Evolution of Ectopic Lymphoid Neogenesis and In Situ Autoantibody Production in Autoimmune Nonobese Diabetic Mice: Cellular and Molecular Characterization of Tertiary Lymphoid Structures in Pancreatic Islets

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Evolution of Ectopic Lymphoid Neogenesis and In Situ Autoantibody Production in Autoimmune Nonobese Diabetic Mice: Cellular and Molecular Characterization of Tertiary Lymphoid Structures in Pancreatic Islets

Elisa Astorri,*1 Michele Bombardieri,*1 Silvia Gabba,* Mark Peakman,† Paolo Pozzilli,‡ and Costantino Pitzalis*†

A pivotal role for tertiary lymphoid structures (TLSs) in promoting Ag-specific humoral responses during chronic inflammation is emerging in several autoimmune conditions, including rheumatoid arthritis, Sjogren’s syndrome, and autoimmune thyroiditis. However, there is limited evidence on the cellular and molecular mechanisms underlying TLS formation and their contribution to autoimmunity in the pancreas during autoimmune insulitis. In this study, we performed a detailed and comprehensive assessment of the evolution of TLSs during autoimmune insulitis in 126 female NOD mice from 4 to 38 wk of age. We demonstrated that during progression from peri- to intrainsulitis in early diabetic mice, T and B cell infiltration follows a highly regulated process with the formation of lymphoid aggregates characterized by T/B cell segregation, follicular dendritic cell networks, and differentiation of germinal center B cells. This process is preceded by local upregulation of lymphotoxins α/β and lymphoid chemokines CXCL13 and CCL19, and is associated with infiltration of B220+/IgD+/CD23+/CD21− follicular B cells expressing CXCR5. Despite a similar incidence of insulitis, late diabetic mice displayed a significantly reduced incidence of fully organized TLSs and reduced levels of lymphotoxins/lymphoid chemokines. Upon development, TLSs were fully functional in supporting in situ autoreactive B cell differentiation, as demonstrated by the expression of activation-induced cytidine deaminase, the enzyme required for Ig affinity maturation and class switching, and the presence of CD138+ plasma cells displaying anti-insulin reactivity. Overall, our work provides direct evidence that TLSs are of critical relevance in promoting autoimmunity and chronic inflammation during autoimmune insulitis and diabetes in NOD mice. The Journal of Immunology, 2010, 185: 000–000.

Type 1 diabetes (T1D) is characterized by the gradual destruction of insulin-producing β cells located in the pancreatic islets of Langerhans (1, 2). Together with autoreactive T cells, which have long been known pathogenic in patients with and animal models of T1D (3), the hallmark of autoimmune insulitis is the presence of humoral immune response against islet Ags, such as anti-insulin Abs (IAAs) and glutamic acid decarboxylase (4). In addition to autoantibody production, autoreactive B cells infiltrate in large number the islets of NOD mice, a robust model of T1D (5), during the inflammatory process, where they have been shown to be required for disease initiation and progression (6). In particular, autoreactive B cells specific for islet Ag play a fundamental role in Ag processing and presentation (i.e., IAAAs) (7, 8) in the context of appropriate MHC II alleles (9) and are crucial in promoting T cell priming and activation within the target organ (10). Recent and very compelling data in patients with T1D further highlight a central pathogenic role for B cells, as demonstrated by evidence that more infiltrating B cell is associated with a more severe and destructive insulitis (11) and that B cell depletion in T1D significantly preserves β cell function over a period of 1 y (12). Thus, understanding the cellular and molecular mechanisms underlying B cell activation and autoantibody production in T1D is of critical importance.

In physiological conditions, B cell activation and differentiation into Ab-producing cells take place in secondary lymphoid organs (SLOs) (13–15), where B cells enter the germinal center (GC) and undergo the processes of somatic hypermutation (SHM) and class switch recombination (CSR) of the Ig genes (16, 17). Both processes are critically dependent on the expression of the enzyme activation-induced cytidine deaminase (AID) in the context of follicular dendritic cell (FDC) networks (18). Increasing evidence demonstrates that lymphoid structures resembling SLOs develop in the target organ of autoimmune/chronic inflammatory diseases (19). We and other authors (19–25) have demonstrated that tertiary lymphoid structures (TLSs) in the target tissues of several human diseases including rheumatoid arthritis (RA) synovium and lungs, Sjogren’s syndrome salivary glands, and autoimmune thyroiditis are functional and support AID expression, SHM/CSR, and in situ autoantibody production. Although lymphoid aggregates resembling TLSs have been identi-
fied during autoimmune insulitis in NOD mice (26–30), the cellular and molecular mechanisms regulating their development and their functionality have not been fully characterized. In addition, little is known about the B cell subpopulations infiltrating the pancreatic islets at different stages of the autoimmune process.

In this study, we provide systematic evidence that functional TLSs progressively develop within islet infiltrates already at early stages of the autoimmune process, with the highest incidence in early diabetic mice. This process is characterized by progressive acquisition of features of SLOs, such as T/B cell segregation, differentiation of FDC networks within the B cell area, and generation of a GC response characterized by GC B cells and expression of AID. This process is accompanied by the de novo expression of genes regulating the initiation, progression, and maintenance of TLSs, such as lymphoid chemokines, lymphotaxis (LTs), and B cell survival and proliferating factors. Finally, we provide evidence that these structures contribute to the autoimmune process through in situ differentiation of autoantibody-producing cells.

Materials and Methods

Mouse samples

NOD mice came from the NOD/Ba colony, established in 1987 at St. Bartholomew’s Medical College (London, U.K.), originally derived from Dr. E. Leiter’s laboratory (Bar Harbor, ME) (31). There is a stable cumulative incidence of diabetes of ~80% in female and 15% in male mice at 30 wk of age (32). The colony is housed in a purpose-built area and maintained strictly according to international (33) and United Kingdom guidelines for animal care. All mice are maintained under pathogen-free conditions. Breeding and all procedures are performed according to the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (PPL 70/6109) (34). Diabetic status of the mice was initially determined rabbit anti-rat Ig secondary Ab (Ab dilution 1:300) and the streptavidin Alexa-555 Ab (1:20 dilution) for 1 h, followed by 1-h incubation with the biotinylated rabbit anti-rat Ig secondary Ab (dilution 1:300) and streptavidin Alexa-488 (dilution 1:300). After washing, B220-PE Ab (dilution 1:100) incubation was then performed for 1 h. Slides were stained with DAPI for 10 min, washed, and mounted with Mowiol.

Histological evaluation of functional ectopic GC-like structures. Staining for GL7 (marker for mouse GC B cells) and FDC was performed on sequential sections of NOD pancreatic tissue to evaluate the presence of GCs. Such stainings were correlated with the histological characterization of the lymphoid aggregates. Frozen sections were processed as above, and GL7-FITC Ab (dilution 1:100) incubation was performed at 4°C overnight. For FDC identification, FDC-M1 Ab (1:100) incubation was performed for 1 h and followed by 1-h incubation with the biotinylated rabbit anti-rat Ig secondary Ab (dilution 1:300) and streptavidin Alexa-555 (dilution 1:300) 1-h incubation. FDC networks were also evaluated using standard immunohistochemistry with peroxidase staining to confirm the localization of the cells on bright field. In this case, after the secondary biotinylated Ab, slides were washed and incubated with streptavidin HRP-ABC complex (DakoCytomation) for 30 min. After washing, color reaction was developed using diaminobenzidine solution (DakoCytomation), and slides were counterstained with hematoxylin, dehydrated, and mounted in DePex.

As AID is expressed transiently and exclusively in B cells undergoing SHM/CSR, staining for AID was performed to evaluate the functionality of the TLS. AID is expressed transiently and exclusively in B cells undergoing SHM/CSR, stained the mouse GC B cells with AID Ab (1:20 dilution) for 1 h, followed by 1-h incubation with the biotinylated rabbit anti-rat Ig secondary Ab (Ab dilution 1:300) and the streptavidin Alexa-555 for 1 h.

Characterization of B cells within the infiltrates. Immunofluorescence was performed to evaluate the subset of B cells infiltrating the islets. It has been demonstrated that B cells may belong to different subpopulations (35). Follicular mature B (FoB) cells in the spleen are situated in white pulp follicles and are characterized by a B220+, IgDlow, CD23high, CD21low, IgMhigh, CD1dhigh, CXCR5low, CD1dlow, CXCR5high phenotype. Conversely, marginal zone (MZ) B (MZB) cells are located between the white and red pulp, close to the marginal sinuses, and exposed to blood Ags. These cells are characterized by a B220+, IgDlow, CD23low, CD21high, IgMhigh, CD1dhigh, CXCR5low phenotype (36). Thus, to identify whether FoB and/or MZB cells were present within infiltrated islets, double immunofluorescence stainings on sequential sections were performed for IgD/Gal, IgD/Cd1D, CD21, CD23, IgD/IgM, and CXCR5/B220. Staining was first optimized on spleen sections and then performed on pancreatic tissue.

Table I. Primary and secondary Abs used for immunofluorescence

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<tr>
<td>R6-60.2</td>
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<td>CXCR5</td>
<td>PE</td>
<td>Rat</td>
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Rabbit anti-rat biotin IgG

Streptavidin Alexa-488

Streptavidin Alexa-555

Rabbit: 1:300

DakoCytomation

Invitrogen (Paisley, U.K.)

Invitrogen
Incubation was performed for 1 h. Incubation in avidin-biotin and protein block. Alexa-555 (dilution 1:300) was performed overnight at 4˚C on slides prior to sequential sections. Biotinylated anti-CD138 Ab incubation (dilution 1:10) was performed for 1 h on acetone-fixed slides. Prior to incubation, slides were blocked for 1 h with DakoCytomation protein block. To confirm specificity of the binding, sections were preincubated with unstained insulin prior to the FITC-conjugated insulin incubation. After washing in TBS, there were DAPI 10-min incubation and final fixation with Mowiol. Peripancreatic lymph nodes and infiltrated salivary glands were included as positive (37) and negative controls, respectively. In order to identify intra- (IL-4, IFN-γ, AID, and cytokines involved in regulating B cell class switching lymphoid chemokines (CXCL12, CXCR4, CXCL13, CXCR5, CCL19, CCR7), AID, and cytokines involved in regulating B cell class switching (IL-4, IFN-γ, IL-13). The real-time PCRs were run in triplicate using the ABI Prism 7900HT instrument, and results were analyzed after 40 cycles of amplification using ABI Sequence Detection System version 2.1 (SDS 2.1). Relative quantification was measured using the comparative threshold cycle method. Two different endogenous controls (mouse β-actin and mammalian 18S) were used to normalize for the CDNA of each sample.

### Statistical analysis

Differences in quantitative variables were analyzed by the Mann-Whitney U test when comparing two groups, and by the Kruskal-Wallis with Dunn’s posttest when comparing multiple groups. χ² test with Yates’ correction when required or Fisher’s exact test when appropriate were used to evaluate associations of qualitative variables in the different groups. All the statistical analyses were performed using GraphPad Prism version 3.03 for Windows (GraphPad, San Diego, CA). A p value of 0.05 was considered statistically significant.

### Results

**Histological characterization of pancreatic inflammatory infiltrate development**

We first characterized the prevalence and the progression of inflammatory infiltrates within pancreatic islets of NOD mice between 4 and 38 wk of age. H&E and CD3/CD220 staining were performed to identify progression from peri- to intrainsulitis and presence of T/B cell aggregates, respectively (Fig. 1A, 1B). As expected, initial infiltrates characterized by T and B lymphocytes were observed from week 6 onward and were mostly organized as peri-islet aggregates (peri-insulitis; Fig. 1A, 1C). With increasing age, larger T and B cell aggregates developed and progressively colonized the whole islet with disappearance of β cells (intrainsulitis; Fig. 1B, 1C). Thus, as expected, infiltrate evolution displays a trend from prevalent peri-infiltration to prevalent intra-infiltration throughout mice life/disease, although different stages of infiltration coexist within the same pancreas.

**Pancreatic islet infiltrates progressively acquire features of TLSs: T/B cell compartmentalization and formation of FDC network**

We next evaluated the progressive acquisition of features of SLOs, such as T/B cell segregation and differentiation of FDC networks (Fig. 2).

Sequential section analysis of pancreatic infiltrates was performed using immunostaining for CD3/CD220 and FDC-M1 to assess the presence of T/B cell compartmentalization and FDC networks. As shown in Fig. 2, inflammatory aggregates clearly display various degrees of cellular organization with initial infiltrates mostly characterized by absence of T/B cell segregation, followed by the development of highly organized lymphoid structures with T and B lymphocytes localizing in discrete areas (Fig. 2A–C). Thus, inflammatory cells enter the islets first with a nonsegregated pattern (nonsegregated insulitis), and then they gradually gain an organo-

### Table II. Genes, specific primers, and probes used for RT-PCR

<table>
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Source of all primers and probes: Applied Biosystems.
nized segregated disposition within the islets (segregated insulitis). In the context of segregated infiltrates, a further degree of lymphoid organization characterized by the differentiation of FDC networks was observed during the evolution of insulitis (Fig. 2E,2F). FDC networks closely colocalized with the B cell-rich areas, as shown by sequential section analysis (Fig. 2D–F). Interestingly, the analysis of the prevalence of FDC-positive infiltrates in different age groups of NOD mice demonstrates that early-onset diabetic mice displayed the highest percentage of FDC+ aggregates (>65%), whereas mice developing diabetes at a later stage displayed a progressive decrease in the prevalence of FDC networks (Fig. 2F), despite a similar incidence of intrainsulitis. Such data are consistent with the presence of a more aggressive phenotype, characterized by evolution of TLSs.

**AID expression and differentiation of GC B cells support functionality of TLSs developing within pancreatic islets**

Following demonstration of acquisition of features of TLSs, we next examined whether ectopic lymphoid structures in the pancreas of NOD mice display characteristics of functionality typical of SLOs.

In SLOs, B cells enter the GC, where they undergo affinity maturation and clonal diversification, followed by differentiation into memory B cells and plasma cells that leads to the production of high-affinity Abs. The above phenomena are critically dependent on the processes of SHM and CSR of the Ig genes. Both processes require the presence of the enzyme AID, which initiates these processes (38, 39). Because AID is exclusively and transiently expressed in B cells undergoing SHM and/or CSR, identification of AID+ B cells allows the evaluation of their functionality (21, 22). Thus, to characterize the functionality of TLSs in NOD insulitis, we performed analysis for GL7 (marker for mouse GC B cells) and AID on sections sequential to those stained for FDC. As shown in Fig. 3, aggregates characterized by FDC network invariably display the presence of GL7+ GC B cells expressing high levels of AID. Conversely, no AID expression was observed in B cells localizing outside FDC networks. In agreement with the evidence that AID expression requires FDCs, quantitative gene expression analysis (Fig. 3D) demonstrated that the highest expression of AID synchronized with the peak of FDC prevalence (Fig. 2F). Overall, these data strongly suggest that TLSs developing during the course of

**FIGURE 2.** Development of ectopic lymphoid structures characterized by T/B cell segregation and FDC networks within pancreatic infiltrated islets of NOD mice. A–C, Immunofluorescence staining for CD3 (T cells, green)/B220 (B cells, orange) in pancreatic islet showing progressive acquisition of T/B cell segregation. Intrainsulitis with: nonsegregated T/B cells (A); partially segregated T/B cells (B); complete segregation of T/B cells (C). D–F, Representative microphotographs of sequential sections of NOD pancreas showing colocalization of ectopic lymphoid structure markers. D, Immunofluorescence staining for CD3 (T cells, green)/B220 (B cells, orange) showing an islet with intrainsulitis characterized by T/B cell segregation. E and F, Sequential sections stained with FDC-M1 (FDC network) using immunofluorescence (E, red) and peroxidase on bright field (F, brown) showing exclusive colocalization of FDC networks within the B cell area. G, Graph enumerating the prevalence of mice displaying FDC+ islets in the different age groups. A–F, Original magnification ×20.

**FIGURE 3.** TLS in NOD islets display a GC response with expression of AID as marker of functionality. A–C, Immunofluorescence staining of pancreatic sequential sections of NOD mice. A, Staining for FDC-M1 (red). B, Staining for GL7 (green), marker for mouse GC B cells. C, Staining for AID (red), which is required for and exclusively expressed in B cells undergoing SHM and/or CSR. A–C, Original magnification ×20. D, Quantitative RT-PCR for AID gene expression in NOD and BALB/c mice pancreas.
autoimmune insulitis support a GC response characterized by B cell activation and differentiation. Importantly, as expected, no organized follicular structures or expression of AID was detected in the pancreas of BALB/c control mice.

Progressive evolution of TLSs is associated with local overexpression of genes regulating ectopic lymphoid neogenesis and B cell functionality

Evolution of TLSs is dependent on the expression and function of a critical set of genes, such as LTs and lymphoid chemokines (19). In addition, B cell survival, proliferation, and function require the presence of a number of cytokines/growth factors, which bind to specific receptors on the cell surface of B lymphocytes. Thus, we examined whether development of TLS in NOD mice pancreas was associated with a concomitant upregulation of such genes. As shown in Fig. 4, lymphoid chemokines and their cognate receptors CXCL13/CXCR5, CCL19/CCR7, as well as CXCL12/CXCR4, which is known to regulate localization of GC B cells (40), were all upregulated in NOD mice, as compared with BALB/c, already at early stages of the autoimmune process and preceded disease onset. Consistent with the histological detection of TLSs, the highest expression level of these molecules was observed in the

![Graphs showing quantitative mRNA expression of TLS-related genes.](http://www.jimmunol.org/)

**FIGURE 4.** Quantitative mRNA expression of TLS-related genes. Quantitative TaqMan real-time evaluation of mRNA expression levels of TLS-related genes in pancreas of NOD and BALB/c mice. Lymphoid chemokines and their specific receptors CXCL13/CXCR5 (A, B), CCL19/CCR7 (C, D), CXCL12/CXCR4 (E, F), as well as LTα (G) and LTβ (H) are shown. Data are presented as the mean ± SEM. *p < 0.05; **p < 0.01 between NOD and BALB/c.
pancreas of NOD mice with earlier onset of diabetes. In agreement with the upregulation of CXCL13 and CCL19, both LTα and LTβ, which are known to be critical for the induction of lymphoid chemokines, were highly expressed in NOD, but not BALB/c mice, and displayed a similarly consistent trend (Fig. 4G, 4H).

Development of B cell-rich aggregates in the pancreas of NOD mice was associated with the in situ upregulation of the B cell survival and proliferating factors BAFF and April (Fig. 5A–C) as well as of IL-4, which are critical activators of AID and class switching in B cells (Fig. 5D). This is consistent with the observed pancreatic expression of AID at both mRNA and protein levels (Fig. 3C, 3D).

CXCR5⁺ follicular B cells are the main subset infiltrating pancreatic islets and localize within ectopic follicles

Two main subsets of mature B cells have been identified in rodents. FoB, which localize within B cell follicles in the spleen and other SLOs, mainly undergo a classical T-dependent GC reaction during which diversification and affinity maturation of the BCR take place allowing the generation of a high-affinity response to cognate Ags. Conversely, MZB cells localize at the periphery of the B cell follicles in association with the marginal sinus of the spleen, where they provide a T-independent first line innate defense against blood-borne pathogens (35, 41). A number of cell surface markers allow the identification of these B cell subsets being FoB B220⁺/IgD⁺/CD23⁻/CD21⁻/IgM⁻/CD1d⁻/CXCR5⁺, whereas MZB are B220⁺/IgD⁻/CD23⁻/CD21⁺/IgM⁺/CD1d⁺/CXCR5⁻ (36). Thus, to characterize the B cell subsets infiltrating TLSs we performed double staining in sequential sections for IgD/B220, IgD/Cd1D, CD21/CD23, IgD/IgM, and CXCR5/B220. Stainings were first performed on spleen sections to validate the procedure. As shown in Fig. 6, double and sequential staining for IgD/Cd1D and CD21/CD23 clearly identify the two populations in NOD spleen. Sequential section analysis of pancreatic tissue demonstrated that the majority of B cells infiltrating TLSs were characterized by a B220⁺/IgD⁺/CD23⁻/CD21⁻ phenotype consistent with a FoB cell subset. CD21 expression in the pancreas was restricted to FDC networks, as demonstrated by positive sequential staining for GL7 (Fig. 6M). Interestingly, only in occasional large infiltrates was it possible to identify a subset of IgD⁺/CD1d⁺ B cells localizing at the periphery of the B cell area, possibly consistent with a transitional 2/MZ phenotype (Fig. 6J–L).

Consistent with the prevalent FoB cell phenotype, the vast majority of islet-infiltrating B220⁺ cells also displayed high expression of CXCR5, with a pattern highly reminiscent of that observed in the follicular area of the spleen (Fig. 7). This suggests that the ectopic expression of CXCL13 is functional in attracting CXCR5⁺ cells in the infiltrated islets.

TLSs sustain in situ production of IAAs

Recent evidence demonstrated that B cells isolated from NOD pancreas (and peripancreatic lymph nodes) display reactivity of the BCR against insulin, suggesting that autoreactive B cells are preferentially recruited to the inflammatory site (28). However, it is not known whether these autoreactive cells are actually generated and localized in association with functional TLSs. To provide direct evidence of this possibility, we performed staining with FITC-conjugated insulin and demonstrated specific binding to infiltrating cells in close association with the B cell area of the aggregates (Fig. 8A, 8B). Of relevance, no binding was ever observed in noninfiltrated islets of both NOD and BALB/c mice nor in highly inflamed salivary gland aggregates of NOD mice, suggesting that generation of an anti-insulin response in this strain takes place exclusively during insulitis (Fig. 8C, 8D, respectively). Conversely, as expected, anti-insulin reactivity was detected in reactive peripancreatic lymph nodes of NOD mice (Fig. 8E, 8F). In all cases, preincubation with unconjugated insulin strongly reduced immunoreactivity (data not shown). To characterize the cells displaying autoreactivity against insulin, we performed double staining for B220 and CD138 (Fig. 8B, 8G), markers of B cells and plasma cells, respectively. Our analysis demonstrated that

**FIGURE 5.** Evaluation of B cell proliferating and survival factor gene expression. Quantitative TaqMan real-time evaluation of pancreatic mRNA expression levels in NOD and BALB/c mice. Gene expression was evaluated for BAFF (A), BAFFFr (B), April (C), and IL-4 (D). Data are presented as the mean ± SEM. **p < 0.01 between NOD and BALB/c.
anti-insulin autoreactive cells are of plasma cell origin (CD138+ and B2202) and are closely associated with the B cell area, suggesting local differentiation in the context of TLSs. This suggests that TLSs can promote humoral autoimmunity against islet Ags over and above SLOs.

**Discussion**

Development of TLSs is a common feature of several chronic inflammatory diseases (19), and in particular, those characterized by organ-specific autoimmunity, such as Hashimoto’s thyroiditis (42), RA (43–46), and Sjogren’s syndrome (22, 40, 47–50). It has been known for some time that in these conditions ectopic lymphoid tissues acquire several features characteristic of SLO, such as segregation of T and B cells in distinct areas, formation of high endothelial venules, development of FDC networks, and expression of lymphoid chemokines CXCL13, CCL19, and CCL21. More recently, we and others have demonstrated that these GC-like structures not only recapitulate the cellular organization of SLOs, but are also capable of supporting activation of the molecular machinery responsible for the in situ production of high-affinity class-switched autoantibodies, as demonstrated by expression of AID, ongoing CSR, and somatically hypermutated Ig V genes (21, 22, 24, 51). Thus, it is now evident that TLSs represent preferential niches for the accumulation and functional activation of Ag-specific autoreactive B cells, contributing to the autoimmune process over and above SLOs.

T1D is an autoimmune disorder in which T and B cells gradually infiltrate pancreatic islets (insulitis), eventually leading to the destruction of insulin-producing β cells. In comparison with the above-mentioned autoimmune diseases, however, the complexity of obtaining human pancreatic tissue in vivo has hampered a systematic investigation of this phenomenon in T1D insulitis. Thus, studies of the cellular dynamics involved in the development of autoimmune insulitis have been largely derived from the NOD mouse strain, a most robust model of T1D. Studies in this model have revealed that B and T lymphocytes invade the islets simultaneously (52), progressing from an initial peri-insulitis toward a destructive intrainsulitis.

Furthermore, although T cells no doubt have a fundamental pathogenic role, there is compelling evidence that B lymphocytes play a nonredundant function in the autoimmune process and disease development. B cells are required for the initiation and progression of the insulitis in NOD mice, as depletion of B cells completely abrogates the development of autoimmunity in this model (6). In addition, it has been shown that B cells must display autoreactive specificities toward islet-associated Ags (i.e., IAAs) in the context of appropriate MHC-II alleles to prime islets CD4+ T cells (7–9), suggesting that B cells exert a critical role in Ag presentation, a process that requires an organized lymphoid setting.
to promote T–B cell interactions (10, 53). The translational relevance of these observations has been recently confirmed in phase II clinical trials showing that B cell depletion achieved with a short course of the anti-CD20 mAb rituximab is capable of significantly preserve β cell function over a period of 1 y in patients with newly diagnosed T1D (12). Thus, although a key role for B cells has been identified, the cellular and molecular mechanisms leading to their activation within islet infiltrates are poorly understood.

In this study, we provide the first in-depth demonstration that the development of inflammatory infiltrates within pancreatic islets of NOD mice is a highly organized process leading to the progressive acquisition of features of SLOs and the formation of functional lymphoid aggregates. First, using double immunofluorescence on sequential sections for CD3/B220, we clearly demonstrated that during the evolution from peri-insulitis to intrainsulitis, T/B cell aggregates display progressive acquisition of T/B cell segregation. This process further allows the development of FDC networks, which were exclusively present within the B cell area and required the presence of segregation of T and B cells in distinct areas. Interestingly, despite a similar prevalence of insulitis, the highest incidence of both T/B cell segregation and FDC network formation was observed in NOD mice developing diabetes early (average 14 wk) as opposed to late-onset diabetic mice, suggesting that evolution of TLSs is associated with a more aggressive phenotype. This evidence is a confirmation in an animal model of recent data obtained from postmortem evaluation of patients with recent-onset T1D in which it was shown that islets with a high degree of destruction and loss of β cell are characterized by a large accumulation of B lymphocytes (11). In addition, this is in keeping with demonstration in other chronic autoimmune conditions, such as RA, that B cell infiltration and TLS formation are associated with a more severe clinical phenotype and are more likely to be resistant to anti-TNF therapy (54).

Importantly, we also provide original demonstration that the development of TLSs in autoimmune insulitis in NOD mice is paralleled by the in situ overexpression of a critical set of genes that are known to be master regulators of physiological as well as ectopic lymphoid organogenesis (55). In particular, we clearly showed that the ectopic expression of lymphoid chemokines CXCL13 and CCL19 was apparent already at 4 wk of age and preceded the pancreatic infiltration of immune cells bearing the specific receptors CXCR5 and CCR7. In addition, the peak of expression of these factors was in keeping with the histological detection of fully formed TLSs within the islets. The expression of lymphoid chemokines in embryonic lymphoid organogenesis by subsets of VCAM-1+ ICAM-1+LTβR+ mesenchymal cells is critically dependent on the signaling through the LTβR upon binding by the membrane-bound heterotrimeric member of TNF family LTβ (LTβR2), which, in embryonic development, is provided by CD3+CD4+CD45+IL-7Ra+ receptor activator of NF-κB+ lymphoid inducer cells that also express CXCR5 (56, 57). In keeping with this evidence, we showed that the TNF family members LTα and LTβ are significantly upregulated in NOD mice in parallel with the induction of CXCL13/CCL19, suggesting that the LTβ/lymphoid chemokine-positive feedback loop is active and functional and precedes the development of TLS during the autoimmune insulitis in NOD mice. These data support previous observations that blockade of LTβ with a LTβR-Ig fusion protein completely prevents diabetes in NOD mice (58).

The importance of this functional pathway was further strengthened by evidence that the vast majority of B cells infiltrating pancreatic islets express CXCR5 and belong to the subpopulation of follicular B cells, as shown by a B220+/IgD+/CD1d+ phenotype. Conversely, only in occasional large infiltrates was it possible to identify a subset of B220+/IgD+/CD1d- B cells localizing at the periphery of the B cell area, possibly consistent with a transitional 2/MZ phenotype (59).

It has been recently demonstrated that the Ig V L chain gene repertoire of islet-infiltrating B cells is highly diversified and differs from that observed in regional lymph nodes (28), suggesting clonal diversification within islet infiltrates. However, this work could not rule out the possibility that B cells prediversified elsewhere and simply accumulate during the course of the insulitis. Moreover, formal demonstration that B cells can activate in situ the molecular machinery to support SHM/CSR of the Ig genes has been missing. In this study, we provide clear evidence, both at mRNA and protein level, that in the pancreas of NOD mice immune cell infiltrates characterized by T/B cell segregation, FDC networks, and differentiation of GL7+ GC B cells invariably express AID, the enzyme responsible for SHM and CSR of the Ig genes (17). AID initiates SHM by introducing single-point mutations in WRC (W = A/T, R = A/G) motif hot spots within the Ig V genes, which encode for the Ag-

![FIGURE 7. Infiltrating B cells display CXCR5 within NOD inflamed islets. A–C, Double immunostaining for CXCR5 (A, green), B220 (B, red), and CXCR5/B220 (C, superimposed) in NOD spleen sections. D and E, Double immunostaining for CXCR5 (D, green), B220 (E, red), and CXCR5/B220 (F, superimposed) in NOD pancreas with intrainsulitis. Original magnification ×20.](http://www.jimmunol.org/DownloadedFrom)
that TLSs in NOD islets provide niches for differentiation of islet-autoactive B cells and contribute to autoimmunity over and above SLO. These data are in agreement with similar evidence showing reactivity of isolated B cells toward the same Ag in fluid phase (28).

Overall, our results provide strong evidence that supports the concept that: 1) TLSs progressively evolve in pancreatic insulins in NOD mice following an extremely tight cellular and molecular organizational program; 2) this process is associated with a more aggressive phenotype; and 3) once formed, these structures display the capacity to activate the molecular machinery to sustain in situ IgV repertoire diversification, class switching, and differentiation of B cells into autoantibody-producing cells.

Disclosures
The authors have no financial conflicts of interest.

References
21. Humber, F., M. Bombardieri, A. Manzo, S. Kelly, M. C. Blades, B. Kirkham, J. Spencer, and C. Pitzalis. 2009. Ectopic lymphoid structures support ongoing binding region of the Ab (60, 61). In addition, AID regulates CSR via its C-terminal region (62) by inducing double-strand breaks and recombination of Ig switch regions of DNA, followed by excision of switch circles within the constant H chain region. As AID is only transiently and exclusively expressed by B cells undergoing SHM and/or class switching, its detection has allowed us to directly demonstrate the capability of TLSs to support activation and differentiation of B lymphocytes to Ab-producing cells within the pancreatic tissue, a possibility further supported by evidence that TLSs are surrounded by CD138+ plasma cells.

Importantly, we have shown that a proportion of these CD138+ plasma cells displays reactivity against insulin, strongly indicating

FIGURE 8. In situ autoantibody production in NOD mice pancreas islet infiltrates. Stainings of NOD mice pancreas, submandibular glands (as negative control for islet-associated autoimmunity within TLS), and peripancreatic lymph nodes (positive control for autoreactive B cells) with FITC-conjugated insulin. A and B, Representative example of TLSs pancreatic islets, showing cells with reactivity for FITC-conjugated insulin alone (A, green) and in double staining with B220 (B, red). C and D, Representative examples showing that neither pancreatic islets without inflammatory cells (C) nor submandibular glands with large inflammatory aggregates (D) displayed reactivity against FITC-conjugated insulin. E and F, Reactive peripancreatic lymph node showing several GL7+ GCs (E, green) and displaying numerous FITC insulin-reactive cells (F, green). G and H, Sequential staining in NOD islets for CD138 (G, red) and CD138/FITC-conjugated insulin (H, red/green) showing recolocalization of the reactivity toward FITC insulin with CD138+ plasma cells. Original magnification ×20 (A–E) and ×40 (F–H).

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