Loss of IL-4 Secretion from Human Type 1a Diabetic Pancreatic Draining Lymph Node NKT Cells

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Loss of IL-4 Secretion from Human Type 1a Diabetic Pancreatic Draining Lymph Node NKT Cells

Sally C. Kent,† Yahua Chen,‡ Sue M. Clemmings,† Vissia Viglietta,* Norma Sue Kenyon,‡ Camillo Ricordi,‡ Bernhard Hering,† and David A. Hafler*

Altered frequency and function of peripheral invariant NKT (iNKT) cells have been implicated in the regulation of murine and human type 1 diabetes. To examine regulatory cells from the site of drainage of autoimmune inflammatory tissue and autoantigenic T cell priming in diabetes, we directly cloned iNKT cells from human pancreatic draining lymph nodes (PLN). From 451 T cell clones from control and diabetic PLN, we derived 55 iNKT cells by two methods and analyzed function by cytokine secretion. iNKT cell clones isolated from control PLN secreted IL-4 and IFN-γ upon TCR stimulation. For type 1a diabetic subjects, PLN iNKT cell clones from three samples secreted IFN-γ and no IL-4. In a recent study of diabetic sample with islet-infiltrating CD4+ T cells, the phenotype of PLN iNKT cell clones was mixed. From normal and diabetic PLN, one-third of CD1d tetramer+sorted T cell clones were reactive with CD1d transfectants or proliferated/secreted cytokine in response to α-galactosylceramide-pulsed PBMCs; tetramer-staining T cell clones from diabetic PLN did not secrete IL-4. This is the first report directly examining iNKT cells from lymph nodes draining the site of autoimmunological attack in humans; iNKT cells were altered in cytokine secretion as previously reported for circulating iNKT cells in human type 1 diabetes. The Journal of Immunology, 2005, 175: 4458–4464.

Natural killer T cells have been implicated in protection from a variety of autoimmune diseases, transplantation rejection, response to microbial pathogens, as well as tumor surveillance, thus indicating their key role as regulators of immune responses. NK1.1+ T cells were first identified in the mouse as T cells with unusual properties (1–3). Murine NK1.1+ T cells expressing an invariant Vα chain (Vα14Ja281) in the TCR with little or no N region nucleotide additions are thought to be a major early source of IL-4 and IFN-γ in response to CD1 on APCs. CD1 is a class I-like molecule containing β2-microglobulin (3). The crystal structure of murine CD1 has been solved and the space available for occupancy in the Ag-binding groove accommodates a long chain fatty acid (4). The Ag recognized by TCR α-chain invariant NKT (iNKT) cells in the context of CD1 is most likely an extremely hydrophobic peptide (5) or lipid in nature: cellular phospholipids (6), or as a model Ag, the marine sponge-derived α-galactosylceramide (α-GalCer) (7), or as yet unidentified microbial or cellular lipids.

Type 1 diabetes is an autoimmune disease in which activated autodestructive T cells infiltrate the islets and specifically destroy insulin producing β-islet cells (8–10). The autoreactive T cells are thought to be Th1 in cytokine secretion phenotype. The skewing of the phenotype of autoreactive T cells could be mediated by the dysfunction of regulatory cell subsets, of which NKT cells are potentially members. Importantly, these cells have been implicated in the progression to disease in several murine models of autoimmune diabetes. Function of iNKT cells in autoimmune disease has been modulated by altering frequency, cytokine secretion, CD1d expression, and administration of α-GalCer in vivo. In NOD mice, adoptive transfer of NK1.1-like T cells or NKT cells delayed the onset of diabetes and there are several reports of decreased frequency and altered function of NKT cells in the NOD model (11–14). Overexpression of iNKT cells in a NOD transgenic mouse found NKT cells abundant in pancreatic lymph nodes and present in islets with increased IL-4 mRNA in islets of nondiabetic mice. Treatment with IL-12 or anti-IL-4 Ab abolished the diabetes protection (15). In addition, using TCR Vα14 or Vα8 chain knockout mice, the presence of NKT cells resulted in a change of function and induction of anergy of naive islet-reactive T cells, but not activated islet-reactive T cells (16). The altered function of iNKT cells may be the result of either changing the type of cytokine effector function or by changing the frequency of the cells so that less functional cytokine is available to influence the function of other cell types. As compared with other strains, a decrease in NKT cell frequency in the periphery of NOD mice was not seen, but a decrease in NKT cell frequency was seen in NOD in spleen, thymus, and liver (17), indicating that site-specific frequency of NKT cells is crucial. However, in Idd congenic mouse strains that are diabetes-resistant, frequency and loss of IL-4 secretion was comparable to the NOD strain (18).

Enhancing iNKT cell function can be accomplished by administration of α-GalCer to wild-type NOD mice to prevent disease and recurrence of diabetes (19, 20). The interaction and intercellular regulation in the pancreatic draining lymph nodes (PLN) of NKT cells, dendritic cells, and autoreactive T cells are crucial events in the disease process. NKT cell activation by CD1d-expressing cells (dendritic cells) presenting α-GalCer results in NKT cells producing soluble factors that induce dendritic cell maturation and accumulation in the PLNs (21) followed by recruitment and tolerization of T cells (22). In CD1d-deficient NOD mice, several reports indicate that treatment of mice with α-GalCer increases disease severity; this indicates that a disruption of the
α-GalCer-induced function of NKT cells in the NKT cell-DC-T cell circuit and can result in a heightened disease state (21, 23, 24). In one report, α-GalCer administration had a protective effect in CD1d-deficient mice (19), but this may be due to differences in congenic mouse strains, as discussed in Wilson and Delovitch (25). Interestingly, CD1d overexpression in NOD islets under the control of the insulin promoter changed the islet microenvironment by increasing NKT cell-related IL-4 production and resulted in protection from diabetes (26).

We examined the cytokine phenotype of Vα24JαQ T cells from identical twins and triplets discordant for type 1 diabetes and found that Vα24JαQ T cell clones from MHC-matched normal control individuals and long-term nonprogression to diabetic individuals secreted both IL-4 and IFN-γ while clones from diabetic individuals secreted IFN-γ and no detectable IL-4 (27, 28). To directly examine the site of immune response regulation and autointerferon T cell priming in diabetes, we assayed the phenotype and function of NKT cells derived from normal and type 1 diabetic PLNs. We have used three methods of isolation of NKT cells. Specifically, we performed single-cell sorting of NKT cell clones by: 1) using anti-Vα24 mAb where cells were cultured onto α-GalCer-pulsed feeders; 2) sorting Vα24+ T cells as previously described (27); or 3) by using CD1d tetramer loaded with α-GalCer. Two methods generated classical iNKT cells while some CD1d tetramer-isolated cells were reactive with α-GalCer or CD1d, but were not classical iNKT cells. However, similar results were obtained with all three methods comparing controls to diabetic PLN. From control PLN, we have found that iNKT cell clones secrete IL-4 and IFN-γ upon TCR activation. In contrast, iNKT cell clones from three type 1 diabetic PLN samples secreted IFN-γ and little IL-4. In a rare recent onset diabetic sample with active inflammation in islets, the cytokine secretion from the iNKT clones was a mixture of the normal control and diabetic phenotype. Moreover, of the CD1d tetramer-derived clones from the diabetic PLN samples responding to α-GalCer, none secreted IL-4. These data indicate that that NKT cells derived from the site of autoimmune inflammatory drainage are altered in their ability to secrete Th2-type cytokines and potentially create a Th1-like environment in situ for priming of autoreactive T cells.

Materials and Methods

Subjects

PLNs (superior and inferior pancreaticoduodenal and supra/infrapancreatic lymph nodes) were harvested from normal controls (NC) and type 1a diabetic (S). For NC and two diabetic, PLN were harvested from brain dead, multiorgan donors. For type 1 diabetic subjects, two samples were obtained from live donors (S1 and S2) and two from brain dead, multiorgan donors (S3 and S5). For diabetic subject S1, one PLN was obtained during solid pancreas transplant removal. For diabetic subject S2, one PLN was obtained during pancreatectomy. All PLN are listed in Table I. All nodes were received within 72 h of autopsy, made into single-cell suspensions and cryopreserved in 10% DMSO/FBS. All tissues were obtained with appropriate Internal Review Board approval.

Cloning and culture of conventional T cell clones and iNKT cell clones

Lymph node cells from normal controls and diabetics were cloned for iNKT cells in three ways. Cells were stained with mAbs Vα24-PE (Beckman Coulter), CD4-FITC, and CD8-FITC (BD Pharmingen). Vα24+ CD4+ CD8- cells were single-cell sorted (FACS_Vantage; BD Biosciences) onto irradiated (5000 rad) allogeneic feeders (150,000/well), 1 μg/ml phytohemagglutinin-P (Remel), and 20 U/ml recombinant human IL-2 (Tecelexin; National Cancer Institute, Frederick, MD) in 96-well plates (Costar). Vα24+ CD4+ CD8- cells were single-cell sorted onto α-GalCer (KRN7000 and vehicle, provided by Kirin Brewing Japan) pulsed allogeneic irradiated PBMCs at 100 ng/ml (1 × 105 feeders/well) and IL-2. Media was 5%, heat-inactivated 5% human male AB serum (Omega Scientific) in RPMI 1640 with 10 mM HEPES buffer, 2 mM l-glutamine, and 100 U/100 μg/ml penicillin/streptomycin (Cambrex). Cultures were fed every 2–3 days by media replacement and addition of IL-2. Positive growth wells were split as necessary. Lymph node cells from normal controls and diabetics were cloned for conventional T cells by limiting dilution at 0.3 cells/well with PHA, irradiated allogeneic feeders and IL-2 as described previously.

Single-cell cloning of α-GalCer-loaded CD1d-tetramer sorted T cells

Murine CD1d-biotin monomers at 10 μg/ml (generously provided by Dr. L. Teyton, The Scripps Research Institute, La Jolla, CA) were loaded with α-GalCer at 100 ng/ml overnight in PBS and then complexed with streptavidin-PE (BD Pharmingen) at 5 μg/ml for 30 min (29, 30). A total of 5 × 104 cells are then stained with the CD1d-loaded tetramer on ice for 2 h and then with anti-CD3-FITC (BD Pharmingen) for an additional 15 min and then washed. Cells were single-cell sorted onto irradiated allogeneic feeders and cultured as above with IL-2.

Functional analysis of T cells for α-GalCer and CD1d reactivity

NKT cells were assayed for recognition of CD1d and the lipid Ag, α-GalCer. Recognition of α-GalCer was examined by pulsing irradiated, allogeneic PBMCs with 10 μg/ml α-GalCer in complete media for 2–3 h, then washing and then plating PBMCs at 5 × 105/well with 2 × 105 T cell clones/well. After 48 h, supernatants were collected for cytokine ELISA and wells pulsed with 1 μCi [3H]thymidine/well (DuPont NEN) for an additional 24 h. Cells were harvested and counted for thymidine incorporation (PerkinElmer Wallac).

For CD1d reactivity, the C1R cell line transfectant cells with CD1 isoforms (31) were fixed with 0.03% glutaraldehyde (Sigma-Aldrich) for 30 s, neutralized with 0.2 M l-lysine and washed. A total of 5 × 104 CD1/C1R transfectant cells were plated with 5 × 105 T cell clones with the addition of

<table>
<thead>
<tr>
<th>Table I. Subject information*</th>
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<tbody>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>NC1</td>
</tr>
<tr>
<td>NC2</td>
</tr>
<tr>
<td>NC3</td>
</tr>
<tr>
<td>NC4</td>
</tr>
<tr>
<td>NC8</td>
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<tr>
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</tr>
<tr>
<td>S1</td>
</tr>
<tr>
<td>S2</td>
</tr>
<tr>
<td>S3</td>
</tr>
<tr>
<td>S5</td>
</tr>
</tbody>
</table>

* PLNs harvested from normal controls and type 1 diabetics from multiorgan donors with approval for research or, for S1 and S2, during surgery. The average age of the normal controls was 43.5 ± 11.5 years and of the diabetics was 27.0 ± 7.7 years. Diabetic S5 was a recent onset diabetic with CD4+ lymphocytic infiltrate in islets with negative staining for insulin (34).
of 1 ng/ml PMA (Sigma-Aldrich). As a positive control, T cells at 5 × 10⁴/well were incubated on plate-bound anti-CD3 (OKT3, 1 µg/ml) or an isotype control Ig (BD Pharmingen). Supernatants were collected for cytokine ELISA after 48 h and wells were pulsed with tritiated thymidine for 18 h and then harvested and counted. As an alternate method for assaying CD1d reactivity, CD1d monomer was used to coated wells of 96-well round-bottom plates (Costar) at 5 µg/ml in PBS or 4 h then washed with PBS. Cells, either iNKT or conventional CD4⁺ T as a control, were plated at 5 × 10⁴/well in complete media for 48 h and supernatant was collected for cytokine ELISA as described in the next section.

**Phenotypic analysis of T cells clones by flow cytometry and cytokine secretion analysis by ELISA**

T cell clones were characterized by flow cytometry (FACSort; BD Biosciences) using Vβ11 and Vα24 Abs from Beckman Coulter and CDR161, CD4, and CD8 Abs from BD Pharmingen. Clones were assayed for CD1d tetramer binding as described for sorting with the CD1d tetramer but as a control, clones were stained with DMSO vehicle-loaded tetramer and analyzed by flow cytometry. Clones expressing the Vα24+ TCR by flow cytometry and using the invariant Vα24+QCDR3 TCR sequence were assayed for cytokine secretion. Cells were stimulated (25,000/well) with plate-bound anti-CD3 at 1 µg/ml (OKT3) or control isotype Ab for 4 and 24 h. Supernatants were collected and assayed for IL-4 and IFN-γ by capture ELISA (paired Abs were from BD Pharmingen) and the assay sensitivity for each was 25 pg/ml. After 24 h, 1 µCi/well of [³H]thymidine was added for 24 h and incorporation was measured by scintillation counting.

**Determination of the TCR α-chain CDR3 region**

The TCRs transcripts of individual Vα24⁺ T cell clones were amplified by RT-PCR (27, 32, 33). PCR DNA products were sequenced on an ABI 373A Automated DNA Sequencer (Brigham and Women’s Sequencing Facility). T cell clones with the Vα24jQα-TCR α-chain with the invariant CDR3 region are designated iNKT cells.

**Statistics**

The Student t test was used to compare relative cell cloning frequencies.

**Results**

**Cloning frequency of NKT and CD4⁺ T cells from normal and diabetic PLN**

To examine the iNKT cell function from the site draining inflammation, PLN were harvested from brain-dead, multiorgan donors and two subjects undergoing surgery. The time of harvesting of the PLN to acquisition and processing of the PLN was variable (between 24 and 72 h). All PLN are listed in Table I with HLA information. The average age of the normal controls was 43.5 ± 11.5 years (three out of seven female, four out of seven male) and of the diabetics was 27.0 ± 7.7 years (one out of four male, three out of four female). Diabetic subject S5 was a recent onset diabetic (1.5 years disease onset) with a CD4⁺ lymphocytic infiltrate in islets with negative staining for insulin and positive staining for glucagon (34). To assess the viability of the samples and the relative frequency of iNKT cells and T cells from these samples and to molecularly characterize each cloned, putative iNKT cell for invariant Vα-chain usage, conventional T cells and iNKT cells were cloned from each of the lymph nodes. As shown in Table II, the relative cloning efficiency of conventional T cells from the normal controls and the diabetics was similar (p = 0.094) while the relative cloning efficiency for iNKT cells from diabetics was less as compared with the controls, but was not significant (p = 0.057).

**iNKT cells from type 1 diabetic PLN have a loss of IL-4 secretion**

To clone and characterize iNKT cells from the PLN, several sorting methods were used. First, to capture cells reactive with α-GalCer, Vα24⁺CD4⁻CD8⁻ cells were single-cell sorted onto irradiated allogeneic PBMC that were pulsed with α-GalCer. Expanded clones were sequenced for the TCR α-chain. In Fig. 1 (hatched symbols), three iNKT cell clones (all CD4⁺) were isolated from two control PLN by this method and these clones secreted both IL-4 and IFN-γ upon CD3 stimulation at 4 h (and 24 h, data not shown). From iNKT cells cloned from two diabetic PLN and assayed in this manner, six clones were isolated that were unable to secrete IL-4 upon CD3 stimulation.

Next, to further examine iNKT cells in the PLN samples, the single-cell sorting method used previously (27) was used. From seven control PLN, single cells sorted as Vα24⁺CD4⁻CD8⁻, 44 clones were derived that used the iNKT cell invariant Vα24 TCR chain sequences and 40 of 44 clones were able to secrete IL-4 upon CD3 ligation (Fig. 1, solid symbols). For the diabetic PLN, using the relative cloning efficiency of conventional T cells from the normal controls and the diabetics was similar (p = 0.094) while the relative cloning efficiency for iNKT cells from diabetics was less as compared with the controls, but was not significant (p = 0.057).

### Table II. Relative cloning efficiencies of T and iNKT cells from normal control and diabetic PLN

<table>
<thead>
<tr>
<th>T Cells</th>
<th>iNKT Cells Derived from Vα24⁺ T Cells on α-GalCer Pulsed Feeder</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. cells plated</td>
<td>No. growth positive wells</td>
</tr>
<tr>
<td>NC1</td>
<td>300</td>
</tr>
<tr>
<td>NC2</td>
<td>300</td>
</tr>
<tr>
<td>NC3</td>
<td>nd</td>
</tr>
<tr>
<td>NC4</td>
<td>600</td>
</tr>
<tr>
<td>NC5</td>
<td>600</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
</tr>
</tbody>
</table>

### Diabetics

| No. cells plated | No. growth positive wells | % T cells | No. iNKT cells | % iNKT cells | No. cells grown | % iNKT cells | Mean ± SE |
| S1 | 480 | 21 | 4.4 | nd | 200 | 17 | 8.5 | 1 | 0.50 | 0.50 |
| S2 | 600 | 30 | 5.0 | 240 | 10 | 2 | 0.833 | 0.833 |
| S3 | 900 | 27 | 3.0 | nd | 225 | 2 | 0.89 | 0 | 0.833 |
| S4 | 900 | 27 | 3.0 | 300 | 12 | 4.0 | 1 | 0.030 | 0.030 |
| S5 | 1200 | 400 | 3.3 | 360 | 55 | 4 | 1.11 | 0.455 | 1.57 |
| Mean ± SE | | | | | | | 0.246 | 0.733 | (0.232) | (0.56) |

*To assess viability of the lymph node tissue and relative cloning efficiency of iNKT cells from normal controls and diabetics, conventional T cells were cloning by limiting dilution (0.3 cells/well) with PHA and irradiated allogeneic PBMCs. iNKT cells were cloned in two ways as described. The mean percentage with SE of T cells cloned from normal controls (1.78 ± 1.81 SE) reflected the variable viability of these tissues and this was similar to the mean seen from the diabetics (p = 0.094). Variability in cloning efficiency was also seen in the total iNKT cells cloned from normal controls and diabetics and no difference in cloning efficiency is seen (p = 0.057), though there is a trend for fewer iNKT cells from the diabetic PLN. nd, not done.
maintaining their ability to secrete IFN-γ. iNKT cell clones displayed a loss of IL-4 secretion after CD3 ligation while PLN. The percentage of CD1d^+ H11022 50 pg/ml IL-4 and IFN-γ secreted by single-cell cloning were V_{	ext{TCR}} \alpha-chain and clones that expressed the invariant Vα24αQ sequence are shown. IL-4 and IFN-γ secretion assessed by cell stimulation (50,000 cell/well) with plate-bound anti-CD3 or control Ig with supernatants collected for 4 h for cytokine ELISA. All clones in Fig. 1 are CD4^-+. The majority of iNKT cell clones (40 of 44) from control PLN secreted >50 pg/ml IL-4 and IFN-γ. From diabetic PLN S1, S2, and S3, the iNKT cell clones displayed a loss of IL-4 secretion after CD3 ligation while maintaining their ability to secrete IFN-γ while recent onset S5 PLN clones had a mixed phenotype of IFN-γ/IL-4 and IFN-γ alone secretion.

frequency in that only 11 clones could be derived from the four PLN. Moreover, these clones from three of the four diabetic PLN displayed a loss of IL-4 secretion after CD3 ligation while maintaining their ability to secrete IFN-γ. Interestingly, 4 of 7 iNKT cell clones from the recent onset PLN, S5 (as shown in Fig. 1) were unable to secrete IL-4 after CD3 ligation while 3 of 7 clones secreted IL-4 upon CD3 ligation. In summary, the majority of iNKT cells derived, with both methods, from control PLN (41 of 44) were able to secrete IFN-γ and IL-4 upon stimulation while iNKT cells from diabetic PLN displayed a loss of IL-4 secretion and iNKT cells from the recent diabetes onset PLN were of a "mixed" phenotype.

**CD1d-tetramer isolated NKT cells from PLN**

As we wanted to confirm our results in another way, we used a complementary method of isolating NKT cells with murine CD1d tetramers that are reported to bind to human iNKT cells (29, 30). A representative sample of CD1d tetramer binding to an iNKT cell clone is shown in Fig. 2A. In addition, plate bound CD1d monomers were able to activate iNKT cell clones to secrete IFN-γ (Fig. 2B). These CD1d tetramers were used to isolate NKT cells by single-cell sorting CD1d tetramer-binding CD3^-+ T cells from PLN. The percentage of CD1d^+ CD3^-+ T cells from normal and diabetic PLN is shown in Table III and ranged from 0.015–0.085% of PLN cells in both normal and diabetic (S3 and S5) PLN. All of the clones isolated from normal or diabetic PLN were CD4^-+ and, interestingly, Vα24 negative. This is in marked contrast to CD1d tetramer-binding clones isolated from the periphery of normal control subjects where T cells binding to the CD1d tetramer and isolated by single-cell cloning were Vα24^-+ (data not shown).

The T cell clones isolated by CD1d tetramer binding from control and diabetic PLN were examined for cytokine secretion after CD3 ligation (Fig. 3). From the normal control PLNs, the CD1d tetramer-isolated clones from normal PLNs, in general, secreted ~1 log less IL-4 and IFN-γ at 4 h after CD3 ligation as compared with iNKT cells (Fig. 1). When CD1d-tetramer isolated clones from S3 diabetic PLN were analyzed, one clone did not secrete IL-4 and one secreted 30 pg/ml IL-4 upon CD3 ligation. The CD1d tetramer-isolated clones from diabetic PLN S5 were analyzed and approximately one-half of clones possessed the loss of IL-4 secretion phenotype.

**Functional activity of CD1d-tetramer isolated NKT cells**

It was of interest to examine the function of the CD1d tetramer-isolated cells as this method has been recently used to evaluate NKT cell function in patients with diabetes (30). Two methods were used. We examined whether these clones were responsive to α-GalCer-pulsed allogeneic PBMCs and whether these clones could respond to C1R cells transfected with CD1a or CD1d. As a comparison, iNKT cells were used as positive controls. Two iNKT cell clones from two normal control PLNs were assayed for proliferation in response to α-GalCer-pulsed allogeneic-irradiated PBMCs (Fig. 4A). Both iNKT cell clones proliferated in response to α-GalCer and secreted IL-4 (both secreted IFN-γ, data not shown).

In contrast, only one of seven randomly selected CD1d tetramer^-+ sorted clones responded to α-GalCer. CD1d tetramer^-+ sorted clones from diabetic PLN S5 were assayed for reactivity with α-GalCer. As a positive control, an iNKT cell from PLN S5 was used in this assay and responded to α-GalCer by proliferation, but not IL-4 secretion (Fig. 4B). Three of seven randomly selected CD1d tetramer-binding clones from S5 were assayed for α-GalCer reactivity and responded to α-GalCer by IFN-γ (not shown), but no IL-4 secretion (clones 5.7, 5.10, and 5.17). It is noteworthy that from diabetic S5, one-half of the clones secreted IL-4 in response to anti-CD3 stimulation (Figs. 3 and 4B) and when seven clones were further tested, clones 5.7 and 5.17 secreted IL-4 in response to anti-CD3 stimulation, but did not secrete IL-4 in response to α-GalCer stimulation (Fig. 4B).

Another set of CD1d-isolated clones from normal PLN were assayed for response to CD1d transfectants (Fig. 4C). Two iNKT cell clones were used as positive controls in this assay and both responded with IFN-γ and IL-4 secretion to the CD1d transfectant,
but not the CD1a transfectant. In contrast, two of six randomly selected CD1d-isolated clones from two normal control PLNs responded to the CD1d transfectants with IFN-γ and IL-4 secretion (3.4 and 3.12). Interestingly, one-third of the clones derived with the CD1d tetramer from normal and diabetic PLN assayed positively for functional reactivity with α-GalCer or reactivity with CD1d.

Discussion

A major difficulty in the investigation of human autoimmune disease has been the general necessity of sampling peripheral blood to gain insight into organ-specific inflammatory response. It is known from investigation of the NOD model of diabetes that self-Ags are transported specifically to the pancreatic lymph node where they trigger autoreactive T lymphocytes, thus initiating autoimmune responses and lymphocyte infiltration (35). These data strongly suggest that the PLN is the critical location to examine the pathogenic T lymphocytes in type 1 diabetes and to investigate their regulation.

We have previously observed a decrease in the frequency of presumably regulatory iNKT cells expressing the invariant Vα24αQ TCR in the periphery of patients with type I diabetes. More striking was the loss of IL-4 secretion in these iNKT cell clones. Here, we directly examined the critical question as to whether there were defects in IL-4 secretion of NKT cells at the site of T cell regulation of the autoimmune response. We cloned invariant Vα24αQ T cell clones from control and diabetic PLN and measured the cytokines secreted by these NKT cells. The iNKT cells derived from sites of autoantigen priming in diabetics have a loss of Th2 function as compared with PLN iNKT cells from control subjects, reflecting previous data examining iNKT cells derived from the circulation of diabetics (27, 28). Interestingly, we did not see a clear frequency decrease of iNKT cells between control and diabetic PLN. Change of frequency of iNKT cells in the periphery of mice and humans in the autoimmune state is in question (18, 27, 30) and these data emphasize the need for precise measurements of iNKT cells. These are among the first studies examining PLNs in subjects with diabetes and suggests that a Th1 cytokine milieu may exist in this inflammatory site during priming of autoreactive T cells.

The definition of NKT cells has evolved in recent years to include several phenotypic subgroups. That is, NKT cells can either express CD4+ or can be negative for expression of CD4 and CD8 (1, 33). Reports indicate that the CD4+ NKT cell subgroup may in fact be responsible for the majority of IL-4 production from NKT cells (1, 29, 36). The use of α-GalCer-loaded CD1d tetramers has led to the detection and recognition of iNKT cells in peripheral subsets. Non-iNKT cells are CD1d-restricted and α-GalCer responsive, but do not express an invariant Vα24 chain in the TCR (37). Several reports in mice of non-iNKT cells recognizing CD1d (38–40) raise the idea of populations of variant NKT cells functioning in disease. In fact, CD1-restricted nonclassical Vα3.2ββ9 NKT cells with higher IFN-γ secretion than IL-4 overexpressed in NOD mice prevented diabetes (41).

In these investigations, we focused on the TCR α-chain invariant NKT cell and NKT cells that bind the CD1d tetramer. Although it is thought that the majority of NKT cells are of the invariant variety, it is intriguing to speculate on the function and recognition of the variant variety of NKT cells we have observed here with the CD1d-binding NKT cells, especially from sites of Ag priming and immune response regulation.

In humans, the decreased frequency of the iNKT cells in the periphery has been implicated in systemic sclerosis (42) and multiple sclerosis (43, 44), and mRNA for the invariant TCR α-chain has been found associated in multiple sclerosis plaques (45) and type 1 diabetes (27, 46). A major question remains regarding the ex vivo measurement of human T cell function, particularly as related to autoimmune disease. We have observed that differences in the strength of signal applied to regulatory T cells has an important impact in creating ex vitro models to examine immune response regulation.

**Table III.** Cloning of CD3+CD1d tetramer+ T cells

<table>
<thead>
<tr>
<th>PLN normal controls</th>
<th>% CD3+CD1d Tetramer</th>
<th>No. Cells Plated</th>
<th>No. Growth Positive Wells</th>
<th>% Growth of Clones</th>
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<td>NC3</td>
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<td>3.33</td>
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<td>NC10</td>
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<td>60</td>
<td>3</td>
<td>5.00</td>
</tr>
<tr>
<td>PLN diabetic</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>S3</td>
<td>0.085</td>
<td>60</td>
<td>2</td>
<td>3.33</td>
</tr>
<tr>
<td>S5</td>
<td>0.025</td>
<td>60</td>
<td>23</td>
<td>38.3</td>
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</table>

*a* CD3+CD1d tetramer+ T cell clones were single-cell sorted from control and diabetic PLN onto irradiated allogeneic feeders and PHA and expanded. PLN cells (5 x 10⁶) from both normal controls and diabetics were stained with α-GalCer loaded CD1d tetramer (PE labeled) for 2 h at 4°C and then stained with CD3-PE for 15 min at 4°C, washed and analyzed. From normal control PLNs, a range of 0.015–0.085% CD1d+CD3+ T cells was seen and from diabetic PLNs, a range of 0.025–0.085% CD1d tetramer+CD3+ T cells was seen. Clones were assayed for surface marker expression by flow cytometry: all clones derived by this method were Vα24+CD4+. Functional data for individual clones is shown in Figs. 3 and 4.
and no IL-4 secretion was detected above assay sensitivity. In cultured with irradiated allogeneic PBMC pulsed with vehicle or 6 CD1d tetramer-derived clones were reactive with C1R-CD1d transfectants. natants were collected for cytokine ELISA. From normal control PLNs, 2 of cultured at 50,000/well with T cells and 1 ng/ml PMA for 48 h and then super- with 0.03% paraformaldehyde for 30 sec, washed extensively, and then cul-

One-third of CD1d tetramer-sorted CD3 \(+\) T cell clones, from either normal control or diabetic PLN, recognize CD1d or are reactive with \(\alpha\)-GalCer. CD1d tetramer-sorted clones were tested for reactivity with \(\alpha\)-GalCer (A and B) or recognition of CD1d (C). Clones (50,000/well) were cultured with irradiated allogeneic PBMC pulsed with vehicle or \(\alpha\)-GalCer (100 ng/ml) or plate-bound anti-CD3, as a positive control, or isotype control (1 \(\mu\)g/ml) for 48 h and supernatant was collected for cytokine ELISA. Wells were pulsed with tritiated thymidine (1 \(\mu\)Ci/well) for an additional 18 h and then harvested and counted. \(\alpha\)KNT cell clones are used in each experiment as positive controls. In A, 1 of 7 normal control CD1d tetramer-derived clones recognized \(\alpha\)-GalCer (clone NC6.2). In B, 3 of 7 CD1d tetramer-derived clones from diabetic PLN S5, S5.7, S5.10, and S5.17, recognized \(\alpha\)-GalCer and no IL-4 secretion was detected above assay sensitivity. In C, \(\alpha\)KNT cells and CD1d tetramer-derived T cell clones (50,000/well) from normal control PLN were cultured with C1R cells transfected with CD1a or CD1d or plate-bound anti-CD3 or isotype control Ab (1 \(\mu\)g/ml). Transfectants were fixed and stained with a murine \(\alpha\)KNT Ab (clone NC6.2) or recognition of CD1d (clone NC6.2). In and then harvested and counted. iNKT cell clones are used in each experiment to define the phenotype of the NKT cells derived by those methods.

Our results indicate that in the PLNs of type 1 diabetics, the iNKT cells isolated were generally unable to secrete IL-4 upon TCR stimulation. We observed a trend, though not significant, of fewer iNKT cells in the diabetic PLN. This may relate to the decreased frequency of iNKT cells in the PLN or the integrity of the tissue postautopsy and receipt of the tissue. We addressed this issue by simultaneously cloning conventional T cells and iNKT cells from each sample and then finding similar cloning efficiencies for conventional T cells and iNKT cells from control and diabetic PLN. Interestingly, all of the iNKT cells isolated from control or diabetic PLN were CD4\(^+\); this is in contrast to the CD4\(^-\)CD8\(^-\) iNKT cells we had observed in the periphery of diabetics (27). This may represent a sequestered specialty subset of iNKT cells. Moreover, the CD1d tetramer-derived NKT cells were of an unusual phenotype: V\(\alpha24\)-, but they bound with CD1d and responded to \(\alpha\)-GalCer/CD1d engagement. The function of these subsets of NKT cells is of interest and further indicates the importance of understanding tissue-specific in situ immune responses related to priming and regulation.

One of the diabetic PLN (S5) was from a recent onset type 1 diabetic subject. These are rarely obtained, and thus it was of interest to compare the results from this PLN to the more chronic disease state-derived PLN. There is a CD4\(^+\) T cell infiltrate within the remaining islets of this subject (34), indicating an ongoing autodestructive immune response. The iNKT cells derived from the PLN of this subject were mixed in the subject’s Th1/Th2 phenotype when isolated by either cloning method. The relationship between the course of diabetes development and alteration of iNKT cell function remains an open point as only longitudinal human clinical trials examining iNKT cell function in preclinical subjects at risk for diabetes development can address this question. However, such a trial can only examine iNKT cells in the periphery. It remains a distinct possibility that development of clinical disease and activation and expansion of pathogenic T cells has a direct/indirect effect on iNKT cell phenotype. However, these data, albeit on one recent onset diabetic PLN, are consistent with the hypothesis that the loss of IL-4 secretion and the general decreases in induced mRNA products on microarray (28) of these diabetic iNKT cells is due to defective Th2 responses and/or clonal exhaustion while attempting to regulate the highly activated autoreactive T cells in situ and we are observing the transition from iNKT cells secreting both Th1 and Th2 cytokines to the loss of IL-4 secretion.
In summary, our data indicate that the majority of iNKT cells and CD1d tetramer-derived NKT cells from the lymph node draining the site of inflammation in human type 1 diabetes have lost the ability to secrete IL-4 upon TCR stimulation. These data indicate it is possible to derive strategies that examine the site of Ag priming and immune response regulation that is crucial to understand the dysregulation of the immune response in autoimmune disease. Moreover, these data confirm that iNKT cell clones in subjects with long-standing type 1 diabetes have defects in IL-4 secretion.

Disclosures

The authors have no financial conflict of interest.

References