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Linda M. Bradley; ... et. al

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Islet-Specific Th1, But Not Th2, Cells Secrete Multiple Chemokines and Promote Rapid Induction of Autoimmune Diabetes[1]

Linda M. Bradley,[2]* Valérie C. Asensio,† Li-Karine Schioetz,* Judith Harbertson,* Troy Krahl,* Gail Patstone,* Nigel Woolf,‡ Iain L. Campbell,† and Nora Sarvetnick*

Migration of CD4 cells into the pancreas represents a hallmark event in the development of insulin-dependent diabetes mellitus. Th1, but not Th2, cells are associated with pathogenesis leading to destruction of islet β-cells and disease onset. Lymphocyte extravasation from blood into tissue is regulated by multiple adhesion receptor/counter-receptor pairs and chemokines. To identify events that regulate entry of CD4 cells into the pancreas, we transferred Th1 or Th2 cells induced in vitro from islet-specific TCR transgenic CD4 cells into immunodeficient (NOD.*scid*) recipients. Although both subsets infiltrated the pancreas and elicited multiple adhesion receptors (peripheral lymph node addressin, mucosal addressin cell adhesion molecule-1, LFA-1, ICAM-1, and VCAM-1) on vascular endothelium, entry/accumulation of Th1 cells was more rapid than that of Th2 cells, and only Th1 cells induced diabetes. In vitro, Th1 cells were also distinguished from Th2 cells by the capacity to synthesize several chemokines that included lymphotactin, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1α, whereas both subsets produced macrophage inflammatory protein-1β. Some of these chemokines as well as RANTES, MCP-3, MCP-5, and cytokine-response gene-2 (CRG-2)/IFN-inducible protein-10 (IP-10) were associated with Th1, but not Th2, pancreatic infiltrates. The data demonstrate polarization of chemokine expression by Th1 vs Th2 cells, which, within the microenvironment of the pancreas, accounts for distinctive inflammatory infiltrates that determine whether insulin-producing β-cells are protected or destroyed. ***The Journal of Immunology*****, 1999, 162: 2511–2520.**

CD4 lymphocytes play a central role in autoimmune diabetes, but the underlying mechanisms that initiate disease in a genetically susceptible individual remain to be elucidated. The nonobese diabetic (NOD)[3] mouse spontaneously develops insulin-dependent diabetes mellitus (IDDM) resembling the human autoimmune disease. Although several cell types participate in IDDM, CD4 cells alone can elicit disease when autoreactive T cells are present in sufficient frequency (1). In particular, Th1 cells that secrete IFN-γ and lymphotoxin (LT) induce disease in both NOD and immunodeficient NOD.*scid* mice (2) largely due to the effects mediated by these proinflammatory cytokines (3–6). In contrast, Th2 cells that secrete IL-4, IL-5, IL-6, and IL-10 fail to transfer diabetes unless recipients are immunocompromised (2, 7) in part due to protection by IL-4 (8–10). Thus, early events leading to differences in the infiltration of autoreactive CD4 subsets into the pancreas may be critical determinants of the course of IDDM in susceptible individuals.

Infiltration of lymphocytes into tissue from blood is regulated by multiple adhesion receptor/counter-receptor pairs and chemokines that mediate sequential, but overlapping, steps to achieve primary adhesion, activation-dependent adhesion, and transmigration (reviewed in Refs. 11 and 12). Initial interactions with vasculature are mediated by selectins expressed on leukocytes (L), platelets (P), and endothelial cells (E), whereas lymphocyte arrest and transmigration are regulated by β_1 or β_7 integrins followed by LFA-1. Integrin adhesiveness is regulated by chemokines (13), which are subdivided into four families based on the arrangement of N-terminal cysteine residues (C, CC, CXC, and CX_3C) (14). Although their involvement in diabetes has yet to be defined, chemokines play a central role in recruiting cells into sites of inflammation through chemoattractant effects (15, 16). Despite tremendous redundancy in the activities of both chemokines and their receptors, several chemokines exert selective effects on subsets of CD4 cells (15, 17, 18), and the production of selected chemokines is typically associated with particular types of lesions in a number of diseases (16, 19). Moreover, Th1 and Th2 cells can be distinguished by responsiveness to different chemokines (20–24) due to differential expression of the receptors CCR5 and CXCR3 vs CCR3, CCR4, and CCR8, respectively (22, 25–28). Selective recruitment of subsets of CD4 effector cells into sites of inflammation may thus contribute to the development of pathogenic vs nonpathogenic infiltrates.

To identify early events in the development of diabetes, we analyzed the abilities of activated islet-specific, TCR transgenic CD4 cells to infiltrate the pancreas and initiate diabetes in NOD.*scid* recipients that have no pre-existing disease. We show that Th1 cells enter and/or accumulate in the pancreas more rapidly than Th2 cells and induce diabetes, but that both subsets induce

Departments of *Immunology and †Neuropharmacology, The Scripps Research Institute, La Jolla, CA 92037; and ‡Department of Surgery, University of California at San Diego, La Jolla, CA 92037

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[2] Address correspondence and reprint requests to Dr. Linda M. Bradley, Department of Immunology, The Scripps Research Institute, IMM-23, 10550 N. Torrey Pines Rd., La Jolla, CA 92037. E-mail address: lbradley@scripps.edu

[3] Abbreviations used in this paper: NOD, nonobese diabetic; IDDM, insulin-dependent diabetes mellitus; LT, lymphotoxin; tg, transgenic; PNAd, peripheral lymph node addressin; MAdCAM-1, mucosal addressin cell adhesion molecule-1; BrdU, bromodeoxyuridine; RPA, RNase protection assay; MIP-1α and -1β, macrophage inflammatory protein-1α and -1β; MCP, monocyte chemoattractant protein; IP-10, IFN-inducible protein-10; LN, lymph nodes; CRG-2, cytokine-response gene-2.

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adhesion receptors on vascular endothelium coincident with their infiltration. In addition to producing typical cytokine patterns, Th1 and Th2 cells are distinguished by their expression pattern of chemokines in vitro, and in vivo, several chemokines are specifically associated with Th1, but not Th2, pancreatic infiltrates. The data reveal partitioning of chemokines and cytokines with the Th cell phenotype, allowing for feedback regulation of cellular infiltration and the potential for tissue destruction.

Materials and Methods

Mice

Female NOD/shi and NOD/Lt-*scid/scid* mice were bred at The Scripps Research Institute (La Jolla, CA). BDC2.5 TCR transgenic (tg) mice (1) that were backcrossed onto the NOD background for 12 generations were a gift from Diane Mathis and Christophe Benoist (Illkirch, France). Mice were used at 8–12 wk of age.

Antibodies

mAb for CD4 cell enrichment, flow cytometry, and immunohistology were generated as previously described (29) and include CD3 (145-2C11), CD8 (3.155), HSA (J11D), B220 (CD45, RA36B2), LFA-1 (CD18, M17 4.4.11.9), ICAM-1 (CD54, FD441.8), and mouse anti-rat κ-chain (MAR.18). mAb specific for PNAd (MECA 79), MAdCAM-1 (MECA 367), Mac-1 (CD11b, M1/70), VCAM-1 (CD106, MVCAM.A), and vβ4 (KT4) and FITC-anti-rat κ chain mAb (RG7/9.1) were obtained from PharMingen (La Jolla, CA). Anti-bromodeoxyuridine (BrdU) was purchased from Harlan Sera-Lab (Sussez, U.K.). Phycoerythrin-anti-CD4 (GK1.5) was obtained from Collaborative Biomedics (Mountain View, CA). Biotinylated anti-rat Ig was purchased from Vector Laboratories (Burlingame, CA). Polyclonal Ab to porcine insulin were obtained from Dako (Carpinteria, CA), and polyclonal Ab to synthetic glucagon were obtained from Chemicon International (Temecula, CA).

Recombinant cytokines and anti-cytokine Abs

Recombinant IL-2, IL-4, and IFN-γ were from X63.Ag8–653 cells transfected with murine cDNA for the respective cytokines (30) and were referenced to standards from R&D Systems (Minneapolis, MN). Murine rIL-12 (sp. act., 5.6×10^6 U/mg) was provided by Dr. Stanley Wolf (Genetics Institute, Cambridge, MA). The anti-cytokine mAb, 11B11 (anti-IL-4), and XMG1.2 and R46A2 (anti-IFN-γ) were purified from ascites. Anti-IL-2 (JES-6 1A12), biotin-anti-IL-2 (JES-6 5H4), and biotin-anti-IL-4 (BVD6) were obtained from PharMingen.

Generation of Th subsets

Resting CD4 cells were enriched from spleens of BDC2.5 mice as previously described (31) by cytotoxic depletion with anti-CD8, -B220, and -HSA (heat stable Ag) mAb followed by anti-rat κ mAb, and then rabbit and guinea pig C (Accurate Chemical, Westbury, NY). In some experiments, high density cells were enriched by Percoll density gradient centrifugation (4 layers: 45, 53, 62, and 80%) and collected from the interface of the 80 and 62% layers. Resulting populations were 95–98% $CD4^+$ cells. Th1 and Th2 cells were induced by culture for 4 days in 25 ml of RPMI 1640 (Irvine Scientific, Santa Ana, CA) containing 7% FCS (HyClone, Logan, UT), 200 μg/ml penicillin, 200 U/ml streptomycin, 4 mM L-glutamine, 10 mM HEPES, and 5×10^{-5} M 2-ME in 75-cm^2 tissue culture flasks (Costar, Cambridge, MA). Flasks were coated with anti-CD3 at 50 μg/ml in 7.0 ml of PBS for 2 h at 37°C and washed before addition of CD4 cells (5×10^5/ml). Cultures were supplemented with 20 ng/ml rIL-2 and 10 μg/ml of anti-CD28, 37.51 (a gift from Dr. J. Allison, University of California, Berkeley, CA). To generate Th1 cells, cultures were supplemented with 5 ng/ml rIL-12 and 10 μg/ml anti-IL-4. To induce Th2 cells, 10 ng/ml rIL-4 and 10 μg/ml anti-IFN-γ were added. After 60 h, the cells were expanded in medium containing rIL-2. At 96 h, the cells were harvested, washed, and injected into NOD.*scid* recipients (see below). To assess cytokine polarization, cells were restimulated in the absence of added cytokines or anti-cytokine mAb with plate-bound anti-CD3 (10 μg/ml) either in 200-μl triplicate cultures in 96-well flat-bottom plates (Costar) at the concentrations indicated in the text to elicit cytokine secretion or in 25-ml volumes at 1×10^6/ml for RNA isolation. Supernatants were harvested 14 h after restimulation of effectors.

Measurement of cytokine production by CD4 cells

IL-2, IL-4, and IFN-γ were detected by ELISA as previously described (31) using the following capture and detecting mAb pairs: IL-2, JES-61A12, and biotin-JES6-5H4; IL-4, 11B11, and biotin-BVD6; and IFN-γ, R46A2, and biotin-XMG1.2. Serial dilutions of test supernatants were referenced to recombinant cytokines.

Analysis of cytokine and chemokine mRNA by RNase protection assay (RPA)

Total RNA was extracted from CD4 cells in suspension and from the pancreas using TRIzol (Life Technologies, Gaithersburg, NY) according to the manufacturer's instructions. Pancreata were flash-frozen in liquid nitrogen and pulverized before addition of TRIzol. mRNA was isolated from the pancreas by $poly(A)^+$ purification as previously described (32). The cytokine probe set detected IL-1α, IL-1β and IL-2, IL-3, IL-4, IL-5, IL-6, IFN-γ, TNF-α, and LT. The chemokine probe set detected lymphotactin, C10, MIP-2, MCP-3, MIP-1β, T cell activation gene 3 (TCA-3), MCP-1, CRG-2/IP-10, MIP-1α, and RANTES. Additional probes for MCP-5 (33) and eotaxin (34) were run separately. A probe for *rpl32* was included to verify the loading of RNA. Details of the method and construction of the probe sets have been described previously (35–37). Probes were labeled with [α-^{32}P]UTP and hybridized with 5 μg of target RNA for Th1 and Th2 cells and 1 μg of RNA for pancreatic $poly(A)^+$ RNA. After digestion of ssRNA, protected fragments were separated by PAGE. Controls included the probe set hybridized to transfer RNA only and to transfer RNA plus an equimolar pool of synthetic sense RNAs complementary to the probe set. For quantitation, gels were exposed by phosphoimaging (Molecular Dynamics, Sunnyvale, CA), and radioactivity in individual bands (after background subtraction) in comparison with *rpl32* was assessed with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Adoptive transfer of CD4 cells

BDC CD4 cells (8×10^6) were injected i.v. into groups of 12 NOD.*scid* mice at 12 wk of age. Recipients were prescreened for the absence of $CD3^+$ $B220^+$ cells in peripheral blood by flow cytometry and for the absence of serum Ig by ELISA as previously described (38). Glucose levels were determined from blood samples using an AccuChek II monitor (Boehringer Mannheim, Indianapolis, IN) daily for the first week after BDC CD4 cell transfer and at 2- to 3-day intervals thereafter. Readings >300 mg/dcl on 2 consecutive days were considered indicative of diabetes. At various times after transfer, recipients were sacrificed, and tissues were evaluated for the presence of v$\beta4^+$ cells by flow cytometry and histology as indicated below.

Analysis of islet-specific CD4 cells by flow cytometry

The tg^+ donor cells were identified by flow cytometry using CD4-FITC and biotin-vβ_4/phycoerythrin-strepavidin. Peripheral LN (pooled inguinal, axillary, brachial, cervical, and periaortic), pancreatic LN, mesenteric LN, and spleen were teased into suspension and stained for both surface markers. Erythrocytes in the spleen were lysed with 0.85% M NH_4Cl. Cells ($2–5 \times 10^5$) were stained for 15 min with 0.5–1 μg of mAb in 100-μl volumes of PBS containing 1% BSA and 0.05% NaN_3. Histograms and dot plots were generated by gating on 5000 lymphocytes cells using a FACScan flow cytometer (Becton Dickinson).

BrdU labeling

NOD.*scid* mice were administered 0.8 mg of BrdU (Sigma, St. Louis, MO) at the time of vβ4 cell transfer and then maintained on drinking water supplemented with 0.8 mg/ml BrdU until sacrifice as previously described (39). Incorporation by cells in the pancreas was determined by staining of paraffin sections (see below), and proliferating cells were quantitated by determining the percentage of positive cells from five randomly chosen infiltrates/pancreas in which >200 total cells were counted.

Histology

One-half of the pancreas from each NOD.*scid* recipient was fixed in 10% buffered formalin and embedded in paraffin. Four-micron-thick sections were stained with eosin and hematoxylin. Paraffin sections were stained with an immunoperoxidase method using Abs to porcine insulin, synthetic glucagon, or BrdU followed by a biotinylated secondary Ab and a biotin-avidin peroxidase complex (both from Vector Laboratories). The other half of the pancreas from each mouse was snap-frozen in Tissue-Tek OTC embedding medium (Sakura Finetechnical Co., Tokyo, Japan). Eight-micron-thick sections were cut on a cryostat, air-dried overnight, and stained with previously titrated mAb to vβ4, CD4, Mac-1, LFA-1, ICAM-1, PNAd, and MAdCAM followed by appropriate biotinylated second step reagents and biotin-avidin peroxidase. After the color reaction with diaminobenzidine, both paraffin and cryostat sections were counterstained with hematoxylin as previously described (8).

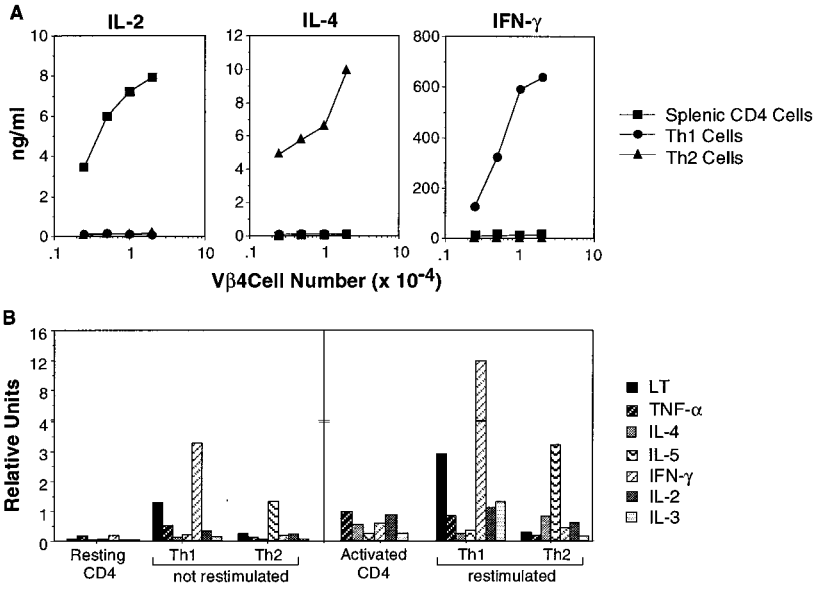

FIGURE 1. Cytokine production by vβ4$^+$ cells before transfer to NOD.*scid* recipients. *A*, CD4 cells from the spleens of BDC mice were cultured with immobilized anti-CD3 and soluble anti-CD28 as described in *Materials and Methods*. Th1 cells were induced with rIL-12 and anti-IL-4; Th2 cells were induced with rIL-4 and anti-IFN-γ. After 4 days the cells were restimulated with anti-CD3 in the absence of cytokines or anti-cytokine mAb, and supernatants were tested for IL-2, IL-4, and IFN-γ by ELISA. *B*, RNA was extracted from resting BDC CD4 cells or Th1 and Th2 cells on day 4 of primary culture (*left panel*) or from the same populations after stimulation for 12 h with immobilized anti-CD3 (*right panel*). Expression of the indicated cytokines was analyzed by RNase protection and was quantitated by comparison to *rpl32* by phosphoimaging.

Results

Generation of Th1 and Th2 cells from CD4 cells of BDC transgenic mice

To study Th1 and Th2 subsets in the initiation of diabetes, islet-specific CD4$^+$ cells were isolated from the spleens of BDC2.5 TCR (vα1, vβ4) tg mice (1). Although BDC mice do not become diabetic (40–42), we showed previously that lymphocytic infiltrates are observed in and around the islets (42). CD4 cells were stimulated with rIL-2, immobilized anti-CD3, and soluble anti-CD28 to generate effector populations that were free of contaminating APC. Th1 cells were induced with rIL-12 and anti-IL-4 mAb. To elicit Th2 cells, rIL-4 and anti-IFN-γ mAb were added. On day 4 both populations demonstrated an equivalent fivefold expansion, were entirely viable, and contained exclusively vβ4$^+$ cells that were activated in appearance and uniformly IL-2R$^+$. Phenotypically, Th1 and Th2 cells were indistinguishable, with low expression of L-selectin, moderate expression of $\alpha_4\beta_7$, and high levels of CD44 and LFA-1 (not shown).

To test the polarization to Th1 or Th2 cytokine patterns, at 4 days the cells were restimulated with immobilized anti-CD3 in the absence of exogenous cytokines, and supernatants were tested for IL-2, IL-4, and IFN-γ. For comparison, splenic vβ4$^+$, CD4 cells from the starting population were stimulated under identical conditions. As shown in Fig. 1*A*, resting CD4 cells exclusively produced IL-2 in response to initial stimulation, whereas this cytokine was not secreted in detectable levels by either Th1 or Th2 cells. Only Th2 cells produced IL-4, and only Th1 cells produced IFN-γ. The data indicate that the effector populations were committed to the Th2 or Th1 phenotype, respectively. To more fully evaluate the cytokine profiles of the Th1 and Th2 cells, we analyzed cytokine mRNA both before and after restimulation with anti-CD3 by RPA. The relative differences in the levels of message were quantitated in comparison with the housekeeping gene, *rpl32* (Fig. 1*B*). Resting CD4 cells contained no detectable cytokine RNA (*left panel*), whereas at the time of harvest from primary cultures, Th1 cells exhibited readily demonstrable RNA for LT, TNF-α, and IFN-γ, while Th2 cells contained IL-5 message but little RNA for other cytokines. At 12 h after activation of resting CD4 cells with anti-CD3 (*right panel*), RNA was detected for all cytokines in the probe set, with the exception of LT. At 12 h after restimulation, Th1 cells had dramatically elevated RNA for LT and IFN-γ and also showed increased RNA for TNF-α, IL-2, and IL-3, but not for the Th2 cytokines, IL-4 and IL-5. In contrast, Th2 cells now contained IL-4 message and higher levels of mRNA for IL-5 than before reactivation, but very low levels of LT, TNF-α, IFN-γ, and IL-3 transcripts. In both populations the polarized cytokine patterns were present by 4 h after restimulation (not shown). The data demonstrate that the Th1 and Th2 cells were polarized to the cytokine profiles typically associated with them.

Chemokine expression by Th1 and Th2 cells

It is now well documented that chemokines play a key, essential step in the infiltration of cells into nonlymphoid tissues. Previous studies indicate that activated T cells and T cell lines produce chemokines (15). We examined BDC Th1 and Th2 cells for synthesis of RNA for the following chemokines (36) belonging to three families by RPA: C: lymphotactin; C-C: MIP-1α, MIP-1β, T cell activation gene 3 (TCA-3), MCP-1, MCP-3, RANTES, and C10; and C-X-C: MIP-2 and CRG-2 (known as IP-10 in humans). As shown in Fig. 2*A*, when Th1 and Th2 effector cells were harvested from primary cultures, chemokine mRNA was not detected. However, by 4 h after restimulation with immobilized anti-CD3, Th1 cells expressed high levels of message for lymphotactin, MIP-1α, MIP-1β, and MCP-1 and low levels of mRNA for CRG-2/IP-10 and RANTES. RNA levels for lymphotactin increased with time after culture, whereas those for other chemokines were sustained throughout the 12-h period. In contrast to the multiple chemokines synthesized by Th1 cells, only MIP-1β was made in high levels by Th 2 cells, and expression was increased with time after stimulation. Identical results were found in three separate experiments.

The differences in chemokine gene expression by Th1 and Th2 cells were quantitated at 12 h, as shown in Fig. 2*B*. Chemokine RNA was not detected in significant amounts in resting CD4 cells or in Th1 or Th2 cells that were harvested from primary cultures but not restimulated (*left panel*). At 12 h after activation of freshly isolated CD4 cells from BDC mice with anti-CD3 (*right panel*) synthesis of lymphotactin, MIP-1α, MIP-1β, MCP-1, and CRG-2/IP-10 was evident. At 12 h after activation of Th1 and Th2 cells, the predominant chemokine detected for both CD4 subsets was MIP-1β. However, 16-fold greater expression was found in Th1 compared with Th2 cells. Lymphotactin and MIP-1α were also

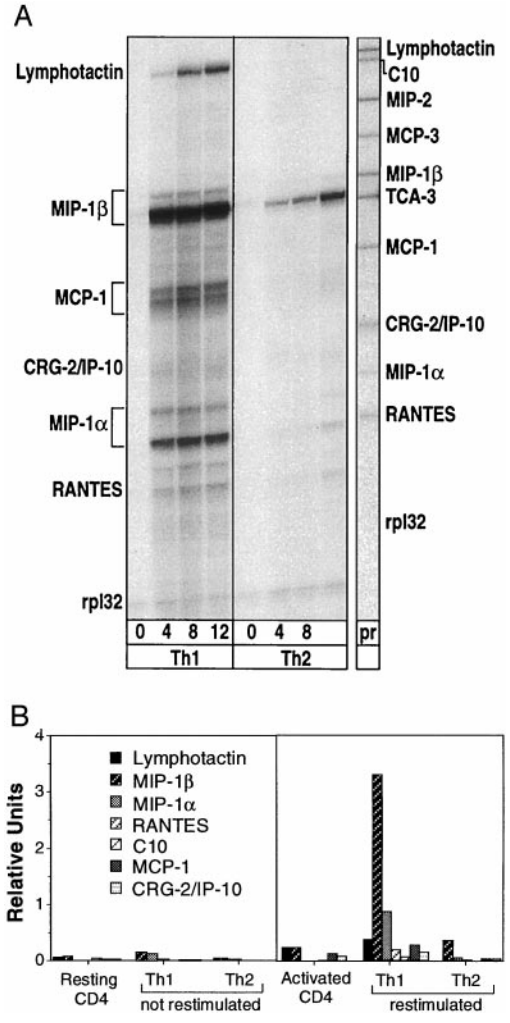

FIGURE 2. Chemokine mRNA detected in Th1 and Th2 cells. *A*, RNA was extracted from Th1 (*left*) or Th2 (*right*) cells on day 4 of primary culture, before stimulation (0), or at 4, 8, and 12 h after stimulation with immobilized anti-CD3 in the absence of added cytokines or cytokine mAb. Expression of the indicated chemokines was analyzed by RNase protection. Individual bands were identified by comparison to the probe set hybridized to transfer RNA (pr). The probes (labeled on the *right*) ran more slowly than the digested fragments from target RNA due to the presence of restriction sites that resulted in lower bands for the individual chemokines in the test samples (labeled on the *left*). *B*, RNA was extracted from resting BDC CD4 cells or Th1 and Th2 cells on day 4 of primary culture (*left panel*) or from the same populations after stimulation for 12 h with immobilized anti-CD3 (*right panel*). Expression of the indicated chemokines was analyzed by RPA and was quantitated by comparison to *rpl32* by phosphoimaging.

highly expressed in restimulated Th1 cells. We also examined Th1 and Th2 cells for synthesis of several other chemokines that included eotaxin, MCP-5, neurotactin, monokine induced by IFN-γ (MIG), exodus, and LPS-induced CXC chemokine (LIX), which were not detected (data not shown). The data demonstrate dramatic differences in the capacity of CD4 subsets to synthesize chemokines and association of multiple chemokines with Th1, but not Th2, cells. Thus, similar to cytokines, there is partitioning of chemokine profiles in Th1 vs Th2 subsets.

*Induction of diabetes BDC Th1 but not Th2 cells after transfer to NOD.*scid *recipients*

To evaluate the capacities of islet-specific CD4 subsets to induce diabetes, Th1 or Th2 cells were transferred to groups of 12

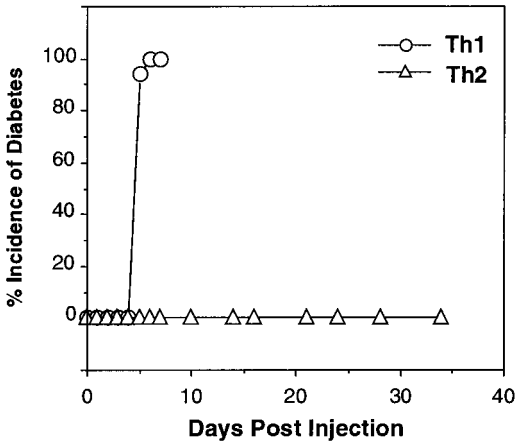

FIGURE 3. Th1, but not Th2, cells induce diabetes in NOD.*scid* recipients. Th1 or Th2 cells were harvested from primary cultures on day 4, and 8×10^6 cells were injected separately into groups of 12 NOD.*scid* mice. Blood glucose levels were measured on the indicated days. Two consecutive readings of >300 mg/dcl were considered indicative of diabetes onset.

NOD.*scid* mice. Recipients were monitored for onset of diabetes by blood glucose levels. As shown in Fig. 3, all mice that received Th1 cells became diabetic by day 6 after cell transfer, whereas recipients of Th2 cells had not developed disease by day 34 after transfer, when the experiments were terminated. As previously reported (42), resting CD4 cells from the spleens of BDC mice also did not induce diabetes (not shown). Identical results were obtained in two other experiments with 36 additional recipients. The Th1 and Th2 cytokine secretion phenotypes were stable after in vivo transfer of the CD4 subsets. On day 3 after transfer, $v\beta4^+$ cells from the spleens of recipients of Th1 cells produced IFN-γ, but not IL-4, in response to stimulation by anti-CD3, whereas recipients of Th2 cells produced IL-4, but not IFN-γ (Fig. 4). $v\beta4^+$ from recipients of either subset secreted IL-2, which is characteristic of cells that have returned to a resting state (43) (not shown).

Analysis of the pancreata by histology at the time of disease onset in recipients of Th1 cells (Fig. 5) showed that transfer of Th1 cells was associated with marked inflammation of the pancreas and extensive damage to the exocrine tissue (Fig. 5, *A* and *B*). There were no visible islets and a complete absence of insulin staining

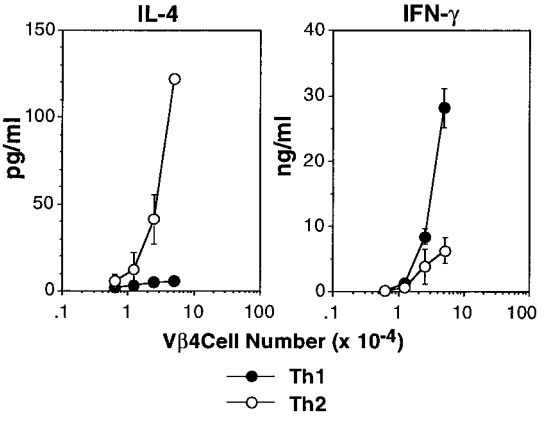

FIGURE 4. Polarization of CD4 subsets after adoptive transfer. Spleen cells from NOD.*scid* recipients of 8×10^6 Th1 or Th2 cells were isolated at 3 days after transfer and stained for the presence of $v\beta4^+$ CD4 cells. The indicated numbers of $v\beta4^+$ cells were cultured on plates coated with anti-CD3 (10 μg/ml) for 36 h, and supernatants were tested for IL-4 and IFN-γ by ELISA.

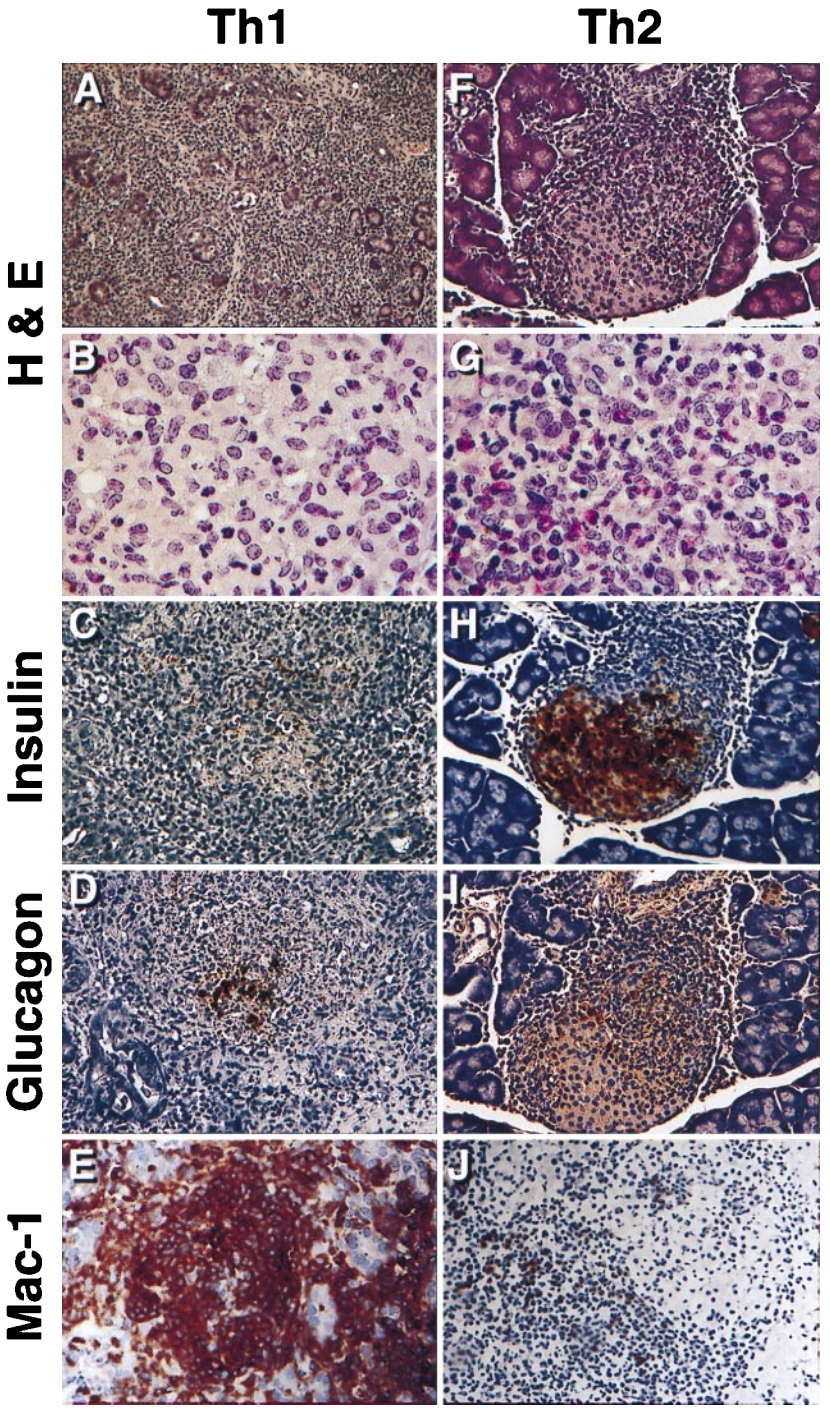

FIGURE 5. Histologic analysis of pancreata from recipients of Th1 or Th2 cells. At 6 days after transfer of Th1 (*left panels*) or Th2 (*right panels*) cells to NOD.*scid* recipients, as described for Fig. 3, 4-μm paraffin sections of pancreata were stained with hematoxylin and eosin (*A*, *B*, *F*, and *G*), insulin (*C* and *H*), and glucagon (*D* and *I*) as indicated in *Materials and Methods*. Eight-micron cryosections were stained with mAb to Mac-1 (*E* and *J*). Tissues are shown at the following magnifications: *A*, ×10; *C–E*, *F*, *H–J*, ×20; and *B* and *G*, ×40.

(Fig. 5*C*), indicating destruction of the β-cells. Sections showed staining for glucagon (Fig. 5*D*), indicating that α-cells were spared. In contrast, although many islets were clearly infiltrated in mice injected with Th2 cells, as shown in *F* and *G*, both β- and α-cells were intact, as indicated by staining with insulin (*H*) and glucagon (*I*), respectively. The infiltrates in recipients of Th2 cells did not progress throughout the 34-day period of observation, and infiltrated islets continued to produce insulin (not shown). There were no visible infiltrates in pancreata of NOD.*scid* mice that were not injected with CD4 cells (not shown).

Although neither Th1 nor Th2 cells homed to the pancreas within 24 h after transfer to NOD.*scid* recipients as determined from the distribution of vβ4$^+$ cells that were labeled with ^{51}Cr or by immunohistology (not shown), marked differences in the pancreatic infiltrates after transfer of Th1 and Th2 cells were seen as early as day 3 after transfer of the two subsets, when vβ4$^+$ cells were found within islets of Th1 recipients, but were not yet detected in Th2 recipients (Fig. 6, *A* and *C*). By day 6 after transfer of Th2 cells, infiltrating vβ4$^+$ cells were readily observed in significant numbers surrounding islets (Fig. 6*D*). In contrast, tg$^+$ cells were now diffusely distributed throughout the pancreas in the recipients of Th1 cells (Fig. 6*B*), where there was extensive infiltration of monocytes/macrophages (Fig. 5*B*) as shown by the prominence of Mac-1$^+$ cells (Fig. 5*E*). Many fewer Mac-1$^+$ cells were present in the pancreata of Th2 recipients (Fig. 5*J*), and in these mice infiltrates were predominantly comprised of polymorphonuclear cells, many of which exhibited characteristics of eosinophils and neutrophils (Fig. 5*G*). The data demonstrate that Th1 cells

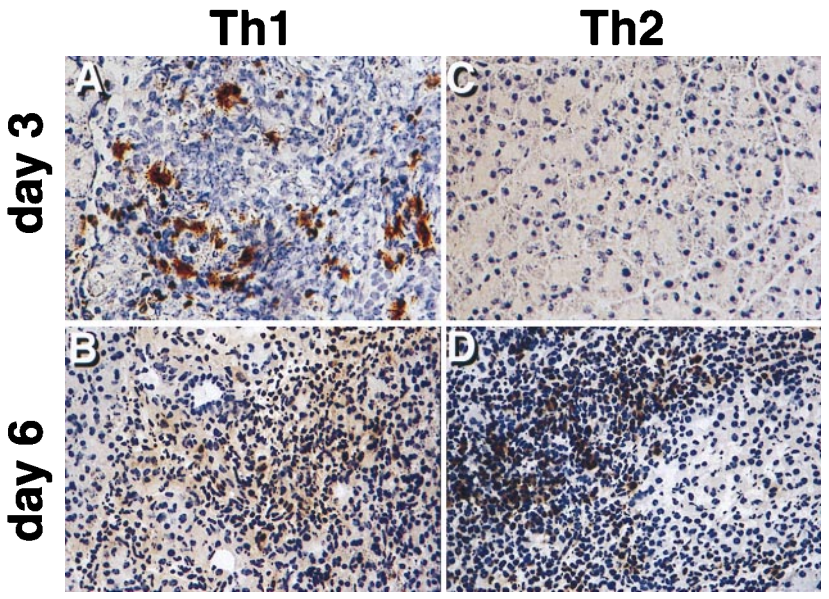

FIGURE 6. Infiltration of the pancreas by Th1 and Th2 cells. Eight-micron cryosections of pancreas were stained with mAb to vβ4 at 3 days (*A* and *C*) and 6 days (*B* and *D*) after transfer of Th1 or Th2 cells to NOD.*scid* recipients. Magnifications are $\times$20.

rapidly infiltrate the pancreas and that destruction of both the endocrine and exocrine tissue occurs as a consequence of their entry leading to diabetes onset. Th2 cells appear in the pancreas with delayed kinetics, and although they become established within the tissue, very different infiltrates result, and β-cells are not destroyed.

The data shown in Table I summarize insulitis indexes for the two groups of mice. In recipients of Th1 cells, the majority of islets were either surrounded (49%, peri-insulitis) or invaded (21%, insulitis) by infiltrating cells. In contrast, in recipients of Th2 cells, 91% of islets were devoid of infiltrates. Only 7% of islets were observed with peri-insulitis, and in only 2% was insulitis established. When dividing cells were labeled by administration of BrdU, no proliferation was visible on day 3 after transfer of Th2 cells when infiltration was very limited, whereas a small number of dividing cells were found in infiltrates around islets in recipients of Th1 cells (not shown). However, on day 5 after cell transfer when both groups of recipients had visible pancreatic infiltrates, Th1 mice showed 38.1 $\pm$ 0.8% BrdU$^+$ cells in infiltrates compared with 9.5 $\pm$ 1.2% for Th2 mice, indicating that cellular expansion occurred in situ in both groups, but was greater in Th1 recipients.

vβ4$^+$ cells were readily detected in lymphoid tissues in recipients of either Th1 and Th2 cells by flow cytometry on day 6 (Fig. 7), particularly in the pancreatic LN. These data indicate that both CD4 subsets had the capacity to reconstitute the NOD.*scid* mice after transfer. However, although both groups of recipients were injected with the same number of vβ4$^+$ cells that were uniformly activated and were indistinguishable in terms of several surface adhesion receptors, fewer tg$^+$ cells were detectable in the recipients of Th2 cells compared with Th1 cells. In particular, in the pancreatic LN, vβ4$^+$ cells were 9 times more numerous in recipients of Th1 cells than Th2 cells. Tg$^+$ cells were also found in nearly twofold greater numbers in the spleens of recipients of Th1 cells and were fourfold more numerous in mesenteric and peripheral LN. The results indicate that Th1 cells become preferentially established in the LN that drain the pancreas.

Induction of adhesion molecules on pancreatic endothelium after transfer of Th1 and Th2 cells

Previous studies demonstrate that multiple adhesion receptors are expressed on pancreatic endothelium of NOD mice when lymphocytic infiltrates are observed (44, 45). On day 3 posttransfer of Th1 cells to NOD.*scid* recipients, when vβ4$^+$ cells were clearly evident in the pancreas, expression of several adhesion molecules was induced on pancreatic endothelium (Table II). Staining was observed with mAb to LFA-1; its counter-receptor, ICAM-1; and the activation-induced vascular adhesion receptor, VCAM-1. PNAd, a major ligand for L-selectin, and MAdCAM-1, the major ligand for $\alpha_4\beta_7$, were also present on endothelium on day 3 after transfer of Th1 cells. In contrast, none of these adhesion molecules was found on the pancreatic endothelium of recipients of Th2 cells when few vβ4$^+$ were detected. By day 6, when vβ4$^+$ cells were numerous in pancreata of Th2 recipients, LFA-1 and ICAM-1 were highly expressed on pancreatic endothelium. VCAM-1, PNAd, and

Table I. *Infiltration of islets posttransfer of Th1 or Th2 cells to NOD.*scid *recipients*

CD4 Subset[a]	No Infiltrate	Peri-insulitis	Insulitis	Total Islets
Th1 (n = 6)	50 (30%)	80 (49%)	34 (21%)	164
Th2 (n = 6)	146 (91%)	11 (7%)	4 (2%)	161
p value Student's t test (paired)	$<$0.001	0.036	0.009	

[a] Th1 and Th2 cells were generated from CD4 cells that were isolated from the spleens of BDC 2.5 TCR transgenic mice by culture in anti-CD3-coated flasks in medium supplemented with rIL-2 and anti-CD28 and either rIL-12 and anti-IL-4, or rIL-4 and anti-IFN-γ, respectively, as described in *Materials and Methods.* After 4 days, 8×10^6 cells of each subset were transferred to groups of NOD.*scid* recipients (n = 6). On day 3 after cell transfer, paraffin-embedded sections of the pancreas were stained with hematoxylin and eosin, and individual islets were scored for the presence of infiltrates. Between 10 and 30 islets per pancreas were analyzed in five levels of sections that differed by 80 μm. For each category, the two groups of recipients were significantly different as calculated using the Student t test (paired).

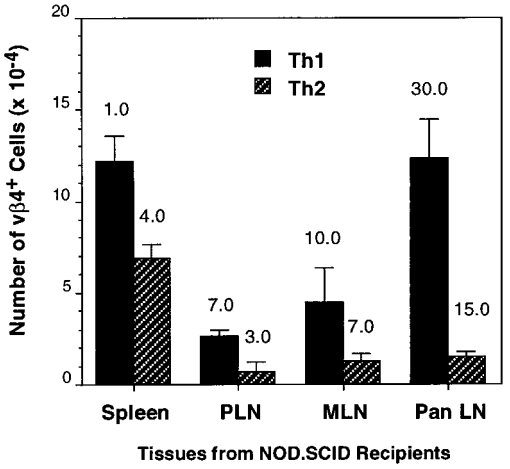

FIGURE 7. Distribution of vβ4$^+$ cells in recipients of Th1 or Th2 cells. On day 6 after transfer of Th1 (■) or Th2 (▨) cells to NOD.*scid* recipients ($n = 13$ and $n = 7$ for Th1 and Th2 recipients, respectively), as described in Fig. 3, vβ4$^+$ cells were quantitated from the percentage of positively staining cells (shown *above each bar*), determined by flow cytometry and the total cells recovered per organ. PLN, peripheral LN; MLN, mesenteric LN; Pan LN, pancreatic LN.

MAdCAM-1 were also detected, although staining was not as extensive as that seen in pancreata from Th1 recipients on day 3 after transfer. Together, the histology data indicate that Th1 cells infiltrate/accumulate in the pancreas more rapidly after adoptive transfer than Th2 cells but that both populations have the capacity to induce expression of several adhesion receptors on pancreatic endothelium that facilitate extravasation of cells from the blood into tissue.

In vivo induction of chemokines in the pancreas after transfer of Th1 or Th2 cells

To identify chemokines that were produced in vivo in recipients of Th1 and Th2 cells, we analyzed expression of pancreatic mRNA isolated on days 3 and 6 after adoptive transfer to NOD.*scid* recipients in comparison with that of NOD.*scid* mice that did not receive CD4 cells and of age-matched NOD mice. As shown in Fig. 8, chemokine gene expression was not detectable in uninjected NOD.*scid* mice, although a low level of C10 mRNA was detected in NOD mice (*left panel*). Chemokine mRNA was not observed until day 6 after transfer of either Th1 or Th2 cells. Several chemokines were then seen in the pancreata of recipients of Th1 cells, including C10, MCP-1, MCP-3, MCP-5, CRG-2/IP-10, and eotaxin, as well as low levels of lymphotactin, MIP-1β, and RANTES. In marked contrast, C10 and low levels of eotaxin were the only chemokines expressed in pancreatic mRNA from recipients of Th2 cells. While lymphotactin is primarily produced by activated T cells, and C10 is produced predominantly by macrophages, the other chemokines that we detected are produced by multiple cells types.

Table II. *Adhesion molecule expression by pancreatic endothelium*

	Th1[a]		Th2	
Marker	Day 3	Day 6	Day 3	Day 6
vβ4	+++	+++	+/−	+++
LFA-1	+	+	−	+
ICAM-1	+	+	−	+
VCAM-1	+	+	−	+
PNAd	+	+	−	+
MAdCAM-1	+	+	−	+

[a] Th1 and Th2 cells were generated from CD4 cells that were isolated from the spleens of BDC 2.5 TCR transgenic mice by culture in anti-CD3-coated flasks in medium supplemented with rIL-2 and anti-CD28 and either rIL-12 and anti-IL-4, or rIL-4 and anti-IFN-γ, respectively, as described in *Materials and Methods*. After 4 days, 8×10^6 cells of each subset were transferred to groups of NOD.*scid* recipients ($n = 6$). At 3 and 6 days after cell transfer, pancreata from recipients were snap-frozen, and 8-μm-thick sections were cut on a cryostat, air-dried overnight, and stained with mAb to vβ4 to identify transferred T cells, and to the indicated adhesion markers followed by appropriate biotinylated second-step reagents and biotin-avidin peroxidase. After the color reaction with diaminobenzidine, sections were counterstained with hematoxylin.

Discussion

In the present study we demonstrate that islet-specific Th1 differ from Th2 cells by their capacity to more rapidly infiltrate and/or accumulate in the pancreas. Considerable evidence indicates that Th1 cells are the primary population that mediates IDDM via production of proinflammatory cytokines, including IFN-γ, LT, and TNF-α (reviewed in Ref. 46), whereas Th2 cells invade the islets but do not typically cause disease, principally due to the protective effects of IL-4 (8–10, 47). Here we show that islet-specific Th1 but not Th2 cells from BDC2.5 TCR transgenic mice cause diabetes in immunodeficient NOD recipients (Fig. 3), supporting previous studies indicating that these subsets can be distinguished by their capacity to promote disease onset in NOD mice (2, 10). Although we did not find that Th2 cells elicited disease in NOD.*scid* mice as was previously reported (7), we used splenic CD4 cells, which in BDC mice are enriched for cells with a memory phenotype (42), rather than thymic CD4 cells, which are naive.

In this study we provide direct evidence that in addition to distinctive cytokine secretion patterns, Th1 cells can be distinguished from Th2 cells by differences in chemokine synthesis (Fig. 2). Th1 cells produced RNA for multiple chemokines, including lymphotactin, MCP-1, MIP-1α, RANTES, and CRG-2/IP-10. MIP-1β was produced by both CD4 subsets and was the only chemokine RNA detected in Th2 cells, although in less abundance than in Th1 cells. Chemokine synthesis was also markedly different in the pancreata of recipients of Th1 vs Th2 cells (Fig. 8). By day 6 after Th1 cell transfer, MCP-3, MCP-5, eotaxin, and CRG-2/IP-10 were detected in addition to lymphotactin, RANTES, and MCP-1, whose synthesis was also associated with in vitro stimulated Th1 cells. In contrast, transcripts for C10 and eotaxin were found in pancreatic mRNA from recipients of either Th1 or Th2 cells, and C10 was the only chemokine mRNA of those we tested to be found in pancreatic tissue from NOD mice. Most of these chemokines are produced by a variety of cell types, including activated macrophages and T cells, and have chemotactic activity for these populations as well (14, 15). However, lymphotactin production as well as its chemotactic effects are primarily associated with activated T cells (48), suggesting that Th1 cells themselves have the capacity to recruit additional Th1 cells into the pancreas.

Although there is considerable redundancy in the use of receptors by chemokines (49), recent studies demonstrate differences in the expression of receptors by Th1 (CCR5 and CXCR3) and Th2 cells (CCR3, CCR4, and CCR8) (22, 25–28). In particular, CCR5 is a receptor for RANTES, MIP-1α, and MIP-1β, which are associated with Th1 responses (50). CXCR3 binds to CRG-2/IP-10 and monokine induced by IFN-γ (MIG), chemokines that attract activated T cells and are induced by IFN-γ (51, 52) as well as 6Ckine (25), a novel CC chemokine with chemotactic effects for resting and activated T cells (17, 53). CCR3 is a receptor for eotaxin (27), while ligands for CCR4 include MIP-1α and MCP-1,

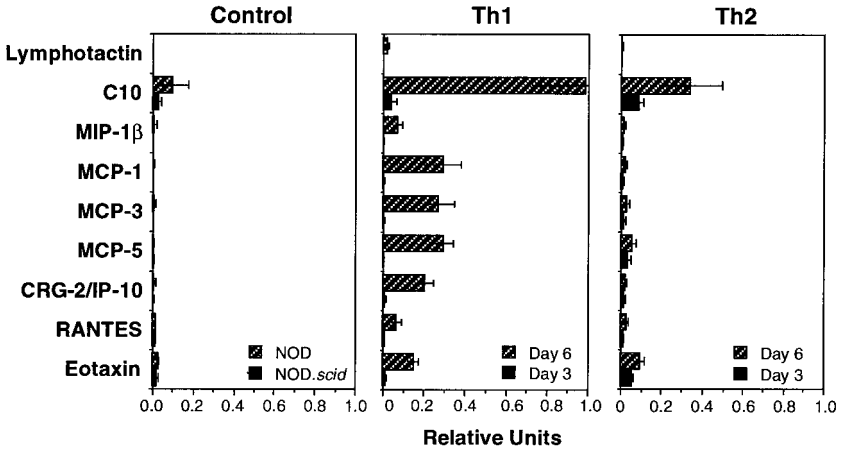

FIGURE 8. Chemokine mRNA detected in pancreata of NOD.*scid* recipients after transfer of Th1 or Th2 cells. mRNA (poly(A) purified) was prepared from pancreata of groups ($n = 3$) of 10-wk-old NOD mice (▨) or NOD.*scid* mice (■; *left panel*), or from the pancreata of recipients of Th1 cells (*middle panel*) or Th2 cells (*right panel*) on day 3 (■) and day 6 (▨) after cell transfer. Expression of the indicated chemokines was analyzed by RPA and quantitated by comparison to *rpl32* by phosphoimaging.

and both receptors bind RANTES (14, 49). In vitro, Th1, but not Th2, cells respond to several chemokines, such as MIP-1α, MIP-1β, RANTES, and CRG-2/IP-10 (21, 22, 24, 26), which become up-regulated in inflamed tissue (14)). In contrast, Th2, but not Th1, cells respond to eotaxin (26, 27). It is noteworthy that CRG-2/IP-10 is synthesized in situ in the pancreata of recipients of Th1 cells. This result establishes a key link between proinflammatory cytokines and chemokines in the induction of pathogenesis by CD4 cells in autoimmune diabetes.

Our finding that Th1 cells produce several chemokines for which they have receptors indicates that autocrine and/or paracrine usage of chemokines contributes to recruitment of T cells as well as other leukocytes into the pancreas. Since many chemokines have chemotactic effects on selected cell types (14, 15), differences in both the production and the response of CD4 subsets to chemokines would profoundly affect recruitment of cells, and as a consequence, the cellular composition of inflammatory infiltrates. Here we show that Mac-1$^+$ cells, which include macrophages and dendritic cells, were associated with Th1-mediated pathology (Fig. 5, *B* and *E*), whereas eosinophils, which are associated with IL-5 production and allergic inflammation (46), were primarily seen after transfer of Th2 cells (Fig. 5*G*). Macrophages represent the initial population present in the pancreas during development of IDDM in NOD mice (reviewed in Ref. 54) and play a key role in the destruction of islets (55, 56). Previous studies indicate that MCP-1, which is produced by Th1, but not Th2, cells (Fig. 2), causes selective recruitment of monocytes/macrophages into the pancreas (57). Mice deficient in the MCP-1 receptor, CCR2, have defective Th1-type responses as well as cellular recruitment to inflammatory lesions (58). Thus, one example of a feedback mechanism that may contribute to the progression of disease is Th1-mediated recruitment of macrophages via secretion of MCP-1, induction of CRG-2/IP-10 production by macrophages in response to Th1-derived IFN-γ, and recruitment of additional Th1 cells via response to CRG-2/IP-10. Additional cytokine/chemokine regulatory loops can be envisioned from the known expression of receptors for these molecules on various cell types.

Several studies have shown that IFN-γ and IL-4, which have counter-regulatory effects in IDDM, can be used in an autocrine manner by Th1 and Th2 cells, respectively, to promote their growth and differentiation (reviewed in Ref. 46). Recent studies indicate that chemokine receptors on activated T cells are modulated by cytokines that include IL-2, IL-4, IL-10, IL-12, TGF-β, and IFN-α (22, 59), revealing an additional level of cytokine-mediated control of T cell recruitment. Further, cytokines may also regulate chemokine synthesis. Thus, although Th2 cells, like Th1 cells, bear the CCR2 receptor for MCP-1 (26), Th2 cytokines can inhibit production of MCP-1 by monocytes/macrophages (60). An implication of our data is that it is the combination of proinflammatory cytokines and chemokines synthesized by Th1 cells within the microenvironment of the pancreas that regulates inflammation leading to destruction of the islets. In contrast, the more limited synthesis of chemokines by Th2 cells combined with down-regulation of inflammatory cytokines by IL-4 results in significantly less inflammation and determines the cellular composition of infiltrates that lack destructive potential. When mixtures of autoreactive CD4 subsets are present, differences in recruitment of inflammatory leukocytes could be a crucial check point for regulation of IDDM.

Our analysis of the homing behavior of islet-specific CD4 subsets demonstrated that vβ4$^+$ cells were first visible in the pancreas and surrounding islets in Th1 recipients on days 2–3 posttransfer, when dividing cells were also seen. In contrast, infiltrates were not prominent until several days later in recipients of Th2 cells. Induction of LFA-1, ICAM-1, VCAM-1, PNAd, and MAdCAM-1 on pancreatic vessels correlated with the presence of vβ4$^+$ cells in recipients of either CD4 subset (Table II). The data suggest that Th1 cells enter the pancreas more quickly than Th2 cells, allowing their local response to islet Ag to outpace that of Th2 cells, leading to increased numbers of vβ4$^+$ cells in various tissues of recipients of Th1 vs Th2 cells by day 6 when recipients of Th1 cells were diabetic (Figs. 6 and 7). Notable was the prominence of Th1 cells in pancreatic LN compared with Th2 cells, suggesting that Th1 cells undergo greater expansion than Th2 cells in response to Ag in the LN that drain the pancreas. Since defective lymphocyte death has been implicated in systemic autoimmune processes (61), it is also possible that Th2 cells are more readily eliminated than Th1 cells after exposure to islet Ag, resulting in fewer cells in both lymphoid tissues and the pancreas.

We envision that activated CD4 cells may typically enter nonlymphoid tissues even when no inflammation pre-exists, as has been proposed to occur during immunosurveillance by memory CD4 cells (62, 63), using adhesion receptors that become elevated in response to initial stimulation by Ag and counter-receptors that are constitutively expressed on endothelium (reviewed in Ref. 11). We presume that this random entry occurs at a low level, since we did not detect vβ4$^+$ cells in tissues such as kidney, liver, or lung after transfer of either Th1 or Th2 cells (not shown). Infiltrating effector CD4 cells have the potential to respond to Ag in nonlymphoid tissues such as the pancreas due to decreased requirements for costimulation, by producing cytokines and chemokines that can then activate the endothelium to express adhesion receptors and secrete additional mediators that regulate the entry of cells into

tissue. The capacity for polarized chemokine expression by Th1 vs Th2 within the microenvironment of the pancreas contributes to the rapid development of distinctive inflammatory infiltrates, setting an irrevocable course leading to the development of diabetes.

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