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Recruitment and Activation of Naive T Cells in the Islets by Lymphotoxin β Receptor-Dependent Tertiary Lymphoid Structure

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Summary

The development of spontaneous insulin-dependent diabetes mellitus is preceded by the organization of tertiary lymphoid organ (TLO) in situ, but its role in the development of tissue destruction and the cytokines that control such structures have not been fully defined. We have now observed that TNF superfamily 14 (TNFSF14) is upregulated in aged nonobese diabetic (NOD) pancreas with the appearance of TLO. Blockade of TNFSF14 signaling caused a substantial reduction in the expression of lymphotoxin β receptor (LTβR)-controlled migration factors within the islets and disrupts organization of tertiary structures, leading to prevention of diabetes. Consistently, enhancing LTβR signaling by transgenic expression of TNFSF14 in the islets of NOD mice rapidly promoted de novo formation of local TLO, resulting in diabetes, even in the absence of draining lymph nodes (LN). Thus, the TNFSF14-LTβR pathway appears to be critical in the development and maintenance of TLO for the onset of diabetes.

Introduction

Although de novo organization of tertiary lymphoid organ (TLO) is known to precede the development of a number of human autoimmune diseases (Aloisi, 2006; Ruddle, 1999), its role in the development of autoimmune diseases is unclear. Insulin-dependent diabetes mellitus (IDDM) is a T cell-mediated autoimmune disease in which the insulin-secreting β cells are selectively destroyed (Bach, 1994; Tisch and McDevitt, 1996). Studies performed in nonobese diabetes (NOD) mice have revealed that the influx of cellular infiltrates migrate to the pancreas starting at 4–6 weeks of age (Bach and Mathis, 1997; Delovitch and Singh, 1997; Green and Flavell, 1999; Ludewig et al., 1998; Tisch and McDevitt, 1996), but it is only with continued progressive inflammation that an organized lymph node-like follicular structure will develop, usually over 2–3 months, culminating in the development of IDDM. In diabetes, the peri-insular infiltration organizes into TLO with cellular infiltrates and increased expression of key chemokines and adhesion molecules (Hanninen et al., 1993; Hjelmstrom et al., 2000; Yang et al., 1997). Although the formation of TLO is a prototypic feature of chronic progressive inflammation (Aloisi, 2006; Ruddle, 1999), the molecular mechanisms by which the TLOs are formed and importantly their role in the pathogenesis of IDDM remains to be demonstrated.

The initiation of the autoimmune response in NOD in the draining lymph node (LN) is essential for priming and clonal expansion of autoreactive T cells poised for islet destruction (Hoglund et al., 1999). The removal of the pancreatic draining LNs prior to a critical time point of autoreactive T cells priming prevents IDDM in NOD mice, demonstrating the absolute requirement of draining LNs in the initial stages of disease induction (Gagnerault et al., 2002; Levisetti et al., 2004). Initiation of the disease process in the draining LN is not sufficient, however, as shown by the fact that diabetogenic T cells isolated from the draining LNs of 10-week-old NOD mice have markedly reduced efficiency for transferring the disease to NOD SCID recipients compared to the spleen (Jaakkola et al., 2003). Similarly, disease incidence is unchanged in NOD mice deprived of pancreatic draining LNs or spleen at 10 weeks compared to control mice with intact secondary lymphoid structures (Gagnerault et al., 2002). The diminished importance of the LNs and the spleen during the effector phase of disease suggests that alternative lymphoid structures may take over their function. However, it is unclear why draining LNs become irrelevant after the initial priming, how the autoreactive response is sustained in their absence, and whether the priming of T cells in an alternative location is a prerequisite for the generation of a sufficiently robust autoimmune repertoire for the development of IDDM.

Membrane lymphotoxin (LT) was initially found to be essential for the development of secondary lymphoid tissues (Fu and Chaplin, 1999). LT, and to a lesser degree tumor necrosis factor (TNF), is sufficient for TLO as well as for the maintenance of the lymphoid structures (Luther et al., 2000; Ngo et al., 1999; Ruddle, 1999). Wild-type (wt) mice treated with lymphotoxin β receptor-immunoglobulin fusion protein (LTβR-Ig) exhibit reduced chemokines and adhesion molecules inside secondary lymphoid tissues, leading to alterations in the lymphoid microenvironment as well as a reduction in the cellularity in the lymph nodes (Browning et al., 2005; Fu and Chaplin, 1999; Mackay and Browning, 1998; Mackay et al., 1997; Ngo et al., 1999; Wu et al., 1999). LTβR-Ig has been shown to alleviate various murine models of autoimmune diseases such as rheumatoid arthritis, colitis, experimental autoimmune encephalomyelitis (EAE) (which is independent of pertuss toxins), and late stage of type 1 diabetes (Fava et al., 2003; Gommerman et al., 2003; Mackay et al., 1998; Wu et al., 2001). Although LT is thought to be responsible for TLO, a recent study indicates that the chronic infection can trigger the same organization in the absence of LT (Moyron-Quiroz et al., 2004). TNF superfamily 14 (TNFSF14), another ligand for LTβR that is commonly known as lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry

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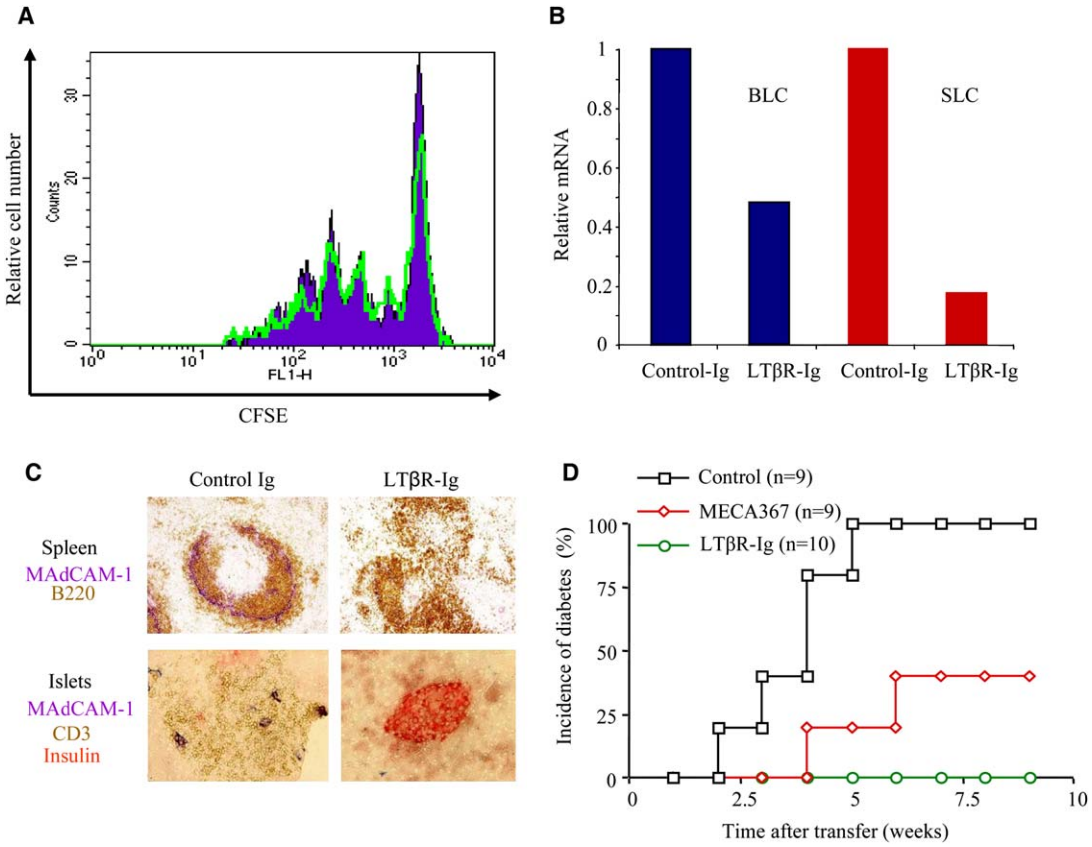

Figure 1. LTβR-Ig Blockade Does Not Perturb Priming, but Reduces Expression of Migration Factors

(A) CFSE-labeled 20 million NOD-BDC2.5 (6- to 7-week-old) splenocytes were adoptively transferred to sublethally irradiated 10-week-old NOD recipients treated with 100 μg of either control h-IgG (Solid) or LTβR-hIgG (Green) on day −7 and day 0. CFSE dilution was determined 72 hr after transfer by FACS analysis after gating on $V\beta4^+CD4^+$ lymphocytes. The data are representative of three independent experiments.

(B) Real-time PCR analysis of cDNA prepared from the pancreatic islets of 10-week-old NOD mice 1 week after last treatment of three weekly treatment with 100 μg of control-IgG or LTβR-IgG. Relative abundance of mRNA of BLC and SLC determined.

(C) 10-week-old NOD mice were treated with 100 μg of control Ig (left) or LTβR-Ig (right) once weekly for 3 weeks. The spleens (top) and the pancreata (bottom) were collected 10 days after the end of the treatment. The splenic sections were stained with B220 (brown) and anti-MAdCAM-1 (purple), whereas pancreatic sections were stained with anti-CD3 (brown), anti-MAdCAM-1 (purple), and anti-insulin (red). Original magnification: ×200.

(D) Sublethally irradiated NOD female mice were injected i.v. with 2×10^7 splenocytes from diabetic NOD mice and treated with a single i.p. injection of 100 μg of Anti-MAdCAM-1 (MECA367), LTβR-Ig, or human Ig. The incidence of diabetes was monitored.

mediator on T cells (LIGHT), can also partially replace the role of LT for the development and maintenance of the lymphoid microenvironment inside the spleen (Gommerman and Browning, 2003; Wang et al., 2002; Ware, 2005). The contribution of LIGHT in the development of TLO during chronic inflammation is, however, unknown. In this study, we uncover the central role of the LIGHT-LTβR in organizing TLO at sites of chronic autoimmune inflammation and the key role these structures play in tipping autoimmunity toward active autoimmune destruction.

Results

LTβR-Ig Treatment Does Not Limit Priming in the Draining LN

We have previously reported that LTβR-Ig treatment in prediabetic NOD mice prevents the development of IDDM, but the mechanism remains unclear (Wu et al., 2001). LTβR-Ig binds not only to heterotrimic membrane bound LTα1β2 (LTβ) on T cells, but also to LIGHT, a costimulatory molecule expressed on T cells and dendritic cells (DC). Because the onset of diabetes is predominantly T cell mediated, it is possible that LTβR-Ig blocks activation of autoreactive T cells primed in the draining LN (DLN). To rule out this possibility, we adoptively transferred 2×10^7 CFSE-labeled splenocytes from 6- to 7-week-old NOD-BDC2.5 T cell receptor (TCR) transgenic T cells to 10-week-old prediabetic NOD recipients that had been treated with LTβR-Ig or control-Ig. BDC2.5 T cells are clonotypic TCRs specific for islet antigens. It has been previously reported that adoptively transferred naive BDC2.5 $CD4^+$ T cells are primed at the pancreatic LNs of NOD recipients 3 days after transfer (Hoglund et al., 1999). Analysis of the donor cells isolated from the pancreatic draining LN 72 hr after adoptive transfer revealed that priming and homing to the DLN remained unperturbed by LTβR-Ig treatment (Figure 1A). These data suggest that the prevention of IDDM by soluble LTβR treatment may be due to mechanisms other than inhibited trafficking and priming of pathogenic T cells in the DLN.

LTβR-Ig Treatment Reduces Expression of Chemokines and Adhesion Molecules inside the Islets

An alternative explanation for the prevention of IDDM via LTβR-Ig administration could be through the prevention of activated lymphocyte trafficking to peripheral target organ. T cells and DCs migrate to the pancreas in response to local CCL21 (SLC) and CCL19 (BLC) expression (Chen et al., 2002; Luther et al., 2000), and homing of early diabetogenic effector lymphocytes to the islets is also dependent on MAdCAM-1 (Yang et al., 1997). To determine whether blocking LTβR signaling disrupted expression of these key lymphocyte migrational cues to the pancreas, real-time PCR of purified pancreatic islets was performed on LTβR-Ig- and control-Ig-treated NOD mice. Mice were treated with 100 μg of either protein once weekly for 3 weeks and sacrificed for analysis 1 week after the last treatment. Relative analysis revealed approximately 80% reduction in the mRNA expression of SLC and to a lesser extent of BLC expression observed in the islets of LTβR-Ig-treated mice compared to that of controls (Figure 1B). Furthermore, immunohistochemical staining of the spleen and pancreas of NOD mice treated with either 100 μg of LTβR-Ig or human-Ig once a week for 3 weeks and sacrificed for analysis 1 week after the last treatment revealed the absence of MAdCAM-1 staining in the LTβR-Ig-treated group. This was in contrast to the abundant staining seen in splenic marginal zones and islets of the control group (Figure 1C, top). Consistently, immunohistochemical staining, with MAdCAM-1, CD3, and insulin, of the pancreatic islet section revealed decreased MAdCAM-1 expression around islets that typically correlated with fewer pathogenic $CD3^+$ T cell infiltrations in the LTβR-Ig-treated group compared to the control group (Figure 1C, bottom). These findings indicate that the expression of MAdCAM-1, SLC, and BLC in the pancreas is markedly perturbed by the blockade of LTβR signaling, resulting in the inhibition of cellular trafficking to the islets. The downregulation of these key adhesion molecule and chemokines in the local microenvironment may be sufficient to prevent disease development even in the presence of primed autoreactive T cells.

To demonstrate that the reduction of LT-mediated MAdCAM-1 expression sufficiently contributes to the reduction of pathogenic insulitis, we transferred 2×10^7 T cells from newly identified diabetic NOD mice (<2 weeks) into sublethaly irradiated 8- to 10-week-old NOD recipients that were concomitantly treated with 100 μg of either anti-MAdCAM-1 (MECA367), LTβR-Ig, or control-Ig once per week for 3 weeks. Then the mice were monitored for incidence of diabetes. Treatment with anti-MAdCAM-1 recapitulated, albeit to a lesser degree, the preventive effects seen with LTβR-Ig treatment (Figure 1D). Taken together, these data suggest that downregulation of LTβR-dependent adhesion molecules and chemokines within the pancreatic islets may contribute to the LTβR-Ig-mediated prevention of IDDM.

Involvement of LIGHT in the Development of IDDM

Stimulation of membrane LTβR on the stromal cells for the expression of chemokines and MAdCAM-1 can occur through two known ligands: LTβ and LIGHT (Gommerman and Browning, 2003; Wang et al., 2002; Ware, 2005). Both ligands are predominantly expressed on activated T cells, but LIGHT can also signal through another TNF receptor family member, HVEM. To unravel the contribution of one or both ligands to the prevention of disease, we treated prediabetic 10-week-old NOD mice with 100 μg of either LTβ antibody to selectively block LT, HVEM-Ig to selectively block LIGHT, LTβR-Ig to block both LTβ and LIGHT, or control-Ig. The mice were injected weekly for 3 weeks and then monitored for incidence of diabetes for 32 weeks. We found that whereas blocking membrane LT had marginal effects in preventing IDDM, blocking LIGHT greatly prevented disease progression (Figure 2A). To exclude the possibility that blocking LIGHT-HVEM merely inhibited costimulatory activation of pathogenic T cell and subsequently prevented diabetes, we examined H&E-stained paraffin sections of pancreatic tissues from HVEM-Ig- or control-Ig-treated NOD mice. Our analysis revealed that HVEM-Ig treatment reduced insulitis in the pancreatic islets by approximately 50% overall compared to the controls (Figure 2B). There was more appreciable reduction of insulitis in the islets with greater than 50% infiltration score in the HVEM-Ig-treated group (Figure 2B). These results suggest that blocking LIGHT interaction with its cognate ligands, by LTβR or HVEM, contributes in ameliorating diabetes pathogenesis.

LIGHT Expression in the Pancreas Accelerates Insulitis and IDDM Onset

We attempt to tease out the mechanism by which LIGHT signaling through its cognate receptors regulates T cell-mediated destruction of β islet cells. First, to demonstrate that the pathological amount of LIGHT in the local microenvironment increases in association with disease progression in the murine model, we measured LIGHT mRNA expression in the pancreas of 5-week-old NOD, prediabetic 20-week-old NOD, and 12-week-old B6 mice as controls. We found that the relative expression of LIGHT in the pancreas increased with age in NOD mice, which in turn correlates with exacerbated insulitis (Figure 3A and data not shown).

Although the age-dependent increase of LIGHT mRNA expressions in the local environment revealed a correlation with the development of diabetes, it did not elucidate the function of LIGHT in disease induction. To further demonstrate the consequence of LIGHT on the islets for propagating the disease, we decided to generate transgenic mice expressing LIGHT in the β cells under the control of the rat insulin promoter (RIP) in the NOD background (see Figure S1 in the Supplemental Data available with this article online). Southern blot analysis as well as RT-PCR of relative LIGHT mRNA expression on the islets of the transgenic (tg) mice were used to detect the presence of the transgene and confirm expression of LIGHT, respectively (Figure S1 and data not shown). The homozygous tg mice we generated possess only one copy of the transgene, thus bearing a closer physiological expression of pancreatic LIGHT in the pancreas of aged NOD mice. In this model, RIP-LIGHT-NOD mice developed the disease at a much earlier age with complete onset compared to the NOD controls (Figure 3B). The disease acceleration was more evident in LIGHT transgenic NOD male mice, as

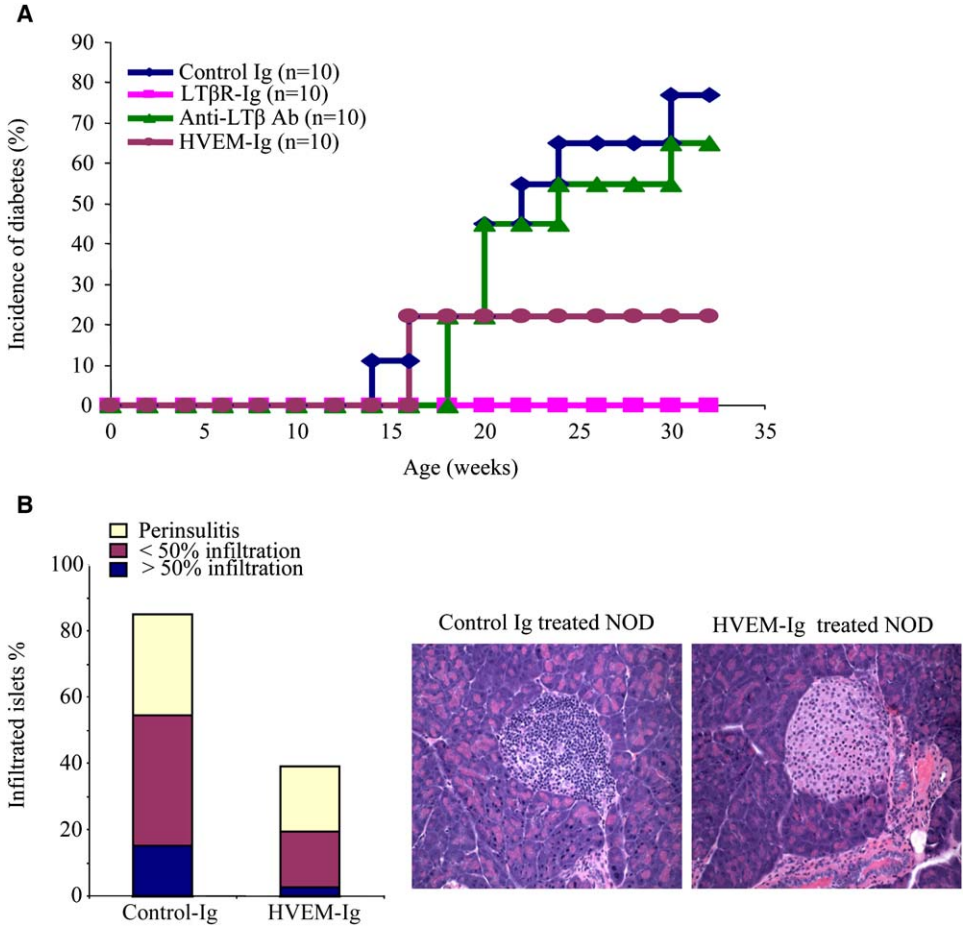

Figure 2. LIGHT Is Required for the Development of IDDM

(A) Blockade of LIGHT via HVEM-Ig prevents the development of IDDM in NOD mice. 10-week-old NOD mice were treated with 100 μg Control Ig, LTβR-Ig, HVEM-Ig, or LTβ Ab once a week consecutively for 3 weeks. Incidence of diabetes evaluated for 32 weeks.

(B) Pancreata stained with H&E 1 week after last treatment with HVEM-IgG or control-IgG. Individual islets from five mice per group were assigned scores for insulitis as measured by cellular infiltration. Original magnification: ×200.

shown by the fact that most developed disease before 18 weeks, whereas none had IDDM in male control; 100% developed the disease before 23 weeks while only 10% developed the disease in the male controls (Figure 3B).

The development of diabetes is often preceded by inflammatory insulitis with subsequent formation of an organized LN-like structure around the islets (Luther et al., 2002; Ruddle, 1999). To investigate whether the higher incidence of IDDM at an early age in the RIP-LIGHT-NOD mice was associated with such trademark characteristics, the pathology of pancreatic tissue was investigated. As anticipated, 4- and 6-week-old RIP-LIGHT-NOD mice displayed severe infiltrates of mononuclear cells of varying sizes, and the large cellular infiltrates in the islets resembled those observed in mice with insulitis (Figure 3C). Minimal infiltration was evident in age-matched NOD mice, although by 6 weeks of age we began to see increased perinsulitis around the islets (Figure 3C). However, we observed that the degree of insulitis in the 4-week-old RIP-LIGHT-NOD mice resembled that of 10-week-old NOD mice, suggesting that local LIGHT expression facilitates homing of activated T cells to the islets.

Islets expressing LT are sufficient to induce TLO (Ruddle, 1999). Our initial observation of increased lymphocytic infiltration to the islets upon LIGHT expression leads us to hypothesize that LIGHT signaling through its receptors can also induce de novo formation of TLO in the pancreas, similar to secondary lymphoid organs. Histological analysis of pancreatic tissue from 5-week-old RIP-LIGHT-NOD mice stained with Thy1.2$^+$ and B220$^+$ showed the presence of an organized T-B cell zone that closely mimicked those found in secondary lymphoid follicles (Figure 3D). Furthermore, similar structured T-B cell zone was observed in the islets of 20-week-old prediabetic NOD mice, yet was conspicuously absent in 5-week-old NOD mice (Figure 3D and data not shown). In addition, both 4- to 5-week-old RIP-LIGHT-NOD mice and prediabetic 20-week-old NOD mice had elevated pancreatic expression of SLC mRNA, a well-characterized chemokine marker known to be critical for the formation of TLO (Fan et al., 2000; Hjelmstrom et al., 2000; Luther et al., 2000), when compared to 4- to 5-week-old NOD mice (Figure 3E). Thus, the increase in pancreatic SLC expression, coupled with the presence of TLO in the islets, indicates that LIGHT signaling with its receptors, likely through LTβR,

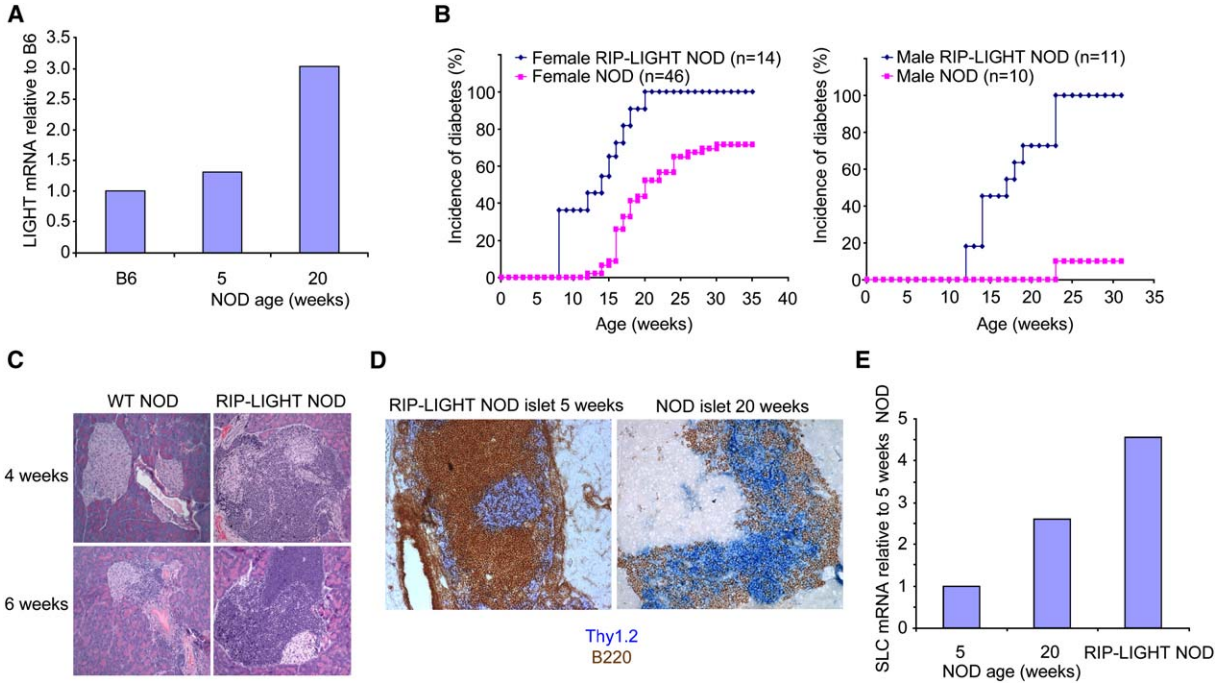

Figure 3. LIGHT Expression on the Pancreatic Islets Exacerbates Diabetes Onset

(A) Real-time PCR analysis of cDNA prepared from pancreas of 5- or 20-week-old NOD and B6 mice. Data are representative of two experiments.

(B) Incidence of diabetes observed for RIP-LIGHT-NOD females and RIP-LIGHT-NOD males with respective NOD wt controls. Mice were considered diabetic with blood glucose concentration >250 mg/dL for two consecutive weeks.

(C) H&E staining of pancreata of 4- or 6-week-old female RIP-LIGHT-NOD and NOD wt mice. Original magnification: ×200.

(D) LIGHT expression on the islets induces TLO similar to the secondary lymphoid organs. Immunohistochemical staining of pancreatic islets of 5-week-old RIP-LIGHT-NOD mice or 20-week-old prediabetic NOD mice; anti-Thy1.2 (blue) and anti-B220 (brown). Original magnification: ×200.

(E) Real-time PCR analysis of SLC expression in pancreas of 5- or 20-week-old NOD mice and 5-week-old RIP-LIGHT-NOD mice. The data are representative of two experiments.

can induce formation of TLO in the pancreas of NOD mice, invariably promoting the development of IDDM.

Naive T Cells Are Attracted to TLO

The hallmark characteristic of secondary lymphoid structures is their exquisite ability to recruit circulating naive lymphocytes and elicit priming and subsequent clonal expansion of antigen-specific T cells (Butcher and Picker, 1996; Cyster, 1999). To confirm that the LIGHT-mediated TLO in the pancreas possess functional characteristics ascribed to secondary lymphoid follicles, we transferred 2 × 10^7 naive CFSE-labeled splenocytes from 7- to 8-week-old BDC2.5 tg mice into sublethally irradiated 4-week-old RIP-LIGHT-NOD and age-matched NOD controls. Immunofluroescent staining of pancreatic tissues at 48 hr after transfer revealed that LIGHT-induced TLO enabled the recruitment of $CFSE^+$ cells to the local islets, whereas only few such cells were detected in age-matched NOD mice (Figure 4A). Our FACS analysis of pancreatic islets, pancreatic DLN, and spleen cells isolated at 24 hr after transfer revealed a substantial population of undiluted CFSE (at 0 cycle)-labeled naive T cells recruited to both the spleen and the pancreatic DLN of all three groups (Figure 4B). However, only the islets of RIP-LIGHT-NOD and 16-week-old WT-NOD mice, which we had previously shown in our earlier data to clearly possess TLO in the islets, contained substantial numbers of adoptively transferred T cells (Figure 4B). Furthermore, at 72 hr after transfer, continued CFSE dilution, which is indicative of further proliferation, was present in the islets of RIP-LIGHT-NOD mice and 16-week-old WT-NOD mice, yet notably absent in young 8-week-old WT-NOD mice. Expectedly, all three groups had proliferation in the pancreatic DLNs by 72 hr after transfer (Figure 4B). Stronger proliferation in the islets than in the draining LN of both 16-week-old NOD mice and 8-week-old RIP-LIGHT-NOD mice may suggest that the islets play a greater role in priming and expanding autoreactive T cells.

To further demonstrate that the cells were proliferating in situ, the recipients were pulsed with 100 μg of 5-bromo-2-deoxyuridine (BrdU) via i.p. at 72 hr after adoptive transfer as described above. The pancreatic tissue was harvested 24 hr later for immunohistology. The serial sections of pancreatic tissue were then visualized with the $CFSE^+$ cells or immunostained with anti-BrdU (brown) and counterstained with hemotoxylin. Our data clearly show that the $CFSE^+$ T cells migrate to the islets of RIP-LIGHT-NOD and the prediabetic 16-week-old NOD mice, and these cells proliferate in situ, as evidenced by BrdU-positive staining within the islets (Figure 5A). These results demonstrate that TLO present in the islets of RIP-LIGHT-NOD and prediabetic NOD mice resembles secondary lymphoid structures in appearance and function, specifically in the recruitment of naive T cells to the target site for activation and proliferation.

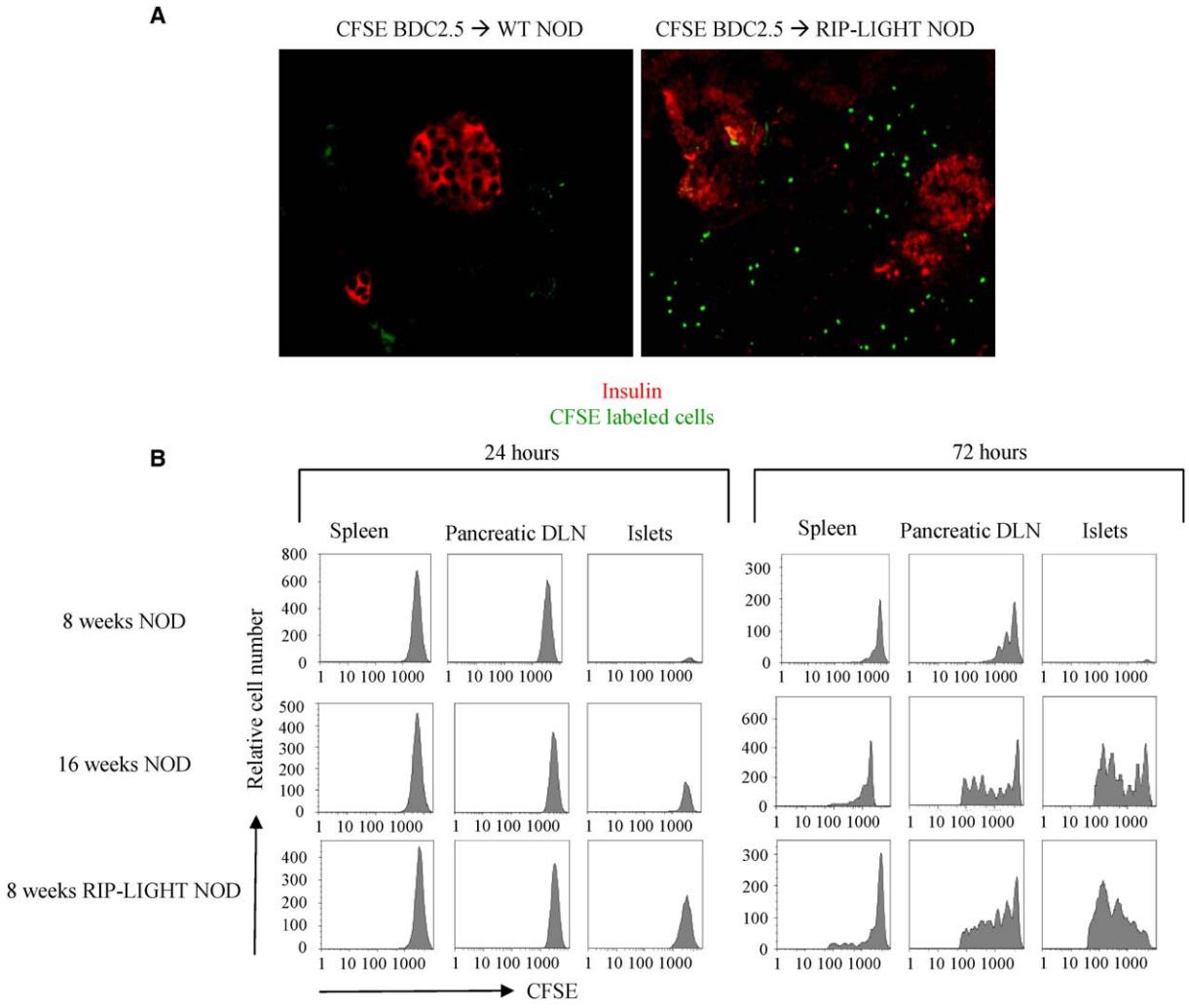

Figure 4. Evidence of Naive T Cells Migrating to and Proliferating at TLO in the Islets

(A) Immunofluorescence staining of CFSE-labeled BDC2.5 splenocytes (2×10^7 cells) adoptively transferred to 4-week-old NOD wt and age-matched RIP-LIGHT-NOD NOD mice. Tissue harvested and fixed 48 hr after cell transfer. Original magnification: ×100.

(B) CFSE-labeled BDC2.5 splenocytes (2×10^7 cells) were adoptively transferred to sublethally irradiated 8-week-old RIP-LIGHT-NOD mice, 8-week-old NOD mice, and prediabetic 16-week-old NOD mice. Islets, PDLN, and spleen were collected 24 hr and 72 hr after adoptive transfer and observed for CFSE dilution on $V\beta4^+CD4^+$ gated lymphocytes. Data are a representation of three experiments with three mice per group.

To ensure that the infiltration and proliferation of T cells in TLO in the islets can translate into the pathogenesis of IDDM, splenocytes from 2×10^7 naive BDC2.5 splenocytes were transferred into sublethally irradiated 4-week-old RIP-LIGHT-NOD and NOD mice (Figure 5B). 50% of the RIP-LIGHT-NOD mice developed diabetes 2 weeks after transfer of BDC2.5 cells, and by 5 weeks after transfer, 100% of the RIP-LIGHT-NOD mice have developed diabetes. In contrast, none (0%) of the irradiated NOD mice developed diabetes even 15 weeks after transfer (Figure 5B). To demonstrate that the observed incidence of diabetes was not caused by the endogenous population of autoreactive T cell in the irradiated LIGHT-expressing mice, 4-week-old RIP-LIGHT-NOD mice were sublethally irradiated and observed for possible disease onset. As we expected, in the absence of adoptively transferred cells, irradiated RIP-LIGHT-NOD mice did not develop diabetes (Figure 5B).

When the 2×10^7 naive BDC2.5 splenocytes were adoptively transferred to irradiated 14- to 16-week-old prediabetic NOD mice (not younger NOD mice), which we have shown earlier to possess TLO in the islets, more than 90% of mice developed IDDM by 12 weeks after transfer, whereas none (0%) developed in mice lacking cell transfer (Figure 5C). The incidence of diabetes development, in this antigen-specific adoptive transfer model, was dependent on LTβR signaling, as shown by the fact that the abrogation of the ectopic structure in RIP-LIGHT-NOD mice by weekly treatment of mice with 100 μg of LTβR-Ig starting at 3 weeks of age for 3 weeks substantially precluded disease onset (data not shown). Earlier, we also provide evidence that the development of IDDM can also be blocked by the use of LTβR-Ig in older (10-week-old) prediabetic WT-NOD mice (Figure 2A). Thus, these results demonstrate the necessity of LTβR signaling-mediated TLO in diabetes

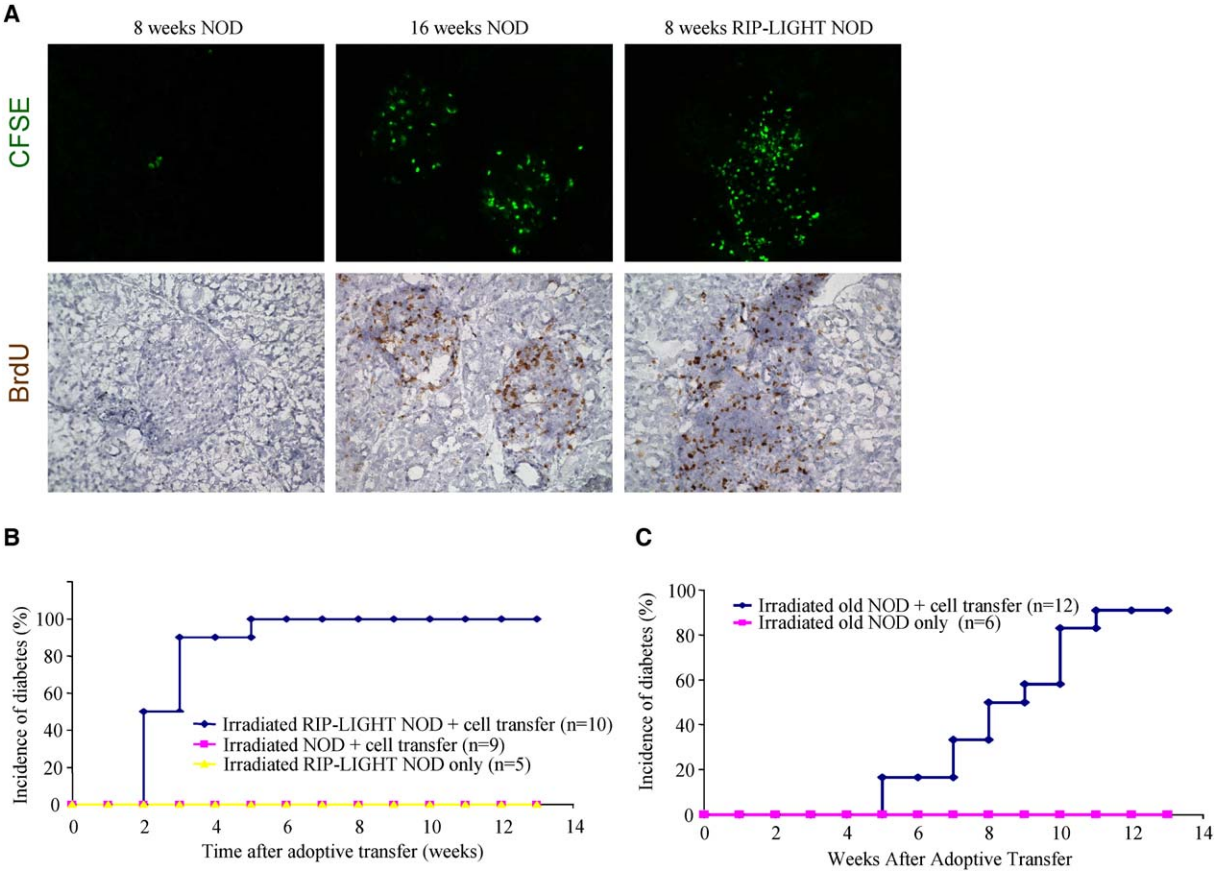

Figure 5. Naive T Cells Are Recruited to TLO and Proliferate In Situ, Inducing Diabetes Onset

(A) BDC2.5 splenocytes (2×10^7 cells) were CFSE labeled and adoptively transferred i.v. to sublethally irradiated 8-week-old NOD, 8-week-old RIP-LIGHT-NOD, and 16-week-old prediabetic NOD mice. Mice were injected with 100 μg of BrdU i.p. at 72 hr after adoptive transfer and pancreas was harvested 24 hr after BrdU injection. Top panel is immunofluroescence of CFSE-labeled (green) naive BDC2.5 splenocytes. The bottom panel is immunohistochemical staining of BrdU (brown) and counterstained with hematoxylin. Each CFSE-labeled tissue section located on the top panel corresponds to its BrdU tissue staining as a serial section. Original magnification: ×200.

(B) BDC2.5 splenocytes (2×10^7 cells) were adoptively transferred to sublethally irradiated 4-week-old RIP-LIGHT-NOD mice and age-matched NOD wt and monitored for incidence of diabetes. Sublethally irradiated RIP-LIGHT-NOD mice without cell transfer included as controls for incidence of diabetes.

(C) BDC2.5 splenocytes (2×10^7 cells) were transferred to sublethally irradiated prediabetic 14- to 16-week-old NOD wt mice and observed for incidence of diabetes.

progression, because the transfer of naive T cells to young (4-week-old) NOD mice with intact DLN could not induce the disease, yet the transfer of naive tg cells to older NOD mice (14- to 16-week-old) was able to cause IDDM.

Tertiary Lymphoid Structure Is Sufficient to Replace Draining Lymph Node

Pancreatic DLN (PDLN) is crucial to prime autoreactive T cells in NOD mice beginning at 3–4 weeks old, because the absence of PDLN protected the mice from developing diabetes (Gagnerault et al., 2002). The same protection was not conferred when the PDLN were ablated at 10 weeks (Gagnerault et al., 2002). We wanted to explore whether the LIGHT-mediated TLO is sufficient to elicit diabetes even in mice lacking PDLN from 3 weeks of age. We propose that in the presence of TLO, even mice lacking DLN could sufficiently develop the disease, because naive T cells can be recruited to the local environment and get primed and proliferate there. To demonstrate this, we surgically excised the PDLN in RIP-LIGHT-NOD and WT-NOD mice at 3 weeks of age and monitored for incidence of diabetes for over 30 weeks. We found that RIP-LIGHT-NOD mice ablated of their PDLNs at 3 weeks were still able to develop IDDM in both males (100%) and females (88%), when only 10% of NOD females and none (0%) of NOD males developed IDDM (Figure 6A).

To formally visualize that TLO can recruit naive T cells into the lesion and proliferate in situ, even in the absence of the draining LN, we transferred CFSE-labeled naive BDC2.5 splenocytes (2×10^7 cells) to 6-week-old RIP-LIGHT-NOD and aged matched NOD mice that had their PDLNs removed. Pancreas tissue was collected and frozen 96 hr after transfer. Naive CFSE-labeled BDC2.5 T cells migrated to the islets even in the absence of PDLN for RIP-LIGHT-NOD mice, whereas infiltrating cells was absent in PDLN-ablated NOD controls (Figure 6B). Similarly, only 4-week-old RIP-LIGHT-NOD mice with PDLN removed showed infiltrating inflammatory cell near the islets in conjunction with in situ proliferation, indicated by the Ki-67 immunohistochemical

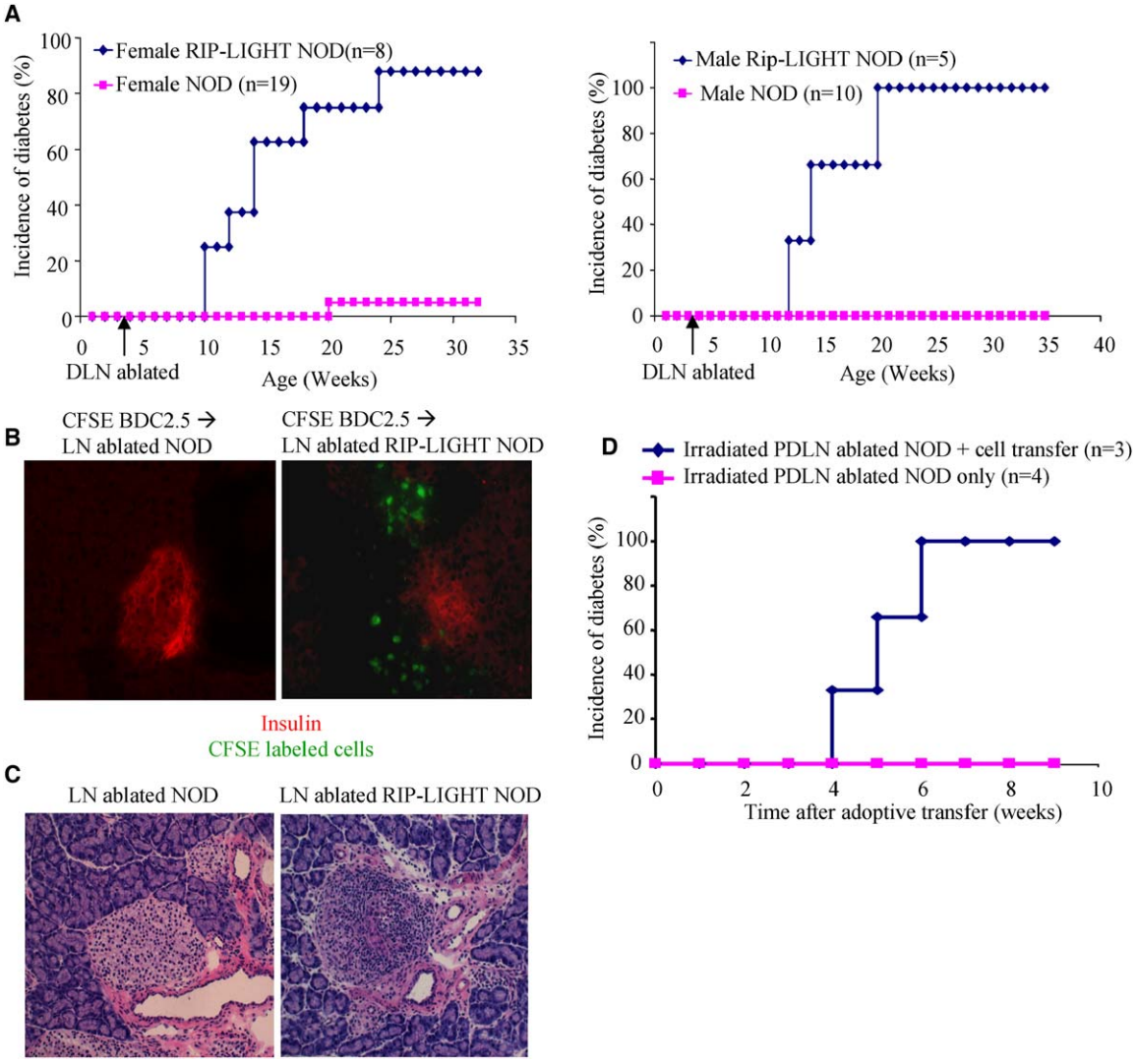

Figure 6. TLO Can Replace the Function of the Draining LN in Recruitment and Activation of Naive T Cell

(A) PDLNs in 3-week-old female or male RIP-LIGHT-NOD mice were surgically excised and spontaneous diabetes development was observed over time.

(B) CFSE-labeled BDC2.5 splenocytes (2×10^7 cells) were adoptively transferred to sublethally irradiated 5-week-old NOD and RIP-LIGHT-NOD mice that have had the DLNs removed. Pancreatic tissues were collected 96 hr after transfer of cells for immunofluoresence staining; insulin (red) and CFSE-labeled transferred cells (green). Original magnification: ×200.

(C) H&E staining of pancreata for 4-week-old female RIP-LIGHT-NOD and NOD mice with PDLN removed.

(D) Sublethally irradiated 36-week-old nondiabetic NOD mice with PDLN removed were adoptively transferred with BDC2.5 splenocytes (2×10^7 cells). Control group did not receive cell transfer. Diabetes development was observed weekly after adoptive transfer.

staining, whereas this observation was absent in NOD controls (Figure 6C and data not shown).

The removal of pancreatic draining LNs in young mice caused them to develop insulitis, albeit to a lesser degree when compared to mice with intact LNs (Gagnerault et al., 2002). To formally demonstrate that TLO present in prediabetic older NOD mice can also prime naive T cells and induce diabetes even in the absence of DLNs, we adoptive transferred naive BDC2.5 (2×10^7 cells) to sublethally irradiated nondiabetic older NOD mice with PDLN removed. Importantly, all the mice (100%) resulted in diabetes onset by 4 weeks post adoptive transfer, whereas none (0%) of age-matched PDLN-ablated NOD controls lacking cell transfer developed diabetes (Figure 6D). As shown earlier, transfer of cells from BDC2.5 mice into younger (4-week-old) NOD mice failed to induce IDDM even when priming occurs unhindered, largely due to the nonappearance of TLO. Collectively, our data suggest that TLO may effectively and sufficiently replace the function of secondary lymphoid tissues for the eventual development of IDDM.

Discussion

The development of spontaneous IDDM is often anticipated by the de novo formation of TLO. The contribution of these structures to disease had been unclear. This study suggests a critical role for these structures in amplifying the adaptive response in situations where effective priming of T cells is hindered because of the paucity

of antigens reaching the secondary lymphoid organs. This phenomenon may be attributed to the complexity of the tissue stroma, which can prevent migration of some antigens to the draining LN (Spiotto et al., 2002), or a failure of indirect presentation upon reaching the LN (Spiotto et al., 2004). Direct recruitment of naive T cells into the lesion permits maximum exposure to highly concentrated and diversified antigens and enhances the activation of more responsive T cells. Therefore, the role of draining LN is diminished as the lesion becomes chronic and TLO increasingly play a role in recruiting and activating T cells, leading to final destruction of tissue.

Membrane LT was initially found to be essential for the development of secondary lymphoid tissues (Browning et al., 2005; Fu and Chaplin, 1999; Mackay and Browning, 1998; Mackay et al., 1997; Ngo et al., 1999; Wu et al., 1999). The use of LTβR-Ig fusion protein has been shown to alleviate various autoimmune diseases (Fava et al., 2003; Gommerman et al., 2003; Mackay et al., 1998; Wu et al., 2001). It has been assumed, but not proven, that LT is the essential ligand for the development of TLO. However, a recent study indicates that chronic infection can trigger the formation of TLO in the absence of LT, raising the possibility that other molecules may replace the role of LT in some conditions (Moyron-Quiroz et al., 2004). Our study suggests that LIGHT may promote the formation of TLO independently of LT. Not only is LIGHT expression prominent in the TLO of aged NOD mice, but blockade of LIGHT by HVEM is effective in reversing insulitis. However, it remains to be determined whether blocking via fusion proteins may function on multiple levels: not only at the local ectopic structure, but also by the disruption of other cellular components involved in disease induction. Consistently, LIGHT rescues disrupted secondary lymphoid structures caused by the absence of LT (Wang et al., 2002), and overexpression of LIGHT at local sites stimulates the formation of TLO leading to the development of IDDM. Our LIGHT tg mice illustrate the efficiency by which an upregulated LIGHT expression in the peripheral tissue can contribute not only to the lymphoid structure but also to the recruitment of naive lymphocytes in the target tissue. It would be interesting to observe whether LIGHT-LTβR signaling plays a similar role in other T cell-mediated autoimmune diseases.

The importance of the secondary lymphoid organs in the initiation and maintenance of autoimmune disease, in particular IDDM, has been suggested by recent studies. Priming of autoreactive T cells in pancreatic draining LNs occurs early in the disease course (Gagnerault et al., 2002; Hoglund et al., 1999; Levisetti et al., 2004). However, removal of the draining LN during the effector phase of the disease fails to inhibit diabetes progression (Gagnerault et al., 2002). Because disease progress rapidly becomes independent of these draining LNs, it is unclear how the autoreactive response becomes self-sustained. Our study shows that in the case of IDDM, a TLO mediated by LIGHT-LTβR signaling develops in the pancreas as the NOD mice age, and this ectopic structure may replace the function of the draining LN for local activation of immune cells. Consistent with data shown by others, we found that resection of PDLN prevented diabetes in NOD mice only if performed prior to 3 weeks of age. Furthermore, the transfer of naive transgenic cells was able to provoke disease in aged nondiabetic PDLN-ablated NOD mice. This suggests that the presence of TLO in the pancreas is sufficient for the recruitment of naive cells and their initial priming, even in the absence of DLN.

Although TLO via LTβR signaling can supersede the function of DLNs after initial priming, cell recruitment alone is not sufficient for autoimmune disease. Earlier studies with RIP-TNFα, RIP-LT Tg, or RIP-SLC and BLC mice in B6 models show clear formation of such structure as well as massive cellular infiltrates, but no tissue destruction (Drayton et al., 2002; Luther et al., 2000; Picarella et al., 1992, 1993). It is likely that expanded autoimmune repertoires as a result of defective central tolerance or enhanced local costimulation may be required. For example, RIP-TNFα x RIP-B7 double transgenic mice lead to IDDM whereas neither single-transgenic mice does (Guerder et al., 1994). The profound exacerbated phenotype of our RIP-LIGHT-NOD mice may result from the dual function of LIGHT in cell recruitment via LTβR signaling and T cell costimulation via HVEM, on an autoimmune prone background.

Our study demonstrates that subsequent to the early initial priming in the PDLN, activated autoreactive cells expressing LIGHT migrate to the peri-insular site to create a TLO. By the expression of chemokines and adhesion molecules downstream of LTβR and costimulation via HVEM, naive T cells are recruited and activated. The LIGHT expression that results from further T cell activation in situ forms a positive loop that becomes self-sustaining and chronic. Such TLOs sufficiently and necessarily induce IDDM and can replace the draining LN by directly recruiting and activating naive diversified T cells into the targeted tissue, ultimately leading to the rapid development of IDDM in the background of autoimmune-prone mice. This important positive feedback loop is necessary for the induction of the chronic disease as the absence of the tertiary structure, as such in young NOD mice, failed to develop the disease even with priming unhindered in the DLN and large number of autoreactive T cells. It is only with the transfer of naive tg cells into old NOD mice manifesting TLO that priming took place in situ, thus making disease induction possible. Resolution of severe insulitis by LTβR-Ig treatment suggests that the migration of inflammatory cells into the islets is a dynamic process. Interference with this dynamic process may reduce inflammation, preventing further activation of autoreactive T cells and tissue damage.

Experimental Procedures

Mice

NOD and BDC2.5 mice were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were maintained under specific pathogen-free conditions. Animal care and use were in accordance with institutional and National Institutes of Health (NIH) guidelines. NOD mice used in this manuscript were female unless otherwise stated. BDC2.5 mice were 6–10 weeks old. All animals were maintained and experiments were performed according to guidelines set forth by the institutional animal care and use committee (IACUC) at the University of Chicago.

Generation of RIP-LIGHT-NOD Tg Mice

A PCR fragment encoding the full-length murine LIGHT was generated with pcDNA-LIGHT (generated in our lab) as the template and

inserted into the RIP transgenic vector at ClaI site, which contains the rat insulin II promoter (RIPII), polyadenylation site, and the first intron of the rat insulin gene (a gift from Dr. Doug Hanahan, UCSF). The RIP-LIGHT-NOD Tg vector was cut by NotI and SalI, and NotI/SalI fragment was purified and used for microinjection. Microinjection of the transgene into NOD mice was performed in the transgenic facility at Jackson Laboratory. The primers used for subcloning of LIGHT into RIP transgenic vector were RIP-LIGHT-NOD1: 5′-TATA TCGATATGGAGAGTGTGGTACAGCC-3′; RIP-LIGHT-NOD2: 5′-TAT ATCGATTCAGACCATGAAAGCTCCGA-3′. Tg founder mice were genotyped by Southern blot as described previously (Wang et al., 2001). The genotyping primers are RIP-forward: 5′-CAACCCTGACT ATCTTCCAGG-3′; RIP-LIGHT-NOD2: 5′-TATATCGATTCAGACCAT GAAAGCTCCGA-3′.

Reagents

The generation and production of recombinant LTβR-human Ig and HVEM-Ig has been previously described (Browning et al., 1997; Wu et al., 1999). Human Ig was obtained from Biogen (Boston, MA) or Sigma (St. Louis, MO). Antibodies to MAdCAM-1 (MECA367), CD3, Thy1.2, and B220 were purchased from Pharmingen (San Diego, CA), and anti-insulin was from Dako Corp. (Carpinteria, CA). Anti-LTβ antibody is a gift from Jeff Browning (Biogen idec, Boston, MA).

LTβR-Ig, HVEM-Ig, Anti-LTβ Treatment and Measurement of Blood Glucose

NOD mice were given 100 μg of fusion protein or antibody once a week for 3 weeks or as indicated. Blood glucose concentration was measured with SureStep strips (Johnson-Johnson, Milpitas, CA). Animals were considered diabetic after two consecutive measurements of $\geq$250 mg/dl of glucose.

Adoptive Transfer

Recipient NOD mice (6–7) were irradiated with 6 Gy (172 rad/min) with a ^{137}Cs irradiator. 2 hr later, the mice were injected intravenously with 2×10^7 splenocytes from diabetic NOD mice in 0.2 ml PBS followed by i.p. injection with 100 μg of LTβR-Ig, anti-MAdCAM-1, or control human Ig. For BDC2.5 transfer experiments, recipient NOD mice (varying ages depending in experiment) were irradiated 6 Gy as mentioned above. The following day, mice were transferred with CFSE labeled 6- to 10-week-old BDC2.5 cells (2×10^7 splenocytes) intravenously. Blood glucose was monitored weekly.

Histology and Immunohistochemistry

10-week-old NOD mice were treated as above. Pancreatic tissue was collected 1 week after the last treatment and fixed in 10% buffered formalin for hemotoxylin and eosin staining and scoring as previously described (Wu et al., 2001), or the pancreata and spleen were frozen at −70°C in OCT compound for immunohistochemical staining with CD3 (L363-29B), MAdCAM-1 (MECA367), anti-insulin (Dako Corp.), B220, or species- and isotype-specific nonreactive control mAbs, followed by biotinylated rabbit anti-rat or goat anti-hamster secondary antibody (Vector Laboratories, Burlingame, CA) and then with streptavidin-HRP complex (Vector Laboratories). For T and B cell zone staining, pancreas sections were incubated with Thy1.2-biotin and B220-FITC followed by streptavidin-AP (Vector Laboratories) and anti-FITC-HRP (Dako Corp.). Color was developed by Vector blue kit (for AP) or Vector DAB kit (for HRP). BrdU, immunohistochemical staining was performed under manufacturer's instructions (Pharmingen). Mice were injected with 100 μg of BrdU via i.p. once, and then after 24 hr pancreas tissue was harvested.

Adoptive Transfer of CFSE-Labeled Cells and Immunofluorescence Staining

BDC2.5 splenocytes were resuspended in PBS at a concentration of 1.8×10^7 cells/ml and incubated with 10 μM of CFSE (Molecular Probes Inc., Eugene, OR) for 30 min at 37°C and then quenched with equal volume of FCS and washed three times with PBS. Cells were i.v. injected at 20×10^6 cells/mouse. At various time points (48–96 hr post transfer depending on experiment or as indicated), spleen, mesenteric LN, pancreatic draining LN, and islets were isolated for analysis of CFSE dilution on $V\beta4^+CD4^+$ gated population by flow cytometry. To visualize CFSE-labeled cells in situ, pancreatic tissues were frozen at −70°C in OCT. Cryostat sections 6 μm in thickness were incubated overnight with guinea pig anti-insulin (Dako Corp.), then were incubated for 1 hr with biotinylated goat anti-guinea pig (Vector Laboratories) followed by a 1 hr incubation with streptavidin-PE-Cy5.

Pancreatic LN Ablation

Two of the pancreatic LNs were removed under a dissection microscope as previously described (Gagnerault et al., 2002).

Islet Isolation

Pancreata were perfused in situ through the common bile duct with collagenase P (0.375 mg/ml; Roche Corp., Basel, Switzerland) and incubated at 37°C for 10 min. Islets were released from the pancreata by gentle shaking. After being washed twice with Hank's balanced salt solution (HBSS), islets were further purified on discontinuous Ficoll gradients. After centrifugation, the islets were harvested from the 1.096/1.069 gradient interface, washed twice in HBSS, and collected under the microscope.

Real-Time RT-PCR

Pancreatic RNA was purified with TRIzol (Invitrogen Life Technologies) and RNA easy kit (Qiagen) according to the instructions provided by the manufacturers. cDNA was synthesized and subject to real-time PCR as described previously (Anders et al., 2005; Lo et al., 2003). Each cDNA sample was amplified in duplex for LIGHT or SLC and GAPDH with the *Taq*Man Universal PCR master mixture according to the manufacturer's instructions (PE Applied Biosystems, Foster City, CA).

Supplemental Data

One Supplemental Figure can be found with this article online at http://www.immunity.com/cgi/content/full/25/3/499/DC1/.

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