice that is extremely potent in transferring disease. As few as 500 CD4^{high} islet-infiltrating CD4$^+$ T cells transferred insulin-dependent diabetes mellitus to CD8 reconstituted NOD-SCID mice within 30 days of transfer. In contrast, CD4^{high} T cells isolated from either NOD spleen or salivary glands did not transfer insulin-dependent diabetes mellitus into similar CD8-reconstituted NOD-SCID recipients. These data indicate that the precursor frequency of NOD islet-reactive, pathogenic CD4$^+$ T cells is much higher in the prediabetic NOD pancreas than in these other organs. The islet-infiltrating CD4^{high} T cells displayed selected memory markers, by cell surface analysis, and displayed a Th 1 phenotype by RNase protection assay, but had a marked decrease in IL-4 mRNA determined by quantitative real time PCR when compared with the less pathogenic CD4^{normal} islet-infiltrating T cells. Use of the CD4^{high} marker to select Ag activated T cells represents a tool to isolate and study pathogenic CD4$^+$ T cells from autoimmune lesions in which the Ag has not been previously defined. *The Journal of Immunology,* **1999, 163: 5708–5714.**

Insulin-dependent diabetes mellitus (IDDM)[3] is an autoimmune disease in which the insulin producing β cells of the islets of Langerhans are thought to be destroyed by cells of the immune system. The nonobese diabetic (NOD) mouse develops IDDM spontaneously, and, due to shared pathophysiology and markers of disease, is widely used as a model for human IDDM (1). Diabetes can be transferred with spleen cells from diabetic NOD mice into young irradiated or immunodeficient NOD recipients (2–4). Normally 10–20 $\times$ 10^6 spleen cells are transferred to recipients for 100% incidence of disease. Adoptive transfers with subsets of lymphoid cells from NOD mice have pointed out a role for both CD4$^+$ and CD8$^+$ T cells in the effector phase of the disease (5–11). However, B cells and (other) APCs play a crucial role in initiating the disease processes (12–16).

We have recently demonstrated that Ag-reactive CD4$^+$ T cells (following challenge with conventional Ag) reside in the CD4^{high} T cell subpopulation (17). In these studies, following stimulation in vitro of the draining lymph node cells from immunized animals, there was a marked up-regulation of CD4 on the Ag-reactive subset of T cells. Limiting dilution analysis (LDA) analyses showed that all of the Ag-reactive CD4$^+$ T cells could be found within the CD4^{high} subpopulation. More importantly, for the studies described below, up-regulation of cell surface expression of CD4 was also demonstrated on CD4$^+$ T cells sorted directly from the draining lymph nodes of Ag-primed mice (17). Using CD4^{high} as a marker, it was possible to enrich the Ag-reactive CD4$^+$ T cell precursor frequency in vivo from $>$1/10,000 to ~1/30 T cells (17).

Previous studies from our laboratory had demonstrated that T cells, isolated from the islets of prediabetic NOD mice, were much more efficient in disease transfer into NOD-SCID recipients than were spleen cells from the same aged prediabetic NOD mice (18). Thus, the precursor frequency of islet-reactive CD4$^+$ T cells was increased in the islets. Applying CD4^{high} as a marker for recent Ag activation in the NOD mouse model, we asked whether we could identify the autoreactive CD4$^+$ T cells within the islets. This approach would allow us to analyze the phenotype of the specific autoreactive CD4$^+$ T cells without any requirement of prior knowledge of the self-Ag recognized.

Materials and Methods

Mice

NOD mice were obtained from Taconic Farms (Germantown, NY) and used between 11 and 13 wk of age. NOD-SCID mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used as recipients between 4 and 6 wk of age. Only female mice were used in this study. All animals were maintained under specific pathogen-free condition in the Department of Comparative Medicine at Stanford University Medical School.

Antibodies

The GK1.5 Ab was purified from GK1.5 hybridoma supernatant on a protein G column (Pharmacia-Upjohn, Uppsala, Sweden) and dialyzed against PBS. R-PE-labeled anti-CD4 was from Caltag (South San Francisco, CA) and PharMingen (San Diego, CA). FITC-labeled anti-CD8 was from

Division of Immunology and Rheumatology, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305

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[2] Address correspondence and reprint requests to Dr. C. Garrison Fathman, Division of Immunology and Rheumatology, R-S021, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305-5111. E-mail address: cfathman@leland.stanford.edu

[3] Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; LDA, limiting dilution analysis; NOD, nonobese diabetic; RPA, RNase protection assay; PI, propidium iodide.

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Caltag. Anti-CD25 FITC, anti-CD69 FITC, anti-CD44 FITC, biotinylated anti-CD45RB, anti-CD62L biotin, and strepavidin-allophycocyanin (APC) were all from PharMingen. Propidium iodide (PI) (Sigma) was used at 1 μg/ml to exclude dead cells.

MiniMACS purification of CD8$^+$ cells and reconstitution of NOD-SCID mice

Single cells suspensions of freshly isolated pooled lymph node (inguinal, mesenteric, and paraaortic) and spleen cells from diabetic NOD female mice were counted and incubated with anti-CD8 magnetic microbeads (Miltenyi Biotec, Auburn, CA) in PBS supplemented with 3% FCS for 20 min at 10–12°C. FITC-labeled anti-CD8 Abs were added for the last 10 min. The cells were washed and purified by passage through magnetic columns according to the manufacturer's recommendation. The enriched population consisted of >90% CD8$^+$ T cells. The cells were resuspended in saline containing 50–100 μg of an anti-CD4 Ab (GK1.5), and 0.5–1 × 10^6 cells were injected i.v. into NOD-SCID recipients. The mice were injected with an additional 50 μg of GK1.5 i.p. for 2 additional days. The mice were allowed to recover for at least 7 days after the last Ab injection and then screened for CD8 reconstitution and CD4 depletion by FACS analysis of PBL before CD4 T cell transfer. Typically, 7 days after reconstitution, 5–10% of the PBL were CD8$^+$ and <0.6% stained for CD4$^+$ cells, a CD4 profile that is similar to the background staining of a nonreconstituted NOD-SCID mouse.

Histological examination

The pancreata and salivary glands from NOD-SCID recipient mice were removed, fixed in 10% formaldehyde, and embedded in paraffin. Thin sections at three levels, 50 μm apart, were cut for staining with hematoxylin and eosin. The severity of infiltration was assessed by light microscopy. At least 20 islets were examined per pancreas.

Lymphocyte preparation

Each donor NOD pancreas was cannulated and perfused with collagenase P (Boehringer Mannheim, Indianapolis, IN) dissolved in HBSS supplemented with penicillin-streptomycin, 4 mmol/liter $NaHCO_3$ and 0.22 mg/liter BSA. Excised pancreata was incubated for 20 min at 37°C, dispersed with a 10-ml pipette, washed, passed through a strainer, and placed on a discontinuous gradient of Ficoll (Sigma, St. Louis, MO) at 27%, 25%, 20.5%, and 11%, to isolate the islets. The islets were removed from the 11–20.5% interface, washed, and handpicked with siliconized pipettes under a dissecting microscope. The handpicked islets were dissociated into single cells by passage through a 18-gauge needle (five times) followed by 21-gauge needle (three times). Salivary gland-infiltrating lymphocytes were isolated by sectioning the donor NOD salivary glands into 3-mm pieces and incubating the segments with collagenase P (Boehringer Mannheim) dissolved in HBSS supplemented with penicillin-streptomycin, 4 mmol/liter $NaHCO_3$ and 0.22 mg/liter BSA. After 20-min incubation the tissue was minced through a steel strainer and, after centrifugation, mononuclear cells was enriched on a Lympholyte-M gradient (Cedarlane, Hornby, Ontario, Canada).

Spleen and lymph node lymphocytes were isolated by mincing the organ with the back of a 6-ml syringe in a dish before passing through a 70-μm nylon mesh cell strainer.

Cell transfer procedure

The sorted cells were resuspended in saline containing 10% normal mouse serum (from NOD) and injected i.v. at the numbers indicated. NOD-SCID recipients were checked for glucosurea using Glucosstix (Boehringer Mannheim) every other day. If glucosurea was observed, the blood glucose was measured with a One Touch II meter (Johnson & Johnson, Milpitas, CA). Mice with glucosurea and a blood glucose >250 mg/dl were considered diabetic. Glucosurea always coincided with high blood glucose levels.

FACS sorting and FACS analysis

The isolated islet cells were resuspended in PBS containing 3% FCS (FACS medium) and stained for two-, three-, or four-color analysis with FITC-, PE-, and APC-labeled Abs using a predetermined optimal concentration for 20 min at 4°C, and washed once in FACS medium. PI was used in all samples to exclude dead cells. Before sorting, the samples were passed through a 70-μm nylon mesh. The sorting CD4^{high} gate for islets and salivary glands was set to include the top 5–10% of all CD4$^+$ cells, an the CD4^{normal} gate was set to include CD4$^+$ T cells whose CD4 expression was just under the mean fluorescence for the entire population (representing 20% of all CD4$^+$ T cells). The yield from twenty 11-wk-old pancreas donors was on average 20,000–30,000 CD4^{high} cells. The yield from twenty 11-wk-old salivary gland donors was, on average, 5000 cells. Sorting CD4^{high} from the spleen was done on the top 3%. This more stringent gate was set to ensure the enrichment of CD4 ^{high} cells with a similar CD4 expression level as on the CD4^{high} cells from the islets.

The cells were sorted for different levels of CD4 expression on a FACStar (Becton Dickinson, Mountain View, CA). Three- and four-color analysis of surface marker expression was done on a FACScan or FACSVantage. All staining and sorting procedures were done aseptically. The data were analyzed using the Herzenberg desk facility (Stanford University), FlowJo 2.7.8 (Tree Star, San Carlos, CA), and CellQuest (Becton Dickinson) on a Power Macintosh G3 (Apple Computer, Cupertino, CA).

Analysis of cytokine mRNA by RNase protection assays (RPA)

Isolated CD4^{high} (1–2 × 10^5) and CD4^{normal} (2 × 10^5) T cells were stimulated with PMA (1 μg/ml) and ionomycin (0.25 μg/ml) (both from Sigma) for 2–4 h in RPMI 1640 medium supplemented with 10% FCS, nonessential amino acids, L-glutamine, sodium pyruvate, HEPES, 2-ME, and penicillin/streptomycin in 37°C in 6% CO_2. After stimulation, the cells were washed once in ice-cold PBS and frozen in −80°C. RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). Probes were labeled with [α-^{32}P]UTP and hybridized with the isolated RNA. The probe set used was mCK-1 (PharMingen) and it detects IL-10, IL-4, IL-5, IL-2, IL-6, IFN-γ, GADPH, and L32. After digestion of single-stranded RNA, the protected fragments were separated by PAGE. Controls included the probe set hybridized to tRNA only, and to tRNA plus a pool of synthetic sense RNAs complementary to the probes set. All steps were performed according to manufacturers' recommendation.

Analysis of cytokine mRNA by quantitative RT-PCR

Isolated CD4^{high} (0.3–2 × 10^5) and CD4^{normal} (0.3–2 × 10^5) T cells were stimulated with PMA and ionomycin for 2 h, and RNA was extracted as mentioned above for the RPA. The RNA sample was treated with DNase to remove all traces of genomic DNA and reverse transcribed with MultiScribe reverse transcriptase (PE Applied Biosystems, Foster City, CA) in the presence of hexamers, according to the manufacturer's instructions.

For IL-4, IFN-γ, and GAPDH, real time quantitative PCR was performed in the ABI Prism 7700 sequence detector, which contains a GeneAmp PCR system R600 (PE Applied Biosystems). Reaction conditions were programmed on a Power Macintosh 7200 (Apple Computer) linked directly to the 7700 Sequence detector. The IL-4 and IFN-γ probes were labeled with the fluorescent reporter dye FAM (6-carboxyfluorescin, covalently linked to the 5′ end of the oligonucleotide) and a quencher, TAMRA (6-carboxytetramethylrhodamine, attached at the 3′ end via a linker arm). The 5′ end of the GAPDH probe was labeled with the fluorescent reporter dye VIC and the 3′ end was linked to the quencher, TAMRA. The primer and probe sequences used were the following: 5′ IFN-γ primer, TCCTGCGGCCTAGCTCTGA; 3′ IFN-γ primer, GCCATGAG GAAGAGCT; IFN-γ probe, ACAATGAACGCTACACACTGCATC TTGGC; 5′ IL-4 primer, CATCGGCATTTTGAA; 3′ IL-4 primer, CGTTT GGCACATCCATCTCC; IL-4 probe, CACAGGAGAAGGGACGCCAT GCA; 5′ GAPDH primer, TGCACCACCAACTGCTTA; 3′ GAPDH primer, GGATGCAGGGATGATGTT; and GAPDH probe, CAGAAGACTGTGGA TGGCCCCTC. All reactions were performed using the TaqMan Gold RT-PCR kit according the manufacturer's recommendation (PE Applied Biosystems). Whole mouse cDNA (PharMingen) was used as standard. The detection limit of IFN-γ and IL-4 in the standard was 1 ng input of the total mouse cDNA. For the different runs cDNA corresponding to 300-5000 cells was used. A normalization to GAPDH was performed for each sample.

Results

CD4 expression of islet-infiltrating T cells of prediabetic NOD mice

Prompted by the observation that CD4^{high} T cells were Ag reactive following immunization with conventional Ag in vivo (17), we asked whether it was possible to isolate this subpopulation from an inflammatory site of an ongoing autoimmune response. To explore this possibility, we isolated islets from prediabetic NOD female mice (age 11 wk) and examined the CD4 expression pattern of islet-infiltrating lymphocytes. As shown by data presented in Fig. 1, there was increased CD4 expression (when compared with lymph node and spleen cells from the same mice) on a small subset

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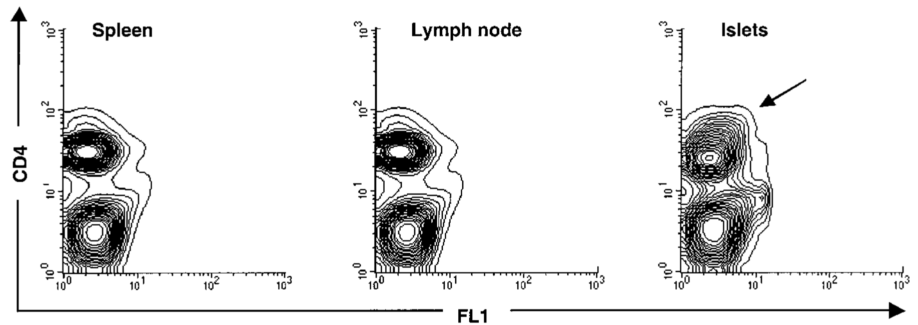

FIGURE 1. CD4 expression on lymph node (mesenteric and inguinal), spleen, or islet-infiltrating T cells. Cells were isolated and pooled from five to twenty 11-wk-old NOD donors as described in *Materials and Methods* and stained with anti-CD4PE Abs and PI. PI-negative cells are displayed in the contour plot. This figure represents a staining pattern that has been reproduced more than 20 times.

of islet-infiltrating lymphocytes. This was demonstrated by comparing the expression of CD4 on lymphocytes from lymph nodes and spleen with the expression on islet-infiltrating T cells. The staining of the lymph node and spleen $CD4^+$ T cells demonstrated a very tight homogenous pattern, whereas the islet-infiltrating T cells displayed heterogeneity in CD4 expression and contained a small population that displayed increased levels of CD4 on their surface. These "$CD4^{high}$ islet-infiltrating T cells" represented ~5% of all $CD4^+$ T cells in the islet-infiltrating population.

Transfer of $CD4^{high}$ T cells to CD8 reconstituted NOD-SCID mice induced diabetes

To assay the pathogenic potential of the islet-infiltrating $CD4^{high}$ T cells, we performed a series of transfers into immunodeficient NOD-SCID mice that had been previously reconstituted with $CD8^+$ T cells from diabetic NOD mice. We reconstituted the recipients with $CD8^+$ T cells from diabetic recipients before cell transfer, because it had been previously shown that both $CD4^+$ and $CD8^+$ cells were required for transfer of diabetes into immunodeficient NOD recipients (5–12). Transfer of 5000 islet-infiltrating $CD4^{high}$ T cells induced diabetes in 12 of 12 of the recipients within 40 days, whereas only 2 of 12 of the recipients of 5000 $CD4^{normal}$ islet-infiltrating T cells developed diabetes during this time period (Fig. 2). Transfer of 1000 $CD4^{high}$ cells proved to be almost as effective as transfer of 5000 $CD4^{high}$ T cells; 9 of 10 recipients of 5000 $CD4^{high}$ T cells developed diabetes within 50 days following transfer. However, only 2 of 12 mice receiving 1000 $CD4^{normal}$ islet-infiltrating T cells developed diabetes within 60 days. (Fig. 2). The difference in diabetogenic potential of these two subpopulations of islet-infiltrating T cells was not due to differences in their "reconstitution" potential; mice receiving $CD4^{high}$ or $CD4^{normal}$ islet-infiltrating T cells displayed equal reconstitution of CD4 T cells in peripheral lymphoid organs of the recipient mice, including the spleen, mesenteric lymph nodes, and pancreatic lymph nodes (data not shown). Transfer of 5000 $CD4^{total}$ cells led to the development of diabetes in 5 of 7 the recipients in 60 days, but with a delayed kinetics compared with the mice that received 5000 or 1000 $CD4^{high}$ T cells (Fig. 2). None of the mice receiving CD8 T cells only (0/18) developed diabetes during the period of observation (56 days).

To determine the lowest number of $CD4^{high}$ islet-infiltrating T cells required for diabetes transfer, we performed a series a transfers using 200–250 or 500 $CD4^{high}$ islet-infiltrating T cells isolated as described above. As shown by data presented in Fig. 3, 1 of 4 of the mice receiving 500 $CD4^{high}$ T cells and 0 of 10 of the recipients transferred with 200–250 $CD4^{high}$ T cells developed diabetes within 60 days of transfer.

As few as 200 $CD4^{high}$ T cells can lead to intraislet infiltration

Although the mice receiving 200–250 $CD4^{high}$ islet-infiltrating T cells did not progress to diabetes during the time course of this study, it was possible that the mice might have developed islet infiltration without β cell destruction and resultant "diabetes." Therefore, we examined by histopathology the pancreata of the NOD-SCID recipients that received 200–250 $CD4^{high}$ islet-infiltrating T cells to ask whether pancreatic-infiltrating-lymphocytes (insulitis) could be found in these mice. As shown by data presented in Table I and Fig. 4, 5 of 10 mice receiving 200–250 $CD4^{high}$ cells had intraislet infiltration. All of the NOD-SCID mice that received 500 $CD4^{high}$ cells (or more) had intraislet infiltration, whereas none of the mice receiving CD8 cells only displayed any signs of pancreatic infiltration. Only 6 of the 10 mice that received

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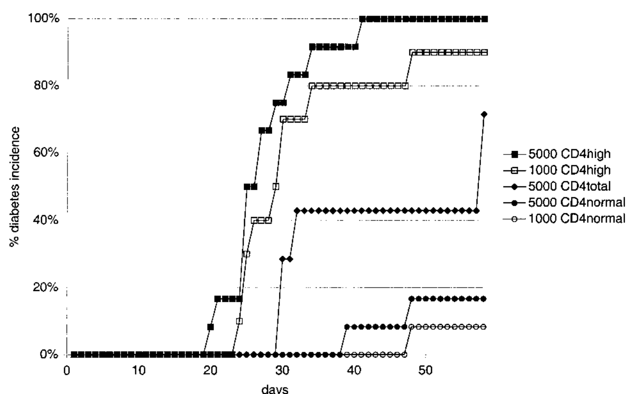

FIGURE 2. Islet $CD4^{high}$ T-cells efficiently transfer diabetes. $CD4^{high}$, $CD4^{normal}$, and $CD4^{total}$ T cells were isolated from 11- to 12-wk-old donors, and aliquots of these cells were transferred into CD8 T cell reconstituted NOD-SCID recipients as indicated.

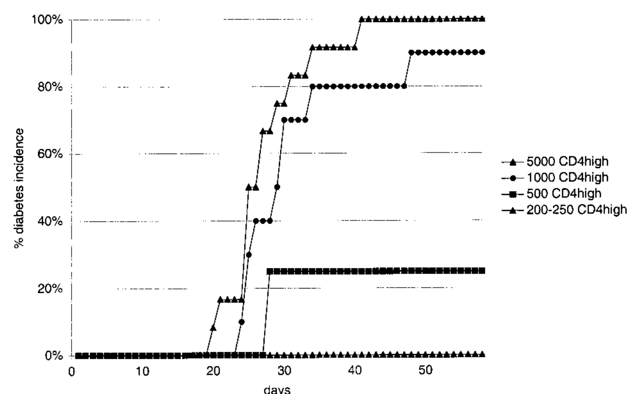

FIGURE 3. As few as 500 $CD4^{high}$ T cells efficiently transferred diabetes. $CD4^{high}$ T cells were isolated from 11- to 12-wk-old donors, and aliquots of these cells were transferred into CD8 T cell reconstituted NOD-SCID recipients as indicated.

Table I. *Frequency of insulitis and diabetes in NOD-SCID recipients*

Source	Subset	Number of Cells	Recipients with Intraislet Infiltration	Recipients with Diabetes
Islets	CD4^{high}	5000	5/5	12/12
		1000	8/9	9/10
		500	3/3	1/4
		200–250	5/10	0/10
Spleen	CD4^{high}	5000	1/4	0/5
Salivary glands	CD4^{high}	1000	1/6	0/6
Islets	CD4^{normal}	5000	6/10	2/12
		1000	1/6	2/12
CD8 only			0/4	0/18

5000 CD4^{normal} and 1of 6 mice that received 1000 CD4^{normal} islet-infiltrating T cells showed any signs of intraislet infiltration. These data confirmed the pathogenic potential of the CD4^{high} islet-infiltrating T cells, and demonstrated that it was dose dependent. Thus, transfer of 200–250 CD4^{high} islet-infiltrating T cells lead to severe inflammation, but at least 500 CD4^{high} islet-infiltrating T cells were required for overt diabetes to occur within the time frame of these studies.

Diabetogenic CD4^{high} T cells can be easily isolated from the pancreas, but not other organs, of NOD mice

Although diabetes can be easily and rapidly transferred to recipient immunodeficient NOD mice with spleen cells from overtly diabetic NOD mice, spleen cells from prediabetic mice are very inefficient in transferring diabetes in the same models (19). It is possible that this inefficiency might be due to the low precursor frequency of islet-reactive T cells in spleens of prediabetic NOD mice. Therefore, we sorted the "brightest" 3% of the CD4^{+} cells from the spleens of 11-wk-old NOD mice and transferred 5000 cells to CD8 reconstituted NOD-SCID mice. As shown by data presented in Table I, none of the mice (0/5) that received CD4^{high} spleen cells developed diabetes within 60 days of observation. Only 1 of 4 mice examined had signs of intraislet infiltration on histopathological examination.

NOD mice develop lymphocyte infiltration in all major endocrine organs including the salivary glands, adrenal glands, and the thyroid. To determine whether NOD CD4^{high} T cells isolated from another site of lymphocyte infiltration were diabetogenic, we isolated and transferred CD4^{high} T cells from inflamed salivary glands of 11-wk-old NOD mice. As shown by data presented in Table I,

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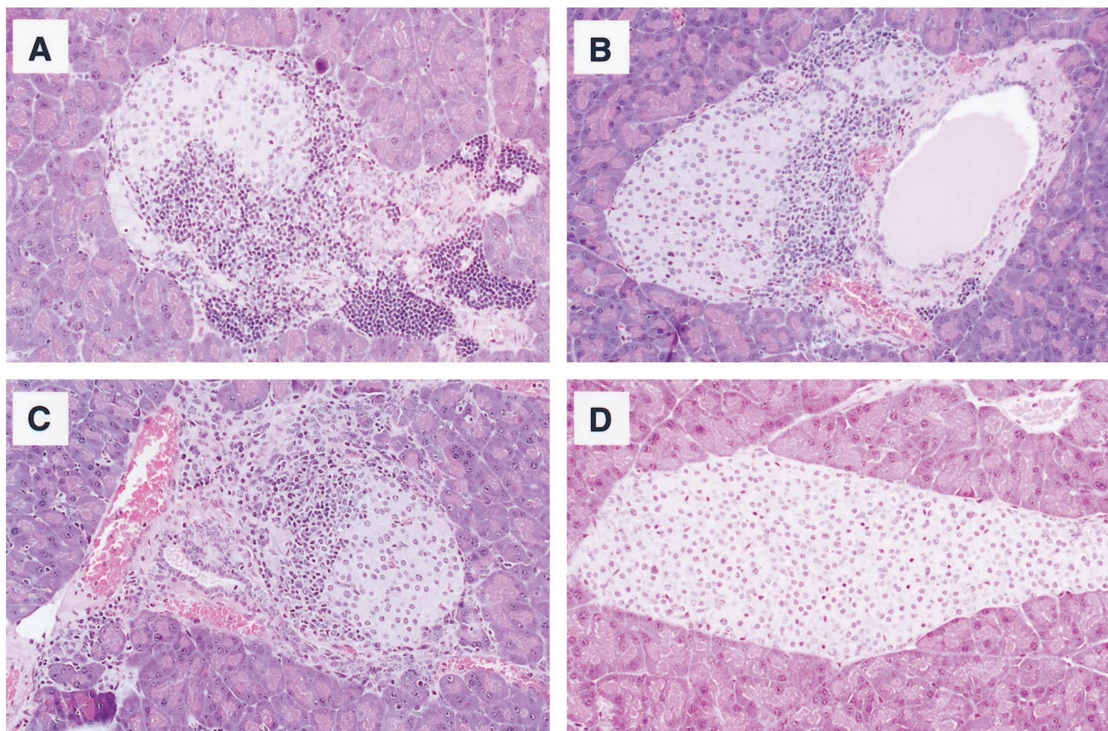

FIGURE 4. Histological analysis of pancreata from CD8 reconstituted NOD-SCID mice receiving CD4^{high} T cells (or no CD4 cells). Mice were sacrificed within 2 wk after the onset of diabetes and their pancreata were fixed with formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin. NOD-SCID mice receiving 5000 CD4^{high} cells (*A*), 500 CD4^{high} cells (*B*), 200–250 CD4^{high} cells (*C*), or no CD4 cells (CD8 reconstituted only) (*D*). All sections are shown at ×20 magnification.

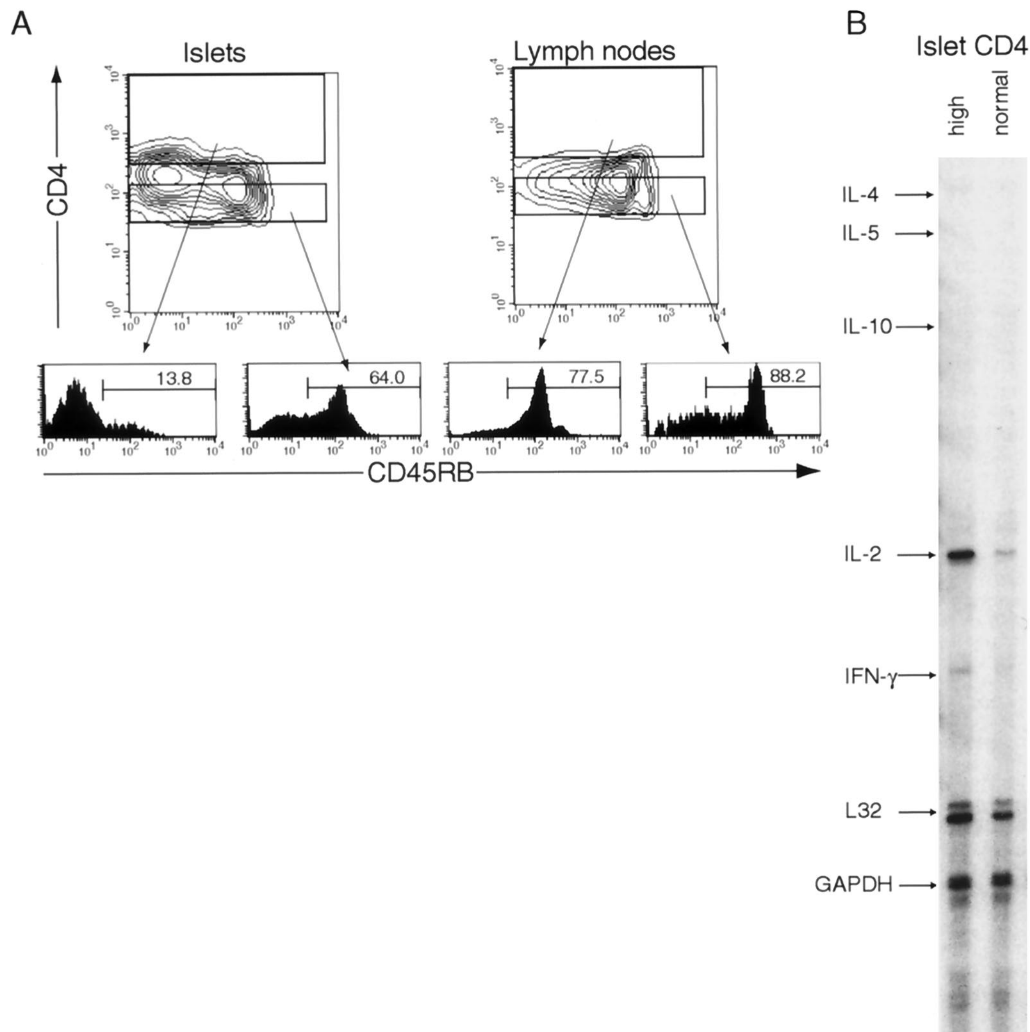

FIGURE 5. Islet-infiltrating CD4^{high} T cells display a memory phenotype and exhibit a Th1-type cytokine profile. *A*, Islet-infiltrating CD4^{+} T cells were stained with selected Abs as indicated. Live (PI-negative) cells are displayed in the figure. *B*, Cytokine mRNA as detected in CD4^{high} and CD4^{normal} T cells isolated from islets. FACS sorted cells were stimulated with PMA and ionomycin for 2 h before RPA. Individual bands were identified with two control sets of labeled probes, one that identifies the labeled probe and the other that gives the length of the protected fragment.

none of the mice (0/6) that received 1000 CD4^{high} T cells from NOD salivary glands developed diabetes within 60 days of transfer. Next, we compared the infiltration pattern of the pancreas and salivary glands of the two groups receiving either 200-1000 islet CD4^{high} cells or 1000 salivary gland CD4^{high} cells. Interestingly, there was a preferential homing to the islets of islet CD4^{high} cells compared with salivary gland CD4^{high} cells. Of the islet CD4^{high} cell recipients 67% displayed intraislet infiltration ($n = 24$) compared with 16.7% ($n = 6$) of the mice receiving salivary gland CD4^{high} cells. Reciprocally, we could detect a preferential homing of salivary gland CD4^{high} cells to the salivary glands. Of the mice receiving salivary gland CD4^{high} cells, 67% ($n = 6$) displayed sialitis, whereas only 25% of the islet CD4^{high} cell recipients showed any signs of salivary gland infiltration.

The islet-infiltrating CD4^{high} T cells display a memory phenotype

In our previous studies of the T cell response to a conventional Ag, we demonstrated that, after challenge with Ag in vitro, there was a marked shift in the cell surface expression of activation/memory markers on CD4^{high} T cells (17). To further characterize the islet-infiltrating CD4^{high} T cells, we stained the islet-infiltrating T cells using a panel of Abs that recognized cell surface markers that are expressed differentially on newly activated or on "memory" T cells. As shown by data presented in Fig. 4, the majority of islet-infiltrating CD4^{high} lymphocytes expressed the cell surface marker CD45RB^{low}, characteristic of memory T cells, compared with lymph node cells isolated from the same animals. Moreover, islet-infiltrating CD4^{high} lymphocytes were also CD62L^{low} compared with lymph node cells isolated from the same animals (data not shown). However, $<$5% of the islet-infiltrating CD4^{high} T cells expressed other activation/memory markers, including CD69, CD25, and CD44 (data not shown).

CD4^{high} islet-infiltrating T cells display a IL-4 low, Th1 cytokine profile

It has been suggested that diabetes in the NOD mouse is a Th1-mediated disease. Islet Ag-reactive T cell clones and lines have been shown to produce Th1 type cytokines. Moreover, T cells isolated from BDC2.5 TCR transgenic NOD mice are highly pathogenic if they have been diverted toward a Th1 pathway before transfer. For these reasons, we investigated the cytokine profile of islet-infiltrating CD4^{+} T cells using an RPA. Islet-infiltrating CD4^{+} T lymphocytes from 11- to 12-wk-old NOD mice were

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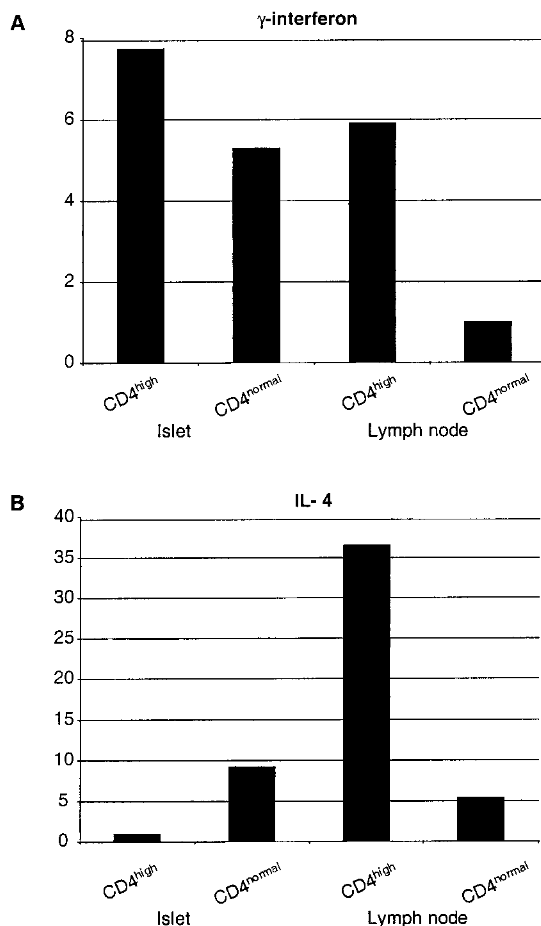

FIGURE 6. Islet CD4^{high} cells display a strong Th1 phenotype. *A*, Relative expression of IFN-γ mRNA in CD4^{high} and CD4^{normal} cells isolated from islets and lymph nodes. *B*, Relative expression of IL-4 mRNA in CD4^{high} and CD4^{normal} cells isolated from islets and lymph nodes. After normalization of the values to GAPDH mRNA expression, the lowest expresser in the group was set as 1, and the other values related to that data set. This figure shows the results of one out of three independent experiments.

isolated and sorted into CD4^{high} and CD4^{normal} populations. The CD4^{high} gate was set to consist of the top 10% of CD4^{+} T cells, and the CD4^{normal} gate was set to include CD4^{+} T cells whose CD4 expression was just under the mean fluorescence for the entire CD4 population. As shown by data presented in Fig. 5*B*, both the CD4^{high} and the CD4^{normal} subpopulations of islet-infiltrating T cells expressed IFN-γ and IL-2 by RPA, but seemed to lack expression of typical Th2 cytokines such as IL-4, IL-5, and IL-10.

Due to the possibility of too low a sensitivity of the RPA to detect low mRNA expression levels, we performed quantitative PCR assays on the CD4^{high} and CD4^{normal} populations isolated from islets and lymph nodes. Islet-infiltrating CD4^{+} T lymphocytes from 11- to 12-wk-old NOD mice were isolated and sorted into CD4^{high} and CD4^{normal} populations, and IFN-γ and IL-4-specific real time RT-PCRs were performed. As an internal control, we used GAPDH mRNA expression. As is demonstrated by data in Fig. 6, the CD4^{high} population made slightly more IFN-γ than did the CD4^{normal} islet-infiltrating population. However, there was a marked reduction in the amount of IL-4 made by the CD4^{high} when compared with the CD4^{normal} islet-infiltrating cells. The lymph node CD4^{high} expressed almost as much IFN-γ as the islet CD4^{high} population, but displayed over 30-fold higher expression of IL-4. The lymph node CD4^{normal} cells had low expression of both IFN-γ and IL-4.

Discussion

Analysis of Ag-reactive CD4^{+} T cells in vivo, in particular, self-Ag-reactive CD4^{+} T cells, has been a major problem in immunology. The frequency of Ag-specific CD4^{+} T cells is quite low, representing less than 1/5–10,000 lymph node cells from primed mice (20–23). The study of the CD4^{+} T cell response in vivo has relied mainly upon adoptive transfer of T cell clones or on studies using T cells from TCR transgenic mice (24–27). In this current study, we demonstrated that it was possible to isolate self-Ag-reactive, pathogenic CD4^{+} T cells, without any prior knowledge of the self-Ag(s) recognized by the T cells. The method of identifying self-Ag-reactive CD4^{+} T cells was derived from our previous demonstration that it was possible to isolate Ag specific CD4^{+} T cells following an immune response to a foreign Ag (myoglobin peptide, residues 110–121), by selecting CD4^{+} T cells that expressed high levels of CD4 (17). Sorting and subsequent transfer of disease with the CD4^{high} subpopulation from inflamed islets of prediabetic NOD mice, as described in this manuscript, demonstrated the potential of using this marker to identify disease specific autoantigen-reactive T cells, isolated from the inflammatory lesions of autoimmune disease.

That some CD8 reconstituted NOD-SCID mice developed diabetes after receiving islet-infiltrating CD4^{normal} T cells could be due to several possibilities. It is possible that the CD4 cell surface expression is not truly bimodal, or there may be some CD4^{+} T cells that are islet-reactive but had not yet up-regulated CD4, or these cells may represent an Ag-specific population that has modulated the expression of CD4 over time. We favor the explanation that the lack of a distinct border between CD4^{high} and CD4^{normal} cells, as separated by the FACS, or the actual contamination of the CD4^{normal} population by a small number of CD4^{high} T cells during the FACS separation procedures, allowed a population of activated T cells to be present in the CD4^{normal} population.

Interestingly, the analysis of the phenotype of the CD4 islet-infiltrating T cells revealed that both CD4^{normal} and CD4^{high} islet-infiltrating T cells displayed a relatively similar phenotype of cell surface markers as well as cytokine profile by RPA (Th1 type). However, the two subpopulations did not have equal pathogenic potential. These data suggested as one possibility that, secondary to an "inflammatory process" in the pancreas and resultant release of cellular attractants, there was a general influx of Th1 T cells into the islets, possibly due to inflamed tissue release of a chemokine that attracted Th1 T cells (28). In the presence of the islet "inflammation" among the Th1 T cells that had been recruited relatively nonspecifically to the islets, it was possible that a small subpopulation expressed islet-reactive TCRs and recognized islet β cell Ags. Due to inflammation and tissue breakdown, and, following Ag recognition and activation, these CD4 positive T cells up-regulated their cell surface expression of CD4. The non-self-Ag-reactive Th1 cells, also recruited to the islets by the inflammatory events, simply represented "innocent bystanders" secunded to the lesion by adhesion (chemokine attraction, and, presumably, could readily migrate out of the lesion) (29). An additional possibility was that the two subpopulations were different in the amount of cytokines they secreted, and that the RPA was not sensitive enough to detect this difference. Therefore, to increase the sensitivity of determination of the cytokine mRNA expression profile, we analyzed CD4^{high} cells and CD4^{normal} cells using real time RT-PCR. Although the IFN-γ mRNA expression was slightly increased in the islet CD4^{high} cells compared with the islet CD4^{normal} cells, the

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amount of IL-4 secreted by the two populations was quite distinct. The CD4^{high} population had a marked reduction in IL-4 secretion when compared with the CD4^{normal} islet-infiltrating population, and both populations secreted less IL-4 than did the lymph node CD4 cells. This suggests that it may be the lack of regulation, and not the existence of "superpathogenic" cells within the islet CD4^{high} population, that makes this population highly diabetogenic.

One unexplained phenomenon of our observations is the homing of the small population (500 cells) of islet-reactive CD4^{high} diabetogenic T cells back to the noninflamed islets in the CD8-reconstituted NOD-SCID recipients. Moreover, we found the islet-infiltrating cells in general preferentially homed to the islets and that the salivary gland-infiltrating CD4$^+$ T cells reinfiltrated the salivary glands. In addition, the CD8 reconstituted mice had no detectable insulitis in the absence of transferred CD4^{high} T cells, thus there was no inflamed endothelium or injured tissue to act as an attractant. This suggests, as one possibility, that CD4^{high} T cells, activated and/or educated in the islets, have acquired a specific islet homing activity that is not dependent upon prior inflammation of the targeted tissue. It is equally possible that the islet-reactive CD4^{high} T cells were activated in another site in the NOD-SCID recipient, expanded, and then homed to the islets. The nature of this potential tissue specific homing is under investigation.

Our studies have provided a novel technique to isolate and study the small population of pathogenic T cells in inflamed organs of tissue-specific autoimmune diseases in the absence of knowledge of the inciting Ag. By isolating these tissue-specific autoantigen-reactive CD4^{high} T cells, it will be possible to study their phenotype and Ag specificity.

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References

1. Makino, S., K. Kunimoto, Y. Muraoka, Y. Mizushima, K. Katagiri, and Y. Tochino. 1980. Breeding of a non-obese, diabetic strain of mice. *Jikken Dobutsu 29:1.*
2. Prochazka, M., H. R. Gaskins, L. D. Shultz, and E. H. Leiter. 1992. The nonobese diabetic scid mouse: model for spontaneous thymomagenesis associated with immunodeficiency. *Proc. Natl. Acad. Sci. USA 89:3290.*
3. Wicker, L. S., B. J. Miller, and Y. Mullen. 1986. Transfer of autoimmune diabetes mellitus with splenocytes from nonobese diabetic (NOD) mice. *Diabetes 35:855.*
4. Soderstrom, I., M. L. Bergman, F. Colucci, K. Lejon, I. Bergqvist, and D. Holmberg. 1996. Establishment and characterization of RAG-2 deficient nonobese diabetic mice. *Scand. J. Immunol. 43:525.*
5. Bendelac, A., C. Boitard, P. Bedossa, H. Bazin, J. F. Bach, and C. Carnaud. 1988. Adoptive T cell transfer of autoimmune nonobese diabetic mouse diabetes does not require recruitment of host B lymphocytes. *J. Immunol. 141:2625.*
6. Boitard, C., A. Bendelac, M. F. Richard, C. Carnaud, and J. F. Bach. 1988. Prevention of diabetes in nonobese diabetic mice by anti-I-A monoclonal antibodies: transfer of protection by splenic T cells. *Proc. Natl. Acad. Sci. USA 85:9719.*
7. Hayward, A. R., and M. Shreiber. 1989. Neonatal injection of CD3 antibody into nonobese diabetic mice reduces the incidence of insulitis and diabetes. *J. Immunol. 143:1555.*
8. Harada, M., and S. Makino. 1986. Suppression of overt diabetes in NOD mice by anti-thymocyte serum or anti-Thy 1, 2 antibody. *Jikken Dobutsu 35:501.*
9. Koike, T., Y. Itoh, T. Ishii, I. Ito, K. Takabayashi, N. Maruyama, H. Tomioka, and S. Yoshida. 1987. Preventive effect of monoclonal anti-L3T4 antibody on development of diabetes in NOD mice. *Diabetes 36:539.*
10. Miller, B. J., M. C. Appel, J. J. O'Neil, and L. S. Wicker. 1988. Both the Lyt-2$^+$ and L3T4$^+$ T cell subsets are required for the transfer of diabetes in nonobese diabetic mice. *J. Immunol. 140:52.*
11. Shizuru, J. A., C. Taylor-Edwards, B. A. Banks, A. K. Gregory, and C. G. Fathman. 1988. Immunotherapy of the nonobese diabetic mouse: treatment with an antibody to T-helper lymphocytes. *Science 240:659.*
12. Forsgren, S., A. Andersson, V. Hillorn, A. Soderstrom, and D. Holmberg. 1991. Immunoglobulin-mediated prevention of autoimmune diabetes in the non-obese diabetic (NOD) mouse. *Scand. J. Immunol. 34:445.*
13. Serreze, D. V., H. D. Chapman, D. S. Varnum, M. S. Hanson, P. C. Reifsnyder, S. D. Richard, S. A. Fleming, E. H. Leiter, and L. D. Shultz. 1996. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice. *J. Exp. Med. 184:2049.*
14. Yang, M., B. Charlton, and A. M. Gautam. 1997. Development of insulitis and diabetes in B cell-deficient NOD mice. *J. Autoimmun. 10:257.*
15. Jansen, A., F. Homo-Delarche, H. Hooijkaas, P.J. Leenen, M. Dardenne, and H.A. Drexhage. 1994. Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and β-cell destruction in NOD mice. *Diabetes 43:667.*
16. Lee, K. U., K. Amano, and J. W. Yoon. 1988. Evidence for initial involvement of macrophage in development of insulitis in NOD mice. *Diabetes 37:989.*
17. Ridgway, W., M. Fassò, and C. G. Fathman. 1998. Following antigen challenge, T cells up-regulate cell surface expression of CD4 in vitro and in vivo. *J. Immunol. 161:714.*
18. Rohane, P. W., A. Shimada, D. T. Kim, C. T. Edwards, B. Charlton, L. D. Shultz, and C. G. Fathman. 1995. Islet-infiltrating lymphocytes from prediabetic NOD mice rapidly transfer diabetes to NOD-*scid/scid* mice. *Diabetes 44:550.*
19. Christianson, S. W., L. D. Shultz, and E. H. Leiter. 1993. Adoptive transfer of diabetes into immunodeficient NOD-*scid/scid* mice: relative contributions of CD4$^+$ and CD8$^+$ T-cells from diabetic versus prediabetic NOD.NON-Thy-1a donors. *Diabetes* 42:44.
20. Ford, D., and D. Burger. 1983. Precursor frequency of antigen-specific T cells: effects of sensitization in vivo and in vitro. *Cell. Immunol. 79:334.*
21. Gebel, H. M., J. R. Scott, C. A. Parvin, and G. E. Rodey. 1983. In vitro immunization to KLH. II. Limiting dilution analysis of antigen-reactive cells in primary and secondary culture. *J. Immunol.* 130:29.
22. Kojima, M., K. B. Cease, G. K. Buckenmeyer, and J. A. Berzofsky. 1988. Limiting dilution comparison of the repertoires of high and low responder MHC-restricted T cells. *J. Exp. Med. 167:1100.*
23. Weyand, C. M., J. Goronzy, M. J. Dallman, and C. G. Fathman. 1986. Administration of recombinant interleukin 2 in vivo induces a polyclonal IgM response. *J. Exp. Med. 163:1607.*
24. Garside, P., E. Ingulli, R. R. Merica, J. G. Johnson, R. J. Noelle, and M. K. Jenkins. 1998. Visualization of specific B and T lymphocyte interactions in the lymph node. *Science 281:96.*
25. Kearney, E. R., K. A. Pape, D. Y. Loh, and M. K. Jenkins. 1994. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity 1:327.*
26. Khoruts, A., and M. K. Jenkins. 1996. Studying immunological tolerance by physically monitoring antigen-specific T cells in vivo. *Ann. NY Acad. Sci. 778:72.*
27. Pape, K. A., E. R. Kearney, A. Khoruts, A. Mondino, R. Merica, Z. M. Chen, E. Ingulli, J. White, J. G. Johnson, and M. K. Jenkins. 1997. Use of adoptive transfer of T-cell-antigen-receptor-transgenic T cell for the study of T-cell activation in vivo. *Immunol. Rev. 156:67.*
28. Bradley, L. M., V. C. Asensio, L. K. Schioetz, J. Harbertson, T. Krahl, G. Patstone, N. Woolf, I.L. Campbell, and N. Sarvetnick. 1999. Islet-specific Th1, but not Th2, cells secrete multiple chemokines and promote rapid induction of autoimmune diabetes. *J. Immunol. 162:2511.*
29. Horwitz, M. S., L. M. Bradley, J. Harbertson, T. Krahl, J. Lee, and N. Sarvetnick. 1998. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat. Med. 4:781.*

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