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CXCL13 Blockade Disrupts B Lymphocyte Organization in Tertiary Lymphoid Structures without Altering B Cell Receptor Bias or Preventing Diabetes in Nonobese Diabetic Mice

Rachel A. Henry and Peggy L. Kendall

Lymphocytes that invade nonlymphoid tissues often organize into follicle-like structures known as tertiary lymphoid organs (TLOs). These structures resemble those found in spleen or lymph nodes, but their function is unknown. TLOs are recognized in many autoimmune diseases, including the NOD mouse model of type 1 diabetes. In some cases, TLOs have been associated with the B lymphocyte chemotactrant, CXCL13. Studies presented in this article show that CXCL13 is present in inflamed islets of NOD mice. Ab blockade of this chemokine unraveled B lymphocyte organization in islet TLOs, without reducing their proportion in the islets. These chaotic milieus contained B lymphocytes with the same distinct repertoire of B cell receptors as those found in mice with well-organized structures. Somatic hypermutation, associated with T–B interactions, was not impaired in these disorganized insulitis lesions. Finally, loss of B lymphocyte organization in islets did not provide disease protection. Thus, B lymphocytes infiltrating islets in NOD mice do not require the morphology of secondary lymphoid tissues to support their role in disease.

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Tertiary lymphoid organs (TLOs) are found in inflamed tissues of multiple autoimmune diseases, including rheumatoid arthritis, Sjogren’s syndrome, experimental autoimmune encephalitis, and the NOD mouse model of type 1 diabetes (T1D) (1–8). These structures mimic secondary lymphoid tissues anatomically, and also share functional characteristics, such as germinal center (GC) reactions, critical for B cell isotype class switching and affinity maturation. In the NOD mouse, these ectopic lymphoid structures, consisting of a central T cell zone surrounded by B cells, begin to coalesce even at the early stage of peri-insulitis. This process occurs for each islet independently, and T cells can be seen grouping together at the islet interface of the lymphocyte attack, with B cells tending to flank them. The lymphocytes fully organize into follicle-like structures that include GCs by the time each islet is fully infiltrated (9). Although many of the cellular and molecular mechanisms underlying the formation of TLOs have been delineated, the role of ectopic lymphocyte organization in disease pathogenesis has yet to be determined. The presence of TLOs in NOD mice can be correlated with disease, which sometimes leads to the assumption that they are critical to disease-related processes (10). However, whether autoreactive lymphocytes must be structurally organized to contribute to disease pathogenesis is still an unanswered question.

Previous studies in our laboratory have shown that B cells in islet TLOs of NOD mice have a skewed repertoire of BCRs compared with the pool of recirculating lymphocytes in secondary lymphoid organs, indicating that a selective process for B cells occurs at the inflamed site. This work also showed the presence of GCs, as well as somatic hypermutation (SHM) of BCRs, suggesting that T–B interactions occur within islet TLOs (9). Because B lymphocytes act as essential APCs to support this T cell-mediated autoimmune disease, such interactions raise the possibility that disease-promoting cellular crosstalk may occur within TLOs in the islets.

CXCL13 (B lymphocyte chemotactrant) is a pivotal chemokine responsible for the formation and maintenance of B lymphocyte follicles and GCs in spleen and lymph nodes (11), and has been identified in inflamed autoimmune-associated TLOs (12–14). Transgenic CXCL13 expression in normal mouse islets is sufficient for full formation of ectopic lymphoid aggregates, a process that is lymphotoxin-dependent (15). Lymphotoxin blockade, in turn, stops the development of diabetes in NOD mice (10, 16) and has been shown to reverse insulitis (10). Therefore, the blockade of CXCL13 could reasonably be expected to disrupt this process in a similar fashion. However, in these studies, the data show the CXCL13 blockade largely disrupted B lymphocyte organizational morphology without altering their recruitment to islets. B lymphocytes in these chaotic milieus maintained the same BCR V gene bias found in untreated mice, and SHM of these genes was robust, indicating that T–B interactions were not effectively disrupted. Diabetes progression was unimpaired. Thus, in NOD mice, B lymphocytes in islets under autoimmune attack do not require widespread duplication of the morphology found in secondary lymphoid tissues to promote disease.

Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232

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Address correspondence and reprint requests to Dr. Peggy L. Kendall, Vanderbilt University Medical School, 1161 21st Avenue South, Medical Center North T3219, Nashville, TN 37232. E-mail address: peggy.kendall@vanderbilt.edu

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Abbreviations used in this paper: GC, germinal center; LC, Ig L chain; NOR, non-obese-resistant; PLN, pancreatic lymph node; SHM, somatic hypermutation; T1D, type 1 diabetes; TLO, tertiary lymphoid organ.

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Materials and Methods

RT-PCR for CXCL13

Islets were isolated from NOD pancreata as previously described (9). Briefly, pancreata were macrocerated with scissors and then agitated at 37°C for 12 min in HBSS containing 3 mg/ml collagenase P. Islets were handpicked using a dissecting microscope and placed in overnight culture so those that extruded lymphocytes could be differentiated from those that did not. RNA was extracted from these isolated islets, and first-strand cDNA was synthesized as previously described and was used as a template in real-time PCR using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) with the cxcl13 primers mentioned previously, and a 56°C annealing temperature, or with primers to amplify hprt as a control: 5′-AGG TTG CAA C GT TG TGC TGG T-3′, 5′-TGA ACT CAT TAT AGT CAA GGG CA-3′, with a 52°C annealing temperature in a MyIQ single-color real-time PCR detection system (Bio-Rad, Hercules, CA). Data were analyzed with MyIQ software (Bio-Rad). The average of triplicate reactions for each individual sample was used to calculate the relative level of cxcl13 to hprt.

Immunohistochemistry and immunofluorescent staining

Frozen sections were obtained as previously described (9). Briefly, pancreata were fixed in 4% paraformaldehyde in a high-phosphate buffer at 4°C for 2 h immediately after removal from 10- to 12-wk-old female NOD mice. This was followed by overnight soak in 30% sucrose, then immersion in OCT (Sakura Finetek, Torrance, CA), and freezing/storing at −80°C. Sections, 5 μm thick, were obtained using a cryostat microtome (Leica Microsystems, Deerfield, IL). For CXCL13 staining, primary Ab was polyclonal, goat anti-mouse CXCL13 IgG (Santa Cruz Biotechnologies, Santa Cruz, CA), followed by biotin-conjugated rabbit-derived anti–goat-IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunofluorescent signals were then converted to red and blue using Adobe Photoshop software (Adobe Systems, San Jose, CA), followed by biotin-conjugated rabbit-derived anti–goat-IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Images were made for analysis of islet organization in anti-CXCL13 treated versus isotype control-treated and untreated controls, images were made of all visible insulitis lesions on immunofluorescently stained slides of frozen sections of pancreata from three mice treated with anti-CXCL13, two isotype treated controls, and two untreated controls. Images were made of all visible insulitis lesions on immunofluorescently stained slides of frozen sections of pancreata from three mice treated with anti-CXCL13, two isotype treated controls, and two untreated controls. Treated groups received 100 μg of anti-CXCL13 or isotype control injected i.p. three times a week from age 3–12 wk. Mice were sacrificed the day after the final injection. Each lesion was classified as disorganized, organized, or intermediate by an independent reviewer who was blinded to treatment group.

Flow cytometry

Lymphocytes suspended at 1 × 10^7/100 μl in a buffer solution were stained with Abs recognizing B220, IgM, and CXCR5 (BD Pharmingen). 7-Aminoactinomycin-D was used to exclude dead cells. Data were obtained using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (TreeStar, Ashland, OR). Lymphocyte gate by size and granularity is used for all plots.

BCR L chain analysis

V_{μ}125NOD mice were treated with anti-CXCL13, three times a week as described previously, from ages 3 to 12 wk. Pancreatic RNA from these mice was reverse-transcribed and amplified using V_{μ} L chain (LC) primers, cloned, sequenced and analyzed for V_{μ} family and CDR mutations as previously described (9). Of note, NOD-specific comparisons were made for analysis of CDR mutations, to differentiate actual mutations from polymorphisms that are strain-specific for NOD κ genes (17, 18). Statistics

Statistics for organization of islets and for LC distributions was performed using Stata Software, version 9 (College Station, TX) to determine p value by Fisher exact test, or Poisson regression, as noted. R software (www.r-project.org) was used to analyze incidence of SHM in islets using negative binomial regression.

Results

CXCL13 is expressed in NOD islets, and its receptor, CXCR5, is found on B lymphocytes in the pancreas

To identify chemotactic factors involved in B lymphocyte traffic to sites of inflammation in the pancreas, we initially used gene chip microarray as a screening tool, and found a 3-fold increase in transcript level for cxcl13 in pancreata from prediabetic NOD females compared with healthy pancreas from nonautoimmune C57BL/6 (not shown). To confirm these pilot studies, individual islets from prediabetic NOD and C57BL/6 females were isolated for examination by RT-PCR. Isolated islets were cultured overnight and then examined for extruded lymphocytes. Inflamed islets from NOD mice were selected for comparison with healthy islets from C57BL/6 mice. RNA encoding for CXCL13 was found to be expressed in inflamed islets isolated from three NOD mice, but not in islets from three C57BL/6 mice (Fig. 1A). Cxcl13 transcript was not detected in isolated NOD islets that were free of infiltrating lymphocytes (not shown).

These studies were extended using real-time PCR for comparisons of cxcl13 levels in islets at 3, 6, and 12 wk of age in NOD mice. Nonobese-resistant (NOR) mice, which are protected against disease, were used as controls. Islets were isolated from three female mice in each group, placed in overnight culture and then examined for extrusion of lymphocytes, prior to RNA extraction, to compare the levels of lymphocytic invasion in our samples. We found a little more than half the islets from 6-wk-old NOD mice extruded lymphocytes (average 54%, SD ± 17%); 12-wk-old NOD mice yielded lymphocytes from 62% (SD ± 9%) of islets and that contrasted with islets from 3-wk-old NOD mice, none of which extruded lymphocytes. NOR mice also showed lymphocyte extrusion in 26% (±14%) of islets, at 14 wk of age. Therefore, nonlymphocyte-extruding islets from additional NOR mice were obtained to provide another control sample. Islets were harvested together with any lymphocytes extruded, and pooled for each mouse, taking all islets, without selecting for inflammation (n = at least 20 islets per mouse). mRNA was obtained from pooled islets, translated to cDNA, and real-time PCR was performed for cxcl13 levels and normalized to hprt levels for each
CXCL13 is expressed in inflamed islets of NOD mice. A. Gel electrophoresis showing RT-PCR products from isolated pancreatic islets using cxcl13-specific primers. First three lanes are from islets of three independent female NOD mice. Last three lanes are from islets of three independent female C57BL/6 mice. Lower lanes are controls using hprt primers from the same islets. B. Real-time PCR showing cxcl13 levels, normalized to hprt, from isolated islets. At least 20 islets were isolated from three mice per group, and pooled for each mouse. Normalized cxcl13 levels are significantly higher in islets from 12-wk-old NOD mice than from any other group, *p < 0.0001, compared with noninfiltrated NOR controls or 3-wk-old NOD mice: p = 0.001 for 6-wk-old NOD mice; p = 0.004 compared with 14-wk-old NOR mice. C. Immunohistochemical staining of frozen NOD pancreatic sections shows CXCL13+ (dark brown) cells among invading lymphocytes in early insulitis. Right panel shows control section processed with secondary reagents, but without primary anti-CXCL13 Ab (original magnification ×20).

Previous studies in our laboratory indicated some B cell-related therapies that are disease-protective are associated with loss of B cells from inflamed islets (20). We hypothesized that CXCL13 drives B lymphocyte recruitment to inflamed islets, and that CXCL13 blockade could reduce their presence there. NOD mice were treated with 100 μg anti-CXCL13 Ab three times weekly, beginning at age 3 wk, a time point at which few, if any, lymphocytes are present in islets, to 12 wk, when insulitis is prominent (21). Pancreata were then harvested and analyzed for alterations in lymphocyte populations by flow cytometry, H&E staining, and immunofluorescent staining of frozen sections. Insulitis development was not impaired. However, immunofluorescent staining showed the architecture of ectopic lymphoctic structures that develop in the islets was strikingly altered. Fig. 3A (and Supplemental Fig. 1) shows normal arrangement of B cells (pink) surrounding T cells (blue) in an islet from a frozen section of the pancreas of an untreated NOD mouse, whereas, Fig. 3B (and Supplemental Fig. 1) shows loss of organization, with B and T cells intermingled in the islet of an NOD mouse treated with anti-CXCL13. Quantification of multiple islets by a blinded, independent scorer indicated significant differences between anti-CXCL13–treated and isotype-treated or untreated controls, with 68% of insulitis lesions from anti-CXCL13–treated mice disorganized, compared with 11% of those from isotype-treated controls, and 5.5% of untreated controls (Fig. 3D). In contrast, 66% of lesions from isotype-treated controls and 72% of those from untreated mice contained well-organized structures, compared with only 12% of those from anti-CXCL13–treated mice. Intermediate levels of organization, in which small areas of T or B cells could be seen grouped together (Supplemental Fig. 1), were seen in 21–22% of insulitis lesions from all groups (CXCL13 versus isotype control p < 0.001; CXCL13 versus untreated control p < 0.001, comparing pooled, independent, islets from: anti-CXCL13 n = 34, isotype control n = 27, untreated n = 18; anti-CXCL13–treated mice n = 3; isotype control mice n = 2; untreated controls mice n = 2).

CXCL13 receptor is expressed on B220+ B cells from pancreas and spleen. Flow cytometry shows CXCRI5 expression on B220+ B cells from pancreas and spleen of the same female NOD mouse. Image is representative of data from six mice.
However, flow cytometry analysis of lymphocytes from pancreata indicates that CXCL13 blockade did not reduce B lymphocyte proportions in pancreatic infiltrates (Fig. 3C). Thus, CXCL13 blockade curtails lymphocytic organization within insulitis lesions, without significantly impeding B lymphocyte presence there.

**Disease-associated B lymphocyte selection into islets occurs despite CXCL13-blockade induced disruption of their structural organization**

NOD mice that harbored an anti-insulin heavy chain Tg have a skewed repertoire of V$x$ genes when recovered from islets. Our previous work showed that the V$x$4 LC family predominates in the pancreata of V$\delta$125/NOD mice, differing notably from spleen and draining pancreatic lymph nodes (PLNs), where, as expected for primary repertoires, the V$x$1 and V$x$9 families predominate (9, 17). To determine whether CXCL13-driven cellular organization contributes to this selection, V$\delta$125/NOD mice were treated with Ab blockade, and the V$x$ composition of islet cDNA libraries was determined, as previously reported for untreated mice (9). Immunofluorescent staining on sections of the same pancreata confirmed that B lymphocyte disorganization also occurred in these mice (not shown). The pattern of LC families expressed in the pancreas (black bars) and draining pancreatic lymph nodes (gray bars) of anti-CXCL13–treated V$\delta$125/NOD mice is shown in Fig. 4A. The V$x$4 family predominated in the pancreata of CXCL13-treated mice, as previously shown in the pancreata of untreated V$\delta$125/NOD mice (9). The V$x$4 family accounted for 70% of LCs identified in this experiment, compared with 26% in draining PLNs from the same mice, a 2.7-fold difference. V$x$1 and V$x$9 families were lower in pancreas, relative to PLNs, 0.38-fold and 0.34-fold, respectively (overall repertoire shift, $p = 0.002$ by Fisher exact test, $n = 53$). The fold changes in the predominant V$x$4 families in the pancreata, relative to draining PLNs, do not significantly differ between untreated and CXCL13-treated V$\delta$125/NOD mice (Fig. 4B). The proportion of V$x$4 family genes is 2.7-fold higher in treated mice, and 3.5-fold higher in untreated mice, whereas, V$x$1 and V$x$9 families are found expressed in pancreata, 0.5-fold relative to PLNs, in treated and untreated mice ($p = 0.066$, 95% CI 0.77–1.01, by Poisson regression; mice $n = 8$, independent clones $n = 104$). Thus, the repertoire shift in the inflamed tissue relative to the secondary lymphoid organs is maintained, despite loss of the tertiary lymphoid, B cell-related morphology, indicating that intact B cell organization in TLOs is not essential for selection of the V$x$4-predominant LC repertoire found to be paired with the V$\delta$125 BCR heavy chain in B cells from the pancreas.

**CDR mutations in islet-derived BCRs, indicative of SHM, occur despite disrupted B lymphocyte organization in islets**

B lymphocytes undergo SHM in response to activation by T cells. Our previously published analyses showed that GCs, the site of SHM, are present within well-organized TLOs in inflamed NOD islets, and that CDR mutations indicative of SHM are present in
Disease studies were performed on VH125/NOD mice treated with Diabetes progresses despite B lymphocyte disorganization in arrangement classically found in well-formed TLOs. The structural support of the follicle-like B cell heavily depend on the T–B interactions in the islets that result in SHM do not indicate that this dose of treatment was adequate to prevent B lymphocyte organization in islets, despite the lack of disease effect.

**Discussion**

This study addresses the role of B lymphocyte organization within TLOs in the autoimmune B cell attack that leads to T1D in the NOD mouse model. Organized tertiary lymphoid structures are found at the site of inflammation in multiple autoimmune diseases. TLOs are typical for fully invaded islets in the NOD mouse model of T1D, but their role in disease has not been defined. Because TLOs have been associated with selection of B cells (9) and recruitment and activation of naive T cells (6), as well as with disease progression in NOD mice, it has been hypothesized that these structures contribute to the disease process. Previous interventions that resulted in disease protection, such as lymphotoxin β receptor blockade, effectively eliminated lymphocytes from islets altogether, but could not address the role of their organization (10). The

![Image](http://www.jimmunol.org/)
CXCL13 and B Lymphocyte Organization in Insulitis

Disclosures

The authors have no financial conflicts of interest.

References