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Tertiary Lymphoidal Structures in the Pancreas Promote Selection of B Lymphocytes in Autoimmune Diabetes

Peggy L. Kendall,* Guowu Yu,* Emily J. Woodward,† and James W. Thomas2*†

Autoimmune diabetes occurs when invading lymphocytes destroy insulin-producing β cells in pancreatic islets. The role of lymphocytic aggregates at this inflammatory site is not understood. We find that B and T lymphocytes attacking islets in NOD mice organize into lymphoid structures with germinal centers. Analysis of BCR L chain genes was used to investigate selection of B lymphocytes in these tertiary lymphoid structures and in draining pancreatic lymph nodes. The pancreatic repertoire as a whole was found to be highly diverse, with the profile of L chain genes isolated from whole pancreas differing from that observed in regional lymph nodes. A Vκ14 L chain predominated within the complex pancreatic repertoire of NOD mice. Skewing toward Vκ4 genes was observed in the pancreas when the repertoire of NOD mice was restricted using a fixed Ig H chain transgene. Nucleotide sequencing of expressed Vκ/H9260 charges. This article must therefore be hereby marked

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Tertiary lymphoid structures (TLS)3 are recognized in multiple inflammatory tissues in both mice and humans, including rheumatoid arthritis (1–4), multiple sclerosis (5, 6), myasthenia gravis (7, 8), and chronic hepatitis C (9, 10). These organized aggregates of lymphocytes are induced by inflammation, unlike those in developmentally programmed secondary lymphoid organs (spleen and lymph nodes) which they resemble. Previous studies have indicated that secondary and tertiary lymphoid structures share many commonalities, such as their organization and underlying chemotactic factors (11–13). Some tertiary lymphoid aggregates have also been shown to contain germinal centers (GC; Refs. 14–16) or to support somatic hypermutation of BCRs (17–19). The role of these inflammatory lymphoid structures in relationship to adjacent secondary lymphoid organs is not known. It could be hypothesized that they simply duplicate the functions of nearby draining lymph nodes or serve as holding areas for lymphocytes which have been primed and activated in those nodes. However, because TLS are present at the site of organ destruction, understanding their unique functions in advancing the immune response may provide targets for disruption of the process.

In type 1 diabetes (T1D), lymphocytic infiltration of islets (insulitis) is a harbinger of disease. Tertiary lymphoid structures have been identified in insulitis lesions (20–22), chiefly in models that express foreign Ags under the insulin promoter, but their nature is not well characterized. In particular, little is known about B lymphocytes within these foci of autoreactivity. Studies on T cell receptors have shown oligoclonality within isolated islets, with a polyclonal repertoire across the pancreas as a whole (23, 24), but their relationship to regional lymph nodes was not explored. Studies of cellular dynamics revealed that B and T lymphocytes invade the islets concurrently (25) and that CD4+ T cell priming and proliferation occur in draining pancreatic lymph nodes before the onset of insulitis (26–28), suggesting a collaborative relationship between these secondary lymphoid organs and TLS in the islets. Lymphocytic invasion of islets, however, is not enough to cause overt disease, because some mice with insulitis fail to progress to diabetes (29). Although autoaggressive forces that drive inflammation within the islets have not been thoroughly explored, the protective action of regulatory T cells was recently shown to occur at the inflamed site (30, 31). Thus, whereas pancreatic lymph nodes have a critical role in pathogenesis, events within islets under attack also govern disease outcomes. Collectively, these data underscore the importance of understanding the nature of the lymphocytic infiltrates in the islets and their relationships to lymphocytes in the regional lymph nodes.

Most studies of islet infiltrates have not included B lymphocytes, although they are known disease promoters. B cells must be present for disease to occur in the NOD model (32–34), must have autoreactive specificities (35, 36), must express appropriate MHCII alleles (37), and are essential for the processing and presentation of at least one islet Ag (38). Thus, a key role for B cells is most likely that of Ag presentation (39, 40), which requires an organized
lymphoid setting to promote T-B cell interactions. Recent studies have also shown that B cells can be engineered to down-regulate T cells via cell to cell contact, offering protection against diabetes (41). Thus, knowledge of the characteristics of B cells populating tertiary vs secondary lymphoid tissues may provide critical clues for disrupting the autoimmune process at the site of attack.

The data presented here show that the lymphocytes infiltrating the islets of NOD mice are organized into tertiary lymphoid structures that contain central T cell zones surrounded by B cell follicles, and include GCs. To delineate the relationship between lymphocytes in this inflamed setting and those populating the draining pancreatic lymph nodes, we analyzed the Ig L chain repertoire expressed by B cells at these two sites in NOD mice. To simplify the analysis while maintaining a diverse repertoire, we also used a disease-promoting transgenic model in which B cells express a fixed H chain, Vg125Tg/NOD (36), that is capable of recombining with a wide range of Vκ (L chain) genes (42). This strategy detects a difference in the repertoire in the pancreas as compared with lymph nodes in both wild-type and HC transgenic NOD mice. Further analyses of nucleotide replacements in Vκ genes from the pancreas support the presence of Ag-driven somatic hypermutation in inflamed islets and are consistent with the presence of GCs by histology. In contrast to the polyclonal repertoire in the pancreas, Vκs isolated from single islets demonstrate oligoclonality. In addition, a large proportion of these Vκ sequences that contain mutations in the CDRs encode for amino acid replacements, consistent with Ag-driven selection of somatically hypermutated light chains. Differences in the expression of B cell Ag receptors in the pancreas and draining lymph node tissues were further validated using flow cytometry to measure B lymphocyte binding of a key autoantigen, insulin. These combined findings indicate that TLSs in islets provide a site at which somatic evolution of selected B lymphocytes diversifies the autoimmune attack and promotes disease progression.

Materials and Methods

Animals

Vg125Tg/NOD mice used in these studies were generated as previously described (36) and are maintained as hemizygotes, backcrossed to wild-type NOD mice (>20 generations). Nontransgenic NOD littermates were also used. Mice are housed in specific pathogen-free conditions. The Institutional Animal Care and Use Committee of Vanderbilt University approved all procedures.

Lymphocyte extraction and islet isolation

Pancreata were harvested immediately after sacrifice and dissected free of contaminating lymph nodes or fat strands. The capsule was injected with 1 mg/ml collagenase P (Roche Diagnostics) in HBSS followed by submersion in 3 ml of the collagenase solution for 30 min at 37°C with agitation. DNase (Sigma-Aldrich; 2.5 mg/ml) was added at 1/100, and the tissue was digested through an 18-gauge needle several times. Islets were isolated using density gradient separation (Isolymph; Gallard-Schlesinger Industries) and centrifugation at 1200 rpm for 30 min at room temperature. The buffy coat, containing islet material as well as lymphocytes, was removed and washed. For islet isolation, pancreata were macerated with scissors in a glass dish in 1 ml of HBSS. Tissue was transferred into a glass tube, and HBSS was added to 4 ml, with 12 mg of collagenase P, and then agitated for 12 min at 37°C. After washing, pancreatic material was placed in 10 ml of HBSS, and islets were hand-picked using a dissecting microscope. Islets were placed in culture overnight at 37°C with 5% CO2 before analysis, and islets with extruded lymphocytes were selected. Control experiments were performed on islets without extruded lymphocytes. Blood mononuclear cells were isolated using density gradient separation as described.

RNA isolation, Vκ amplification, and analysis

Pancreatic lymph nodes, and spleen were harvested immediately after sacrifice and placed directly into RNALater (Ambion.) Islets were isolated as described previously and then placed in RNALater. Germinal center B lymphocytes from pancreatic lymph nodes were sorted with a FACSaria Cell Sorting System (BD Biosciences), gating on GL7, B220+, 7-aminoactinomycin D (7AAD)- cells within the lymphocyte region by size and granularity. RNA extraction was performed with the RNeasy Mini or Micro Kit (Ambion), following the manufacturer’s instructions. For Vκ gene analysis, first-strand cDNA was generated from RNA, combined with Superscript II RN (Invitrogen Life Technologies), and 0.67 μg of oligodeoxynucleotide primer (Amershams Biosciences), in a standard cDNA synthesis protocol. Vκ-Jκc genes were PCR amplified using the following primers: a universal degenerate murine Vκ primer 5'-ATTGTGKTMSCACMCRTCTCA-3' and murine κ constant primer 5'-GGATACTAGTGGTGTGACATC-3'. The PCR reaction included AmpliTaq DNA polymerase (2 μl/reaction) (Applied Biosystems), 200 μM dNTP, 1.25 mM MgCl2, 250 nM κ constant primer, and 250 nM Vκ primer. The reaction was cycled at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min for 40 cycles. PCR product was ligated into pGEM-T Easy linearized plasmid (pGEM-T Easy Vector System I; Promega). An Applied Biosystems 3730xl DNA analyzer (Vanderbilt-Ingram Cancer Center, Nashville, TN) was used to sequence positive clones. Blastn (www.ncbi.nlm.nih.gov/BLAST), the ImMunoGeneTics database (http://imgt.cines.fr/ISt04), and Bio Edit (www.bioedit.org) were used in sequence analysis and germline assignment. Statistical analysis of Vκ profiles was performed using “R” software (R Development Core Team. 2006. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0; http://www.R-project.org).

Flow cytometry

Lymphocytes were suspended at 1 x 10⁶/100 μl in a buffer solution containing 2.5% sodium azide and 3% PBS in PBS. Staining was performed using fluorochrome-conjugated Abs to B220, IgM, IgG, GL7, and CD3 from BD Pharmingen. For detection of insulin, biotinylated human insulin (Becton Dickinson) was used to exclude dead cells; this is especially critical in lymphocytes derived from the pancreas. Biotinylated human insulin conjugated in our laboratory was used at 500 ng/100 μl to identify insulin-binding cells, followed by avidin-FITC. Insulin-biotin conjugates are not hormonally active and do not detect hormone receptors on lymphoblastoid cell lines. Duplicate samples prepared for inhibition studies also contained unlabeled insulin at 500 ng/ml. Labeled cells were analyzed using FACS-Calibur flow cytometer (BD Biosciences) and data analyzed using WinMDI software (J. Trotter, Scripps Institute, San Diego, CA). Lymphocyte gate by size and granularity is used for all plots.

Histology and immunofluorescence

Pancreata were fixed in 10% neutral buffered formalin, dehydrated by graded ethanol series, cleared in xylene, and embedded in paraffin blocks. The blocks were then sectioned at 5 μm and stained with H&E, following standard protocols. Sections were evaluated using light microscopy. For immunofluorescence, freshly harvested pancreata were fixed in 4% paraformaldehyde-0.1 M PBS (12.07 g of Na2HPO4 (dibasic), 2.04 g of KH2PO4 (monobasic), 8.0 g of NaCl, 2.0 g of KCl; pH 7.5, same-day preparation) for 1.5 h at 4°C under mild agitation, followed by four washings in 0.1 M PBS over a period of 2 h at 4°C under mild agitation. Tissue was equilibrated in 30% sucrose in 1 x PBS (Invitrogen) overnight at 4°C until tissue settled to the bottom of the tube. Pancreata were then frozen in OCT (Sakura Finetek), and cut into 8 μm sections using a cryostat microtome (Leica). Sections were rehydrated with PBS for 2 min before blocking for 30 min at room temperature in blocking buffer (5% normal goat serum and 1% BSA in 1 x PBS), then stained with anti-B220-FITC and anti-CD3 PE or anti-B220 PE and anti-GL7 FITC for 1 h at room temperature, washed with PBS, dehydrated, mounted in Fluoromount (Dako). Slides were examined by conventional fluorescence microscopy using an Olympus BX60 epifluorescence microscope. Images were captured using a charge-coupled device camera and MagnaFire software (Optronics) and optimized for signal-to-noise using Adobe Photoshop software (Adobe Systems); this software was also used to pseudocolor images containing anti-GL7, transposing red and green colors.

Results

Lymphocytes in the islets of prediabetic NOD mice form tertiary lymphoid structures

The relative composition of B and T lymphocytes within insulitis lesions in the pancreas was initially investigated using flow cytometry on mononuclear cells isolated from pancreata of prediabetic NOD mice. Fig. 1A shows a dot plot of pancreatic lymphocytes from a 9-wk-old NOD mouse in which B cells (B220+) and T cells...
(CD3+) are found in roughly even proportions (7.9 and 8.7%, respectively, B:T ratio, 0.48). The unstained population in this assay is due to pancreatic tissue components, including islets that segregate with lymphocytes in density, size, and granularity (24). To evaluate the possible contribution of non-islet-invading lymphocytes, such as those from blood vessels in the pancreas, we used nonautoimmune C57BL/6 mice, in which there is no insulitis, as controls. As Fig. 1B illustrates, T and B cell proportions from non-diabetes-prone pancreata are consistent with those found in blood (0.95% vs 0.39%, B:T ratio, 0.29). In absolute numbers, T cells gleaned from NOD pancreas were 137 × 10^3, vs 8 × 10^3 from C57BL/6, whereas NOD B cells numbered 125 × 10^3 vs 3 × 10^3 for C57BL/6. These data are representative of three experiments. We also performed flow cytometry on lymphocytes from whole blood to confirm published reports that circulating B:T ratios in NOD mice are similar to those found in blood (0.95% vs 0.39%, B:T ratio, 0.29). Thus, the large proportion of B and T lymphocytes in the pancreata of NOD mice relative to normal pancreata, as well as a pancreatic lymphocyte ratio that is not reflective of the ratio in circulation, indicates that blood-borne lymphocytes contribute only a small fraction of cells in whole pancreas.

Histology was then used to evaluate the nature of insulitis lesions in prediabetic NOD mice. H&E staining on fixed pancreatic sections (Fig. 2A) illustrates a typical pattern, in which lymphocytes (dark blue) have advanced en masse across the upper half of an islet, before engulfing it entirely. Because H&E staining does not differentiate B cells from T cells, we used immunofluorescence microscopy on frozen sections to ascertain the organization of invading lymphocytes. Immunostaining with GL7 (red) reveals the organization of invading lymphocytes; lighter area is healthy islet tissue. Images are typical. Original magnification, ×20 in A, B, and D; ×40 in C.

FIGURE 1. B and T lymphocytes contribute to insulitis in prediabetic NOD mice. A, Flow cytometry shows the composition of lymphocytes from the pancreas of a 9-wk-old prediabetic NOD mouse. B220+ B lymphocytes comprise 7.9% (125 × 10^3 in absolute numbers) and CD3+ T cells comprise 8.7% (137 × 10^3) of cells within the lymphocyte gate. The large proportion of unstained cells is due to nonlymphocyte pancreatic tissue cells that fall into this region. B, Analysis of a control pancreas from an 8-wk-old nonautoimmune C57BL/6 mouse shows that few lymphocytes are recovered when there is no islet invasion. Proportions of T cells (0.95%; 8 × 10^3) and B cells (0.39%; 3 × 10^3) are consistent with those found in blood. Cells were gated by size and granularity. 7AAD was used to exclude dead cells. Data are representative of three experiments.

FIGURE 2. Lymphocytes in pancreatic islets of prediabetic NOD mice form organized structures. A, Lymphocytes (blue) invade islets in a typical pattern of insulitis, as shown by H&E staining. Arrows indicate invading lymphocytes; lighter area is healthy islet tissue. B–D, Dual staining immunofluorescence reveals lymphocyte organization. B cells (B220, green) surround T cells (CD3, red) beginning with peri-insulitis (B), through partial invasion (C), becoming fully developed tertiary lymphoid structures when islets are fully invaded (D). Central black areas in B and C consist of healthy islet tissue. H&E staining performed on formalin-fixed, paraffin-embedded sections; immunofluorescence performed on paraformaldehyde-fixed, frozen sections. Images are typical. Original magnification, ×20 in A, B, and D; ×40 in C.

FIGURE 3. Inflamed islets contain GCs. Top, immunofluorescence microscopy shows that a highly invaded pancreatic islet (right), contains a GL7+ (red) aggregate within the tertiary B lymphocyte follicle (B220+, green), indicative of GCs. NOD spleen follicle (left) and pancreatic lymph node (middle) are shown for comparison. Central T cell areas are unstained. Bottom, Flow cytometry shows 2.7 ± 1.1% of total pancreatic B lymphocytes are positive for GL7, compared with 10.1 ± 0.9% of splenic B cells, and 16.4 ± 0.6% of B cells in the pancreatic lymph nodes (n = 3). GCs emerge within B lymphocyte zones of tertiary lymphoid structures in the islets

Secondary lymphoid tissues contain GCs in which higher affinity BCR specificities evolve via somatic hypermutation (44). To determine the potential for islet infiltrates to support GC reactions, the conventional GC marker GL7 was used. Spleens and draining pancreatic lymph nodes of NOD mice are known to contain GCs (45) and serve as controls. Immunofluorescence microscopy reveals GL7+ areas (red) within B cell zones (B220+, green) in an inflamed pancreatic islet from a 10 wk old prediabetic NOD mouse (Fig. 3, top right).
B lymphocytes invading the pancreas express endogenous light chains. Unbiased recombination with multiple Vk families was found in both pancreas and draining lymph nodes. Within the pancreas, however, a single L chain gene segment, Vk14–126, was overrepresented, comprising 30% of Vk genes isolated from NOD pancreata. Vk14–126, derived from the germ-line segment called br9, was found in four of six pancreata. In pancreatic lymph nodes, neither Vk14–126 nor any other L chain gene predominated. When nucleotide sequences were analyzed for combinatorial diversity in the joining gene segment, Vk14–126 was found to combine with Jk1 in 12%, with Jk2 in 38%, and with Jk4 in 50% of isolates. These Vk14–126 and J segment combinations were obtained independently in different mice, a finding that indicates the overrepresentation of Vk14–126 is not attributable to expansion of related clones. Furthermore, comparisons of Vk14–126 nucleotide sequences obtained from different mice show sequence conservation, without the mutations which would be expected should this dominance arise from expansion within a germinal center. Thus, a single, unmutated, L chain gene segment, Vk14–126, predominates among B lymphocytes invading the pancreata of multiple pre-diabetic NOD mice.

A single L chain predominates among Vk gene segments expressed by B lymphocytes invading the pancreas, but not adjacent lymph nodes

Secondary lymphoid organs harbor recirculating and resident B cells with a highly diverse repertoire. It is not known whether the same is true of TLS. To investigate the nature of the B lymphocyte repertoire in the pancreas, Vk genes were analyzed. RNA was purified from the pancreata of 9-wk-old, prediabetic NOD mice (n = 6) and used to generate cDNA. Pancreatic lymph nodes were used as controls. As flow cytometry studies indicated that 97% of B lymphocytes in the pancreas express κ L chains (data not shown), variable κ L chain (Vk) regions were amplified from total cDNA using a κ constant region primer and degenerate 5′-primers that anneal to >95% of all Vk gene segments. Nucleotide sequencing was performed on 41 isolates from pancreas and 25 isolates from draining pancreatic lymph nodes of the same mice. L chain identity was assigned based on sequence homology to known germ-line Vk genes published in the ImmunoGeneTics (V-Quest) and NCBI (IgBLAST) databases. Identical clones from a single PCR reaction were counted as one isolate for the purposes of frequency calculations (Fig. 4). A polyclonal repertoire, including members of the Vk1, 3, 4, 9, 12, and 14 families were found in both pancreas and draining lymph nodes. Within the pancreas, however, a single L chain gene segment, Vk14–126, was overrepresented, comprising 30% of Vk genes isolated from NOD pancreata. Vk14–126, derived from the germ-line segment called br9, was found in four of six pancreata. In pancreatic lymph nodes, neither Vk14–126 nor any other L chain gene predominated. When nucleotide sequences were analyzed for combinatorial diversity in the joining gene segment, Vk14–126 was found to combine with Jk1 in 12%, with Jk2 in 38%, and with Jk4 in 50% of isolates. These Vk14–126 and J segment combinations were obtained independently in different mice, a finding that indicates the overrepresentation of Vk14–126 is not attributable to expansion of related clones. Furthermore, comparisons of Vk14–126 nucleotide sequences obtained from different mice show sequence conservation, without the mutations which would be expected should this dominance arise from expansion within a germinal center. Thus, a single, unmutated, L chain gene segment, Vk14–126, predominates among B lymphocytes invading the pancreata of multiple pre-diabetic NOD mice.

Wild-type NOD B lymphocytes utilize a large repertoire of heavy and L chain combinations, thus diluting the impact of analysis of L chain gene expression alone. Therefore, these studies were extended to Vh125Tg/NOD mice, in which the BCR is generated by a fixed anti-insulin IgH chain transgene (Vh125)2, combined with endogenous light chains. Unbiased recombination with multiple Vκs results in a diverse repertoire in these mice, with only 1–3% of splenic B cells binding insulin (42). B lymphocytes in Vh125Tg/NOD mice support the development of T1D, and induce disease at an accelerated rate compared with wild-type NOD mice (36). These B cells have been shown to maintain allelic exclusion of >95% in the spleen, and analysis by flow cytometry shows that this allelic exclusion is preserved among B cells invading the pancreas as well (Fig. 5A). Because the H chain variable region of

FIGURE 4. A single L chain gene predominates among B lymphocytes invading NOD pancreata. Analysis of Vk genes from pancreas (■) or adjacent pancreatic lymph nodes (□) from 9-wk-old, prediabetic NOD mice (n = 6) shows extensive diversity. A single gene sequence, Vk14–126, was identified in 30% of genes sequenced from pancreata. Vk gene sequences from pancreata, n = 26; those from pancreatic lymph nodes, n = 20.

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FIGURE 5. Allelic exclusion and skewing of L chain gene families in the pancreas of Vh125Tg/NOD mice. A, Flow cytometry of B lymphocytes extracted from pancreas shows that Vh125Tg/NOD B cells invade the pancreas and maintain allelic exclusion, 92 ± 2.6% (n = 3). B220+ cells are analyzed for IgM+ transgene (y-axis), vs IgM+ endogenous allele (x-axis). Cells are gated by size and granularity, and dead cells were excluded using 7AAD. B, Analysis of Vk genes from Vh125Tg/NOD mice (n = 5) shows that Vk gene families 1 and 9 predominate (35 and 41% of clones, respectively) in the pancreatic lymph nodes (□). In the pancreas (■), the Vc4 family is overrepresented by comparison (40% of clones). Vk gene sequences from pancreas, n = 35; those from pancreatic lymph nodes, n = 17; p = 0.006 by Fisher’s exact test.
VH125Tg/NOD B lymphocytes does not change, analysis of V\(\kappa\) gene regions in these mice improves accessibility to changes in the B cell repertoire. With the approach described above, the frequency of V\(\kappa\)/H9260 gene family representation in the pancreata and pancreatic lymph nodes of VH125Tg/NOD mice was determined, and the results are depicted in Fig. 5B. In the presence of a restricted H chain, a more substantial difference in V\(\kappa\)/H9260 expression is found when B lymphocytes populating the pancreas are compared with those in the draining lymph nodes. The V\(\kappa\)1 and 9 gene families predominated in the pancreatic lymph nodes of VH125Tg/NOD mice, the same pattern of predominance that was previously reported in VH125Tg/NOD spleens (42). In contrast, a marked shift away from V\(\kappa\)/H92601 and 9 to V\(\kappa\)/H92604 gene families was revealed in the pancreas of VH125Tg/NOD mice (\(p = 0.006\) by Fisher’s exact test). V\(\kappa\)/H92604 genes were obtained 4-fold more frequently in the pancreata than in pancreatic lymph nodes or spleen. V\(\kappa\)/H92604–57 and V\(\kappa\)/H92604–91 predominated among the V\(\kappa\)4 families, comprising 57% of them. V\(\kappa\)/H92604–74, the original insulin-binding partner of VH125, as well as V\(\kappa\)/H92604–50, V\(\kappa\)/H92604–56, and V\(\kappa\)/H92604–70 comprised the remainder. These data from IgH-transgenic mice reinforce the findings in wild-type NOD mice and suggest that selection of certain B cell receptors is part of the autoimmune attack on pancreatic islets in prediabetic NOD mice.

**B lymphocytes in the pancreas of prediabetic VH125Tg/NOD mice demonstrate Ag-driven clonal expansion**

To investigate the clonal relatedness of V\(\kappa\) genes that are over-represented in the pancreas of prediabetic VH125Tg/NOD mice, nucleotide sequences derived from related V\(\kappa\)- genes and their Jk segments were analyzed. Fig. 6 shows four V\(\kappa\)3–4/Jk1 genes from the pancreas of a single VH125Tg/NOD mouse, aligned to the nearest known V\(\kappa\)3–4 germline segment, from non-autoimmune-prone C57BL/6 mice. Multiple nucleotide differences from the nonautoimmune strain that are shared by all NOD V\(\kappa\)4 isolates likely represent polymorphisms (Fig. 6, shaded areas), as we have previously reported in other NOD L chain genes (42). These shared differences were present in all V\(\kappa\)3–4 sequences, including clones from multiple different mice (\(n = 9\), data not shown), consistent with a germline gene origin. In addition, these sequences contain novel nucleotide replacements, often in CDRs (Fig. 6, boxed) and are likely clonally related, in that they share recombination events in the V\(\kappa\)-Jk join. Although the size and diversity of CDRs in L chains is not complex, shared recombination events and novel nucleotide replacements in the CDRs of V\(\kappa\)s are consistent with somatic mutation and Ag-driven selection at the site of inflammation in VH125Tg/NOD mice. These events were not frequent at the prediabetic stage, with only 11% of light chains sequenced from pancreata showing shared mutations, including three from the V\(\kappa\)4 families. However, their presence suggests that the diverse repertoire within inflamed islets has the capacity to evolve.

*Vh* isolates from individual islets are oligoclonal, with multiple mutations in CDRs

Because individual islets are anatomically isolated and invasion is not uniform, tertiary lymphoid structures found in NOD pancreas may each represent an independent site of autoimmune attack. To evaluate the diversity and clonal relatedness among B lymphocytes populating individual islets, we used mechanical separation to isolate islets from both nontransgenic and VH125Tg/NOD pancreata. RNA was then extracted individually from each islet and used to generate cDNA libraries for analysis of V\(\kappa\) genes as above. Eighteen islets from seven different mice yielded 48 isolates, identified...
in Table I. Strikingly, two-thirds of islets generated only single \( V \kappa \) gene segments, usually identified as multiple, identical clones. Two islets produced sequences having the same \( V \kappa \) with differing \( J \kappa \) segments suggesting recurrent selection of similar BCRs into the same site (VH125Tg/NOD #15, islet 2; WT NOD #19, islet 1).

We examined all \( V \kappa \) gene sequences obtained from islets for mutations within the CDRs. Because the NOD L chain locus has not been sequenced and contains polymorphisms compared with other mouse strains, we utilized L chain gene sequences obtained from multiple NOD mice in our own database for consensus alignments. In general, homologous \( V \kappa \) gene sequences obtained from different mice shared near-complete identity, allowing easy observance of rare nucleotide substitutions in specific light chains. Of 27 L chain sequences obtained from the islets, 24 had homologous partners from the database available for consensus analysis. Nine (38%) of islet \( V \kappa \) gene sequences showed mutations within, or flanking, the CDR. Two sequences had three CDR changes, six had two CDR changes, and one had a single CDR change. Eighteen of the 19 mutations encoded for an amino acid replacement. CDR mutations encoding for amino acid replacements are indicative that Ag-driven selection of somatically hypermutated L chains is occurring within individual islets.

These findings differed from data obtained from whole pancreas, in which only 16% of \( V \kappa \) sequences showed CDR mutations, including the shared mutations already mentioned. This relative increase in altered CDRs in the islets raised the possibility that the process of islet isolation selects for lymphocytic infiltrates which have matured and are enriched in GCs. Therefore, we performed flow cytometry for GL7 on lymphocytes from islets isolated from three 9-wk-old prediabetic NOD mice. This assay showed that 53 ± 25% of B220+ cells co-isolated with islets are GL7+ (data not shown). Together, the increase in GL7+ B lymphocytes and evidence of amino acid replacements in CDRs indicate that lymphocytes strongly associated with islets represent a more evolved and Ag-selected repertoire than those from pancreas as a whole.

Differential Ag-binding emerges in islets vs pancreatic lymph nodes

\( V \kappa \) representation suggests that different Ag specificities may be selected into pancreas relative to draining lymph nodes. To directly test that possibility, flow cytometry was used to investigate the binding capabilities of B lymphocytes from a key islet Ag, insulin, in the pancreata of both wild-type and VH125Tg/NOD. Duplicate cell samples were first stained with biotinylated insulin, followed by a streptavidin-fluorochrome to detect binding. A 10-fold concentration of unlabeled insulin was added to one of the samples during primary staining, as a competitive inhibitor, to confirm specificity. Despite having a fixed H chain that is skewed toward insulin binding, the VH125Tg/NOD pancreatic B lymphocytes did not show specific insulin-binding (data not shown). This suggests that other autoantigens may be driving disease in VH125Tg/NOD mice at the prediabetic stage. However, on screening wild-type NOD, insulin-binding pancreatic B lymphocytes did emerge in 30% of animals. As shown in Fig. 7A, 14% of B220+ lymphocytes from the pancreas recognize biotinylated insulin. The binding is specific, given that unlabeled insulin added to a duplicate sample competitively inhibits the interaction (Fig. 7C).

FIGURE 7. Insulin-binding differs between B lymphocytes from pancreas and pancreatic lymph nodes of the same animal. A, Flow cytometry shows 14% of B lymphocytes from the pancreas of a 10-wk-old NOD mouse bind insulin. B, In draining pancreatic lymph nodes from the same animal, only 2% of lymphocytes bind insulin. C and D, Specificity controls performed by adding unlabeled insulin to duplicate samples (competitive inhibition) indicate that biotinylated insulin binding is specific for the Ag in the pancreas (C) but not in pancreatic lymph nodes (D). Cells are gated to lymphocyte population by size and granularity, with dead cells excluded using 7AAD.

<table>
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<sup>a</sup> Numbers in brackets, number of clones.

<sup>b</sup> Contains CDR mutations.

<sup>c</sup> Not identical with adjacent sequence.
Thus, tertiary lymphoid structures at the site of inflammation may play a distinct role in expanding autoreactivity relevant to β cell destruction.

**Discussion**

Tertiary lymphoid structures described in autoimmune and infectious diseases of mice and humans appear histologically to mimic those which arise developmentally in secondary lymphoid tissues, but their function is still under study. The data presented here show that tertiary lymphoid structures form in heavily infiltrated islets of prediabetic NOD mice, as evidenced by microarchitecture consisting of B cell follicles surrounding T cell zones. These organized structures are found consistently in nearby pancreatic lymph nodes, and also in the majority of those that are 25–50% infiltrated. Islets with heavy infiltrates also include markers of GC activity. Molecular analysis of Vκ gene representation, as well as Ag-binding studies with a key islet Ag, insulin, indicate that TLS do not simply mimic regional lymph nodes. Rather, they expand the autoimmune repertoire at the site of attack in T1D, suggesting a unique function for tertiary lymphoid tissues in autoimmune diabetes.

These data suggest two levels of BCR selection occurring within the pancreas: 1) the divergence of L chain usage in whole pancreas, compared with draining lymph nodes and spleen, indicates that this site does not reflect the same general B cell population which resides in and circulates through the nearby secondary organs; 2) the presence of GCs in the islets and shared mutations found among L chain gene sequences from pancreata indicates that the skewed pancreatic repertoire is further honed via Ag-driven somatic hypermutation at the site. CDR replacement mutations in B cells that are associated with individual islets provide further evidence for Ag-driven selection and expansion of B cells in the pancreas.

The precise mechanism of the initial, organ-specific selection of B cells into the pancreas must still be elucidated. One possibility is that most recirculating B lymphocytes traverse the tertiary lymphoid structures, with the rapid egress of those with irrelevant specificities, and the concomitant accumulation of those that have some capacity to recognize Ag. Cognate T-B interactions, presentation of Ag to B cells by resident dendritic cells, or binding of free Ag available within the islets may all be factors contributing to this process. A second hypothesis is that autoreactive B lymphocytes are originally selected in secondary lymphoid tissues, activated there, and induced to respond to inflammatory chemoattractants expressed in the islets. This pathway is supported by the large amount of GC activity in the nearby pancreatic lymph nodes, as well as by evidence for a similar pathway followed by T cells bearing a transgenic receptor (26, 27). However, a preliminary examination of L chain gene sequences from GL7+ B cells sorted from pancreatic lymph nodes did not yield a profile matching that of the pancreata (data not shown.) Furthermore, analysis of the Vκ sequences from whole pancreas indicates that the great majority are in germline configuration, decreasing the probability that they had undergone GC reactions before pancreatic invasion. Therefore, these data do not support Ag-driven selection external to the pancreas as the primary mechanism responsible for the skewing of the repertoire found there.

Data from individual islets, by contrast, suggest that such Ag-driven somatic hypermutation and selection does occur locally, as evidenced by amino acid-altering mutations in a high proportion of CDRs. The high frequency of GL7 expression among these B cells indicates that the islet isolation process provides B lymphocytes enriched for GC cells or activated B cells. This may be due either to death of nonactivated cells in overnight culture or the possibility that nonactivated cells are not integrin anchored within the tissue and are not retained during islet isolation. Therefore, cells obtained by this method provide an opportunity to identify Ag-receptors which appear to have been Ag-selected at the site of islet destruction. Although the Vκ profile found in this small sample of isolated islets does not entirely mirror that of whole pancreas, there are some meaningful commonalities. For example, the Vκ4–57, found frequently in whole pancreas of Vκ125Tg/NOD, is found to contain CDR replacement mutations within individual islets, indicating that it is selected, in unmutated form, as part of the skewed pancreatic repertoire, and is further able to undergo the next level of selection via Ag-driven hypermutation. Thus, we propose that a two-tiered, selective process is at work: the first driving the skewing of the overall, germline-encoded repertoire in the pancreas; and the second, selecting B cells from this repertoire for mutation and expansion within individual islets. This secondary process is T cell mediated. The oligoclonality seen within our data set is consistent with prior studies on TCRs (23, 24), implying that these oligoclonal B and T cell populations in individual islets may arise from a limited number of cognate T-B cell interactions occurring independently in each islet. Additional techniques, such as laser microdissection of islets, are currently under development in our laboratory to better determine relationships between lymphocytes within individual foci of autoreactivity in this disease.

An important question raised by these studies concerns the Ags targeted by B lymphocytes selected to the islets. The L chain structures emerging from these molecular studies open new avenues in the pursuit of critical autoantigens. In addition to Vκ4–57 and others found paired with the VH125 H chain, the Vκ14–126 gene found in wild-type NOD pancreata matches those from a variety of autoantibodies: spontaneous anti-nuclear Abs from NZB congenic mice; anti-bromelain-treated RBC; and anti-PC mAb (46–48). All of these are classic B1a-associated specificities. We and others have previously shown the presence of B1a, or Ly1+ B cells, amid pancreatic infiltrates (25, 49). Furthermore, a treatment targeting this autoreactive subset of B cells in NOD mice has been shown to drastically reduce the numbers of B lymphocytes in the islets, as well as conferring disease protection (49). Therefore, it is possible that the B1a subset is the source of the Vκ14–126-expressing cells. The H chain partner for Vκ14–126 is not yet known. It may be that this L chain pairs with only one or with a variety of heavy chains. The work of identifying one or more H chain partners, along with their target Ag, is under way.

Despite the fact that a Vκ4 family member, Vκ4–74/Jκ5, is the original, insulin-binding, L chain partner for the VH125 H chain, this Vκ was found only once in these studies. Further, pancreatic B lymphocytes in the prediabetic VH125/NOD mice studied did not demonstrate insulin-specific binding as measured by flow cytometry. Clues to the reason for this may be provided by the related Tg125/NOD model, in which both H and L chains of the BCR are transgenic (50). The resulting high affinity insulin-binding B cells in the Tg125/NOD model are anergic; therefore, it is reasonable to hypothesize that tolerance mechanisms skew the repertoire against this specificity when endogenous light chains are utilized in the VH125Tg/NOD model studied here. Because the IgH in the VH125Tg/NOD is capable of interacting with multiple Vκs, perhaps closely related L chains, such as others in the Vκ4 family, may confer low affinity insulin binding that avoid tolerance mechanisms well enough to enter the repertoire and be selected into the islets. Subsequent affinity maturation within GCs in the islets may then generate high affinity insulin-binding...
cells, or drive determinant spread to other islet Ags. Further kinetic and affinity studies are needed to evaluate whether such B cells emerge later in the disease process than the prediabetic time point studied here.

In two different models, we provide evidence for an independent, lymphoid entity in prediabetic pancreatic islets. These tertiary lymphoid structures support a selected B lymphocyte repertoire at the inflamed site and are capable of expanding the evolution of autoreactive specificities as indicated by the presence of GC activity. The discovery of unprecedented large populations of autoantigen-binding B lymphocytes for one key islet Ag, i.e., insulin, in the pancreas, may be consistent with this process for other β cell autoantigens as well. These latter findings are indicative of T-B interactions at the inflamed site, because T cell help is needed to promote these B cell activities, implying that this environment should also be able to support Ag presentation by B cells to T cells. Although B lymphocytes most likely contribute to diabetic pathogenesis via Ag presentation, the location of this presumed activity is not known. Data presented here suggest that tertiary lymphoid tissue in the islets could support Ag presentation by B cells at that location, as well as enriching them for autoreactive specificities. Thus, understanding the factors responsible for creating this environment, as well as the specificities that dominate here, may provide molecular targets for intervention at the inflammatory site.

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Disclosures
The authors have no financial conflict of interest.

References


