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B1 Cells Promote Pancreas Infiltration by Autoreactive T Cells

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Abstract

The entry of autoreactive T cells into the pancreas is a critical checkpoint in the development of autoimmune diabetes. In this study, we identify a role for B1 cells in this process using the DO11 × RIP-mOVA mouse model. In transgenic mice with islet-specific T cells, but no B cells, T cells are primed in the pancreatic lymph node but fail to enter the pancreas. Reconstitution of the B1 cell population by adoptive transfer permits extensive T cell pancreas infiltration. Reconstituted B1 cells traffic to the pancreas and modify expression of adhesion molecules on pancreatic vasculature, notably VCAM-1. Despite substantial pancreas infiltration, islet destruction is minimal unless regulatory T cells are depleted. These data identify a role for B1 cells in permitting circulating islet-specific T cells to access their Ag-bearing tissue and emphasize the existence of multiple checkpoints to regulate autoimmune disease.

It is well established that the existence of self-reactive T cells in the periphery is insufficient to cause autoimmune disease (1). In the case of autoimmune diabetes, two clear checkpoints limit disease onset (2). First, lymphocytes with the relevant specificity must be primed and recruited into the pancreas. Second, the infiltrate must convert from an “innocuous” to an “aggressive” state, triggering β cell destruction and overt diabetes. Several mechanisms are thought to influence the latter checkpoint, including the CTLA-4 pathway (3, 4) and the actions of regulatory T cells (Tregs) (5). However, much less is known about the factors that permit initial tissue entry by autoreactive lymphocytes. Proliferation of autoreactive T cells in the pancreatic lymph node (PanLN) is known to precede pancreas infiltration (6), but it remains unclear whether such priming is sufficient to permit subsequent pancreas entry. Notably, recent data suggest that even preactivated T cells do not constitutively gain entry to nonlymphoid sites and indicate a requirement for local tissue conditioning (7).

Although autoimmune diabetes is a T cell-driven disease, studies using the NOD mouse (8, 9), the BioBreeding rat (10), and the DO11 × RIP-mOVA mouse (11) have shown that B cells, as well as T cells, participate in pancreatic inflammation. Recent data suggest B cells also infiltrate the pancreas in humans with type 1 diabetes (12). Although the requirement for B cells in diabetes is not absolute in mouse (13) or man (14), a large body of evidence suggests an important role for B cells in promoting disease. In particular, pancreas infiltration in B cell-deficient NOD mice has been reported to be virtually absent (15) or significantly suppressed (16). For example, in one study, ~6% of islets were infiltrated in

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10- to 12-wk-old B cell-deficient NOD mice, compared with 61% infiltration in age-matched B cell-replete animals (17). Moreover, B cell depletion using anti-IgM has been shown to completely abrogate insulitis in NOD animals (18). Despite clear indications of the importance of B cells, their mechanism of action is still the subject of debate. Islet autoantibodies predict disease onset in both mouse and human (19) but are not thought to be pathogenic in themselves. The elegant demonstration that B cells that are unable to secrete Ab retain the capacity to promote diabetes (20) supports the idea that the role of B cells extends beyond the provision of circulating Ab. In this regard, several studies suggest B cells play an Ag-presenting role in diabetes (17, 21, 22) or support T cell survival within islets (23). New evidence that B cell depletion may be beneficial in humans with type 1 diabetes (24) as well as mice (25, 26) highlights the potential of this subset as a therapeutic target.

Our understanding of the role of B cells in diabetes to date is based on strategies that ablate both conventional B2 cells and the less prevalent B1 cell subset. Thus, the relative contribution of each subset to disease is not clear. In the current study, we have used the DO11 × RIP-mOVA mouse model to test the role of B1 cells in diabetes induction. Mice bearing transgenic T cells (DO11.10) specific for a pancreas-expressed protein (OVA) develop spontaneous diabetes. The relatively synchronous disease onset in this model has allowed us to show that B1 cells are present in the pancreas before T cells during the initiation of insulitis. In B cell-deficient transgenic animals, islet-specific T cells fail to enter the pancreas, and this can be reversed by restoring the B1 population by adoptive transfer. These data therefore reveal an unexpected role for B1 cells in policing entry of autoreactive T cells into the pancreas.

Materials and Methods

Mice

DO11.10 TCR transgenic and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). RAG2$^{-/-}$ mice were purchased from Taconic Farms (Germantown, NY). Rat insulin promoter (RIP)-mOVA mice on a BALB/c background expressing a membrane-bound form of OVA under the control of the RIP (from line 296-1B) were a gift from W. Heath (Walter and Eliza Hall Institute, Melbourne, Victoria, Australia). Mice were housed at the Birmingham Biomedical Services Unit (Birmingham, U.K.) and used according to institutional guidelines. Blood glucose was measured using an Ascensia Elite XL blood glucose meter (Bayer, Pittsburgh, PA). Mice were considered diabetic following two consecutive readings of >250 mg/dl.

Flow cytometry

Lymphocytes from the pancreas were obtained as described previously (11). Cells were stained with Abs against FoxP3 (FJK-16s; eBioscience, San Diego, CA), CD4-PerCP (RM4-5), CTLA-4-PE (UC10-4F10-11), CD69-PE (H1.2F3), CD19-allophycocyanin (1D3), CD5-biot/FITC/PE (53-7.3), IgM^a-FITC (DS-1), IgD^a-biot (AMS-9.1), B220-PerCP (RA3-6B2), CD11b-Pacific blue (M1/70; eBioscience), B220 PerCP, CD23-FITC, CD21/35-PE, CD8a-FITC (53-6.7), α_4 integrin (R1-2), $\alpha_4\beta_7$ (DATK32), pan (Ha2/5), and activated (9EG7) β_1 integrin. All Abs were purchased from BD Pharmingen (San Diego, CA) unless otherwise indicated. For intracellular FoxP3 staining, cells were fixed and permeabilized, according to the manufacturer's instructions (eBioscience). Seven-color analysis was performed using a Dako Cyan-ADP (DakoCytomation, Fort Collins, CO).

Adoptive transfer

Peritoneal cells (5×10^6 cells/mouse) or sorted cells were injected i.p. B1a and $CD8^+$ T cells were purified by high-speed cell sorting (MoFlo; DakoCytomation) by selecting $CD19^{hi}CD5^+CD11b^+$ cells from the peritoneal lavage ($2–4 \times 10^6$ cells injected/mouse) or $CD8^+$ cells from the spleen of BALB/c mice (5×10^6 cells injected/mouse). B2 cells (5×10^6 cells injected/mouse) were purified from BALB/c LNs (to reduce contamination with B1 cells, which are absent from LNs) by cell sorting ($CD19^+CD11b^-$ IgD^{hi}) or MACS with equivalent results (latter shown). Sorted populations were routinely >97% pure. Serum from diabetic DO11 × RIP-mOVA mice was injected i.v. at 200 μl/mouse, twice per week for 6 wk. Blocking Ab or control Ab was injected at 100 μg/mouse twice per week i.p. for 6 wk. Where indicated, mice received two 0.5-mg doses of anti-CD25 (PC61) i.p. 1 wk apart. Where indicated, mice were injected i.p. with 200 μg/kg anti–CD22-calicheamicin or control conjugate (anti-rat VLA-4 calicheamicin) twice per week (days 0 and 3) starting at 2 wk of age.

RT-PCR

B cells were added to a monolayer of endothelial cells (mCEC cell line) for 6 h. mRNA was extracted from endothelial cells and subjected to quantitative PCR (SensiMix No-Ref SYBR Green kit; Quantace, London, U.K.). Where indicated, a neutralizing anti–TNF-α Ab (500-P64; PeproTech, Rocky Hill, NJ) was added to a concentration of 10 mg/ml, or rTNF-α (315-01A; PeproTech) was added to a concentration of 25 ng/ml.

Tissue sections

Seven-micrometer frozen sections were acetone-fixed and stained with rabbit anti-insulin (Santa Cruz Biotechnology, Santa Cruz, CA), KJ-126-biot (Caltag Laboratories, Burlingame, CA), goat anti-rabbit IgG-HRP, and streptavidin-alkaline phosphatase. For confocal microscopy (LSM 510; Zeiss, Oberkochen, Germany), primary Abs used were purified or Alexa 647-conjugated (Invitrogen, Paisley, U.K.) anti-CD4, anti–IgM-Rhodamine Red (Jackson ImmunoResearch Laboratories, West Grove, PA), rat anti–VCAM-1, rabbit anti-insulin, hamster anti-CD11c, and anti–Foxp3-FITC. Rat Abs were detected using tetramethylrhodamine isothiocyanate-conjugated goat anti-rat IgG (Southern Biotechnology Associates, Birmingham, AL). Rabbit Abs were detected with Alexa 488- or 7-amino-4-methylcoumarin-3-acetic acid–conjugated Donkey anti-rabbit IgG (Invitrogen). FITC conjugates were detected with rabbit anti-FITC, followed by donkey anti-rabbit Alexa 488. Hamster anti-CD3 was detected with biotin-conjugated goat anti-hamster IgG followed by Cy-5–conjugated streptavidin (Jackson ImmunoResearch Laboratories).

Results

Lack of pancreas infiltration and diabetes in the absence of B cells

To study autoimmune tissue infiltration, we have developed an animal model in which peripheral CD4 T cells recognize an Ag that is expressed under the control of the RIP in the pancreatic islets. The model is based on the coexpression of the OVA-specific TCR transgene, DO11.10, and the RIP-mOVA transgene. We have previously reported that mice bearing these two transgenes (DO11 × RIP-mOVA mice) develop spontaneous diabetes with 100% penetrance (11). In this study, we show that *rag* gene deficiency abrogates disease in DO11 × RIP-mOVA mice (Fig. 1A). In many mouse models of autoimmunity, *rag* deficiency exacerbates disease because it prevents the development of Tregs bearing endogenous TCR α-chains (27, 28). Our system differs from those models in that the development of Tregs is driven by the DO11 TCR recognizing thymus-expressed Ag (OVA) so that endogenous TCR α-chain rearrangement is not required. Thus, *rag* deficiency does

not interrupt Treg development in our mice. Consistent with this, we have previously shown that both conventional and Tregs continue to be produced in DO11 × RIP-mOVA mice following breeding to a *rag*$^{-/-}$ background (29). Because effector T cells and Tregs are encoded by a transgenic TCR, the major effect of *rag* deficiency in this strain is to abrogate B cell development. To test whether B cell deficiency could account for the lack of diabetes in DO11 × RIP-mOVA mice rendered *rag* deficient, we used a toxin-conjugated CD22 Ab. DO11 × RIP-mOVA mice given twice-weekly injections of CD22-calicheamicin (30) were efficiently depleted of B cells and were prevented from developing disease (Fig. 1*B*–*E*), confirming a requirement for B cells for diabetes development in this model.

We next sought to determine at which point diabetes development was interrupted in DO11 × RIP-mOVA/rag^{-} mice. To test whether islet-specific T cells were exposed to Ag in the PanLN, we compared the expression of activation markers on T cells in the PanLN versus control LN that did not drain the pancreas (inguinal LN [IngLN]). Analysis of CD69 and CTLA-4 expression revealed higher levels on both conventional (Foxp3^{-}) and regulatory (Foxp3^{+}) T cells in the PanLNs of DO11 × RIP-mOVA/rag^{-} mice compared with their counterparts in non-Ag draining LNs (Fig. 2A). This indicated that pancreatic Ag was being presented in the draining LNs, implying that B cells were not essential for the trafficking of OVA from the pancreas to the PanLN. Consistent with this, presentation of pancreas-derived Ag has been shown to be intact in the PanLNs of *rag*-deficient NOD mice (6). We next assessed the ability of T cells to enter the pancreas. Pancreas sections from DO11 × RIP-mOVA/rag^{-} mice were almost completely devoid of infiltrating T cells (Fig. 2*B*). This contrasted starkly with mice bearing an intact *rag* gene (hence B cell sufficient) in which there was extensive infiltration (Fig. 2*B*, Supplemental Fig. 1). Collectively, these data indicated that tissue infiltration and diabetes were prevented in DO11 × RIP-mOVA/rag^{-} mice despite 1) the existence of islet-specific T cells in the periphery and 2) evidence that these were exposed to Ag in the PanLN.

B cells enter the pancreas before T cells in DO11 × RIP-mOVA mice

To further dissect the contribution of B cells to tissue infiltration, we examined B cell recruitment to the pancreas during spontaneous diabetes development. A useful feature of the DO11 × RIP-mOVA strain is that diabetes onset within the colony is relatively synchronous. This facilitated kinetic analysis of B and T cell entry to the pancreas in the early lesion. At most time points examined, pancreas-infiltrating lymphocytes in DO11 × RIP-mOVA mice comprised a roughly equal mixture of T and B cells (Fig. 3*A*). However, very early insulitis, in 3-wk-old animals, was characterized by a clear predominance of B cells (Fig. 3*A*). This suggested that B cell entry into the target organ preceded that of autoreactive T cells. We next examined the phenotype of the pancreas-infiltrating B cell population. Surprisingly, a considerable fraction of the B cells in the pancreas expressed CD5, consistent with a B1a cell lineage (Fig. 3*B*). The CD19^{hi}CD5^{+} staining profile of pancreas-infiltrating B cells closely resembled that of peritoneal B cells (Fig. 3*B*). We used multiparameter FACS analysis to show that the CD5^{+} B cells in the pancreas were phenotypically identical to those in the peritoneum, including IgM^{+}IgD$^{-/low}$B220^{low}CD11b^{+} (Fig. 3C), consistent with the B1a phenotype defined by others (31). Pancreas-resident CD19^{+}CD5^{+} cells also resembled B1a cells rather than B2 cells in their expression levels of MHC class II, CD86, and CD80 (data not shown) and did not express the CD21/CD23 profiles characteristic of follicular (FO) or marginal zone (MZ) B cells (Fig. 3D). The frequency of pancreatic B cells expressing CD5 decreased during the course of disease progression (Fig. 3*E*–*G*) implying B1 cells featured early and FO B cells were dominant at later time points (see Table I). Cells with a MZ phenotype were rare in the pancreas at all time points (Table I and data not shown). The proportion of B1 cells in the peritoneal cavity of DO11 × RIP-mOVA animals was equivalent to that found in nontransgenic controls.

Collectively, these data show that B cells with a B1a phenotype are present in the pancreas prior to extensive T cell infiltration.

Adoptive Transfer of B1 cells can trigger T cell pancreas infiltration

Because B1a cells were early participants in tissue infiltration, and T cells failed to enter the pancreas in mice lacking B cells, we hypothesized that B1a cells were required to permit T cell tissue entry. To test this idea, we reconstituted B1 cells in DO11 × RIP-mOVA/rag$^-$ mice by i.p. injection. As a source of B1 cells, we initially used BALB/c peritoneal lavage cells that comprise ~60% B1a cells. Remarkably, DO11 × RIP-mOVA/rag$^-$ mice given a single injection of peritoneal lavage cells exhibited marked pancreas infiltration 6 wk later (Fig. 4A). Not every islet was affected, but those that were showed dramatic T cell infiltration, the extent of which is readily apparent in low-power histology pictures (Fig. 4*A*, *upper panel*). Confocal microscopy revealed the presence of scattered B cells within the tightly packed sheath of infiltrating T cells (Fig. 4*A*, *lower panel*, Supplemental Fig. 2). To confirm that the cells responsible for triggering tissue infiltration were B1a cells, we injected purified BALB/c B1a cells (CD19^{hi}CD5$^+$CD11b$^+$) into DO11 × RIP-mOVA/rag$^-$ mice. Adoptive transfer of sorted B1a cells was sufficient to trigger T cell infiltration into the pancreas of DO11 × RIP-mOVA/rag$^-$ mice (Fig. 4*B*, Supplemental Fig. 3*A*). Recipients of purified B1a cells had at least as many pancreatic islets infiltrated by T cells as recipients of unseparated peritoneal lavage cells (Fig. 4*B*).

To test whether the ability to trigger pancreas infiltration was specific to B1a cells, we also injected B2 cells into DO11 × RIP-mOVA/rag$^-$ mice. In these recipients, pancreas sections remained clear of T cell infiltration (Fig. 4*B*). In an attempt to control for the fact that B2 cells do not self-renew after transfer (unlike B1a cells), we performed repeated injections of B2 cells (twice weekly for 4wk), but pancreas infiltration was not triggered in the recipient DO11 × RIP-mOVA/rag$^-$ mice (data not shown). Because DO11 × RIP-mOVA/rag$^-$ mice also lack a CD8 T cell population, we also injected them with BALB/c CD8 cells; however, this did not cause pancreas infiltration (Fig. 4B). The presence and phenotype of adoptively transferred cell populations were checked by flow cytometry at the time the mice were culled. As expected, a CD8$^+$ population was only detected in recipients of CD8 cells (Fig. 4*C*, *upper right panel*), and CD19$^+$ cells were detected only in recipients of B1a and B2 cells (Fig. 4*C*, *upper left panel*). Injected B1a and B2 cells maintained their signature CD5 profile (Fig. 4*C*, *lower panels*). Thus, B2 cells and CD8 cells survived after adoptive transfer but were unable to cause pancreas infiltration. Repeated injection of serum from B cell-sufficient DO11 × RIP-mOVA mice was also ineffective at triggering T cell pancreas infiltration (Fig. 4*B*). These findings are consistent with the work of Serreze et al. (21) who showed that reconstitution of B cell-deficient NOD mice with Ig from diabetic animals failed to cause insulitis or diabetes. This contrasts with the situation in Sjogren's syndrome or the K/BxN arthritis model where pathology can be readily invoked by a single injection of IgG (32) or 100 μl serum (33) from diseased animals. Collectively, these data show that reconstitution of DO11 × RIP-mOVA/rag$^-$ mice with B1a cells is sufficient to trigger entry of islet-reactive T cells into the pancreas. Thus, the lack of T cell pancreas infiltration can be attributed to a lack of B cells, rather than to alternative effects of *rag* deficiency (such as preventing the development of T cells with two TCRs).

B1 cells promote expression of vascular addressins in the pancreas; key role for VCAM-1

The lack of B cells in DO11 × RIP-mOVA/rag$^-$ mice did not abrogate the presentation of pancreas-derived Ag in the PanLN. Indeed, DO11 T cells in the PanLN, but not the IngLN, expressed activation markers indicating exposure to OVA (Fig. 2*A*), and proliferation of DO11 cells in response to pancreas-expressed OVA did not require B cells (Supplemental Fig. 4). This is consistent with the identification of the CD11c$^+$CD11b$^+$CD8$^-$ dendritic cell

as the cell responsible for mediating presentation of pancreas-derived Ag (34). Because B1 cells could be found within the pancreas, we postulated that they might play a role at this site, perhaps by acting on pancreatic vasculature. We therefore examined expression of vascular addressins in pancreas sections from DO11 × RIP-mOVA/rag$^-$ with or without B1 cells. We noted a marked upregulation of both VCAM-1 and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on pancreatic vessels in B1 cell-reconstituted animals (Fig. 5*A* and data not shown). To test whether B1 cells can act directly on endothelium, we incubated a mouse endothelial cell line with B1 or B2 cells for 6 h. B1 cells (but not B2 cells) triggered induction of mRNA for both VCAM-1 and MAdCAM-1 (Fig. 5*B*). TNF-α is known to be capable of inducing vascular addressins in endothelial cells, and expression of TNF-α under the rat insulin promoter leads to induction of VCAM-1 in pancreatic islet endothelium (35). Blockade of TNF-α in our coculture system abrogated the ability of B1 cells to induce VCAM-1 and MAdCAM-1 in endothelial cells. This suggests TNF-α is one factor provided by B1 cells to promote addressin induction; however, there are likely to be additional signals involved because provision of TNF-α alone was insufficient to induce MAdCAM-1 in the time frame of our assay. We measured expression of the partner integrins for these addressins on pancreas-infiltrating T cells in DO11 × RIP-mOVA mice (Fig. 5C). The major binding partner for VCAM-1 is $\alpha_4\beta_1$ (VLA-4) whereas that for MAdCAM-1 is $\alpha_4\beta_7$ (which can also bind VCAM-1). Pancreas-infiltrating T cells expressed α_4, but relatively few stained with an Ab recognizing a combinatorial epitope found in the $\alpha_4\beta_7$ heterodimer. Strikingly, all the T cells that had entered the pancreas expressed high levels of β_1 integrin, whereas those in the IngLNs showed no or low staining (Fig. 5*C*). Further staining confirmed the presence of β_1 integrin in its activated conformation. This finding is compatible with the demonstration that T cells responding to islet Ag in the PanLN upregulate β_1 integrin (36). To examine the relative importance of MAdCAM-1 and VCAM-1 in permitting T cells to enter the pancreas after B1 cell reconstitution, we used blocking Abs. Although MAdCAM-1 blockade had a relatively small effect on B1 cell-induced T cell pancreas infiltration, mice receiving anti–VCAM-1 Abs showed markedly reduced infiltration (Fig. 5*D*, Supplemental Fig. 3*B*). Taken together, these data suggest that the ability of B1 cells to upregulate VCAM-1 in the pancreas is likely to be a critical step in permitting T cell pancreas infiltration.

Treg depletion triggers islet destruction

We have shown that DO11 × RIP-mOVA/rag$^-$ mice have islet-specific T cells that fail to enter the pancreas and that insulitis can be triggered in these animals by injection of B1 cells. However, it was notable that despite extensive T cell infiltration in the pancreas, none of the B1-reconstituted animals developed overt diabetes. This was surprising given the sheer number of self-reactive T cells localized in the pancreas of these recipients. Sequential transfer of B2 cells following initial B1 cell reconstitution also failed to induce overt diabetes (data not shown). Because OVA-specific Tregs also develop in these animals, we hypothesized that these might infiltrate the pancreas along with the conventional T cells and regulate their pathogenicity. Indeed, Foxp3$^+$ cells could be identified among the pancreas-infiltrating lymphocytes in DO11 × RIP-mOVA mice reconstituted with peritoneal B cells (Fig. 6*A*). To test whether Tregs were acting to prevent the autoreactive T cells from instigating damage, we used anti-CD25 to deplete this subset. Pancreas sections from treated animals showed effective depletion of Foxp3$^+$ cells (Fig. 6*A*). The combination of peritoneal B cell reconstitution and Treg depletion resulted in aggressive pancreas lesions with marked loss of insulin staining. Blood glucose levels in these animals were significantly higher than in mice that received either treatment alone (Fig. 6*B*). Collectively, these data support a model in which B1 cells promote T cell pancreas entry, but loss of Treg suppression is necessary for tissue destruction to ensue.

Discussion

Pinpointing the factors that dictate whether autoreactive T cells gain access to their target tissue has major implications for our understanding of autoimmunity. Our study has identified a role for B1 cells in this process. The starting point for our investigation was the finding that cells with a B1a phenotype could be identified within the pancreas prior to extensive lymphocyte infiltration in mice developing diabetes. In mice lacking B cells, islet-specific T cells largely failed to enter the pancreas, despite their ready availability and evidence that they had been exposed to Ag in the PanLN. Provision of a single injection of B1a cells was able to reverse this phenotype and trigger extensive T cell pancreas infiltration. These data place B1 a cells as pivotal players in controlling the first checkpoint in the development of pancreas-specific autoimmunity.

Although B1 cell reconstitution provoked extensive insulitis in DO11 × RIP-mOVA/rag^{-} mice, this was not sufficient to cause overt diabetes. Despite the presence of numerous infiltrating T cells following B1 cell reconstitution, there appeared to be limited islet cell destruction. Protection from diabetes was associated with the entry of Tregs, as well as effector cells, into the pancreas following B1 cell injection. The synergistic effect of B1 cell reconstitution and Treg depletion in our model suggests that these cell types act at different stages during the initiation of autoimmunity. Thus, B1 cells promote T cell pancreas entry, but infiltrating lymphocytes remain subject to the control of Tregs. Such data are consistent with the previous demonstration that Tregs in BDC2.5/NOD mice function to keep the intraislet infiltrate in a nondestructive state (5). B cell depletion has previously been associated with an increase in the proportion of CD25^{+}Foxp3^{+} cells within the CD4^{+} population (25, 26). In our study, we did not detect a significant change in the proportion of cells with this phenotype after B cell depletion within the time frame of our analysis (Fig. 1*E*). Nevertheless, our Treg depletion experiment argues for a critical role for Tregs in the context of B cell deficiency.

The most well established animal model of autoimmune diabetes is the NOD mouse. Intriguingly, anti-insulin Abs in this strain have been shown to be low avidity and polyreactive, resembling the natural Ab produced by B1 cells (37). Such Abs exhibit germline-encoded V genes, lack N segments, and are characteristic of the fetal repertoire. Interruption of B1a cell development by Btk deficiency protects NOD mice from diabetes (38), although clearly the development and function of other B cell subsets are also altered in these animals. B1a cells have also been reported to participate in insulitis in NOD mice (9, 39), although this is not a universal finding (40). In one study, preferential depletion of B1a cells by hypotonic lysis altered the ability of B2 cells to enter the pancreas, and this was associated with decreased autoantibody titers and delayed disease onset (39). Although nonspecific effects of hypotonic stress cannot be ruled out, such findings nevertheless support a role for B1 cells in promoting lymphocyte recruitment to the pancreas.

The remarkable heterogeneity of B cell populations in mouse and man is gradually emerging. Indeed, analogous to subdivision within the T cell population it seems that certain types of B cell can inhibit immune responses (41-43) often via their production of IL-10 (44, 45). Conversely, B cells can clearly contribute to autoimmune pathology (15, 16, 18, 33, 46), and B cell depletion with rituximab is beneficial in numerous settings (47) including type 1 diabetes (24). At first glance, the fact that B1a cells produce IL-10 (48) appears to make them unlikely candidates for promoting (rather than regulating) autoimmunity. However, although IL-10 is widely linked with tolerant outcomes, several lines of evidence suggest this is not always the case. It has recently been shown that tolerance induced by anti-CD45RB Ab requires B cells but does not depend on IL-10; instead, IL-10 counteracts such tolerance (49). Likewise, transgenic expression of IL-10 in the pancreas of NOD mice did

not inhibit diabetes but actually accelerated it, even permitting disease to occur in the presence of a normally protective MHC haplotype (50, 51). Pancreas-expressed IL-10 could also overcome the protection conferred by C57BL/6-derived alleles at the Idd3 and Idd10 loci (52). Thus, although IL-10 is unquestionably an important immunosuppressive cytokine, it can also exhibit immunostimulatory properties under certain conditions.

It is not clear whether the role of B1 cells in promoting lymphocyte recruitment is specific to the pancreas, given its anatomical location in the peritoneum. Certainly peritoneal insults can modulate T cell responses to pancreatic Ags, and a lymphocyte trafficking route direct from the peritoneal cavity to the PanLN has been described previously (53). In contrast, the proinflammatory effects of B1 cells may prove more generally applicable. In this regard, B1 cells have previously been shown to recruit T cells during contact sensitivity reactions (54), and overproduction of B1a cells is associated with tissue infiltration and systemic autoimmunity (55, 56).

Interestingly, entry of B1a cells into the pancreas in our study did not depend on RIP-mOVA expression or on the presence of T cells (data not shown); indeed, a small number of B1a cells constitutively trafficked to the pancreas in normal mice. TLR ligation has been shown to promote B1 cell exit from the peritoneal cavity and entry to tissues (57). It is tempting to speculate that augmentation of B1 cell trafficking following infection could provide a mechanism for the observed association between environmental triggers and initiation of autoimmune disease processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

FO	follicular
IngLN	inguinal lymph node
LN	lymph node
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MZ	marginal zone
PanLN	pancreatic lymph node
RIP	rat insulin promoter
Treg	regulatory T cell

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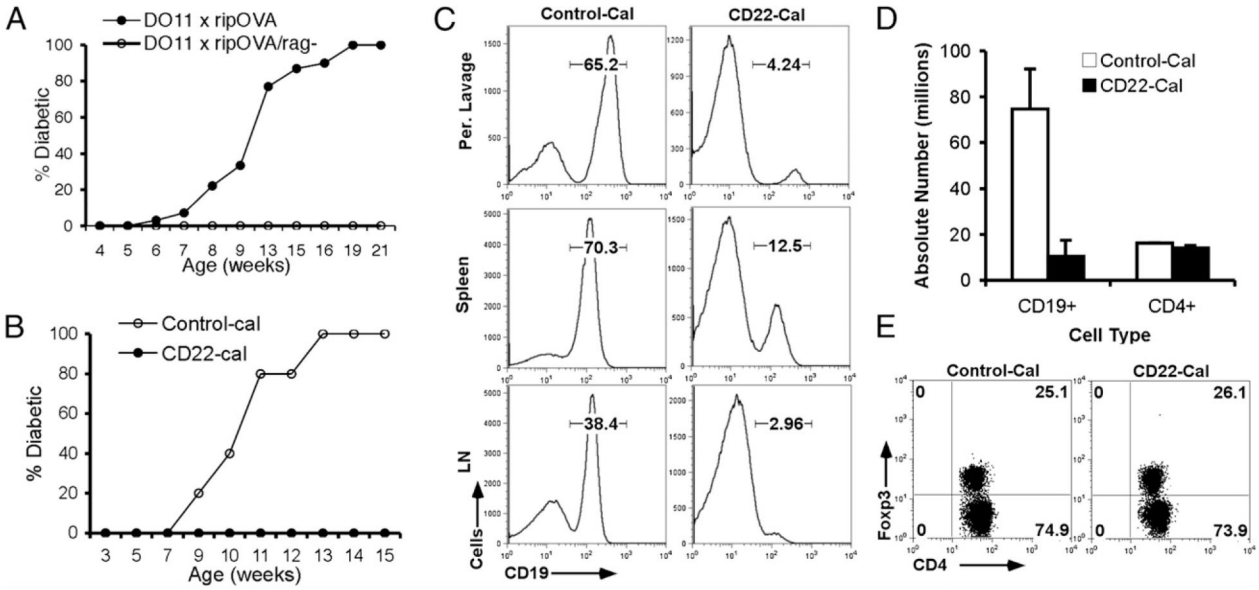

FIGURE 1. RAG deficiency or B cell depletion inhibit diabetes in DO11 × RIP-mOVA mice.

A, Blood glucose of DO11 × RIP-mOVA mice or their rag^{-} counterparts was monitored at various ages, and the percent diabetes was calculated ($n \geq 9$ for each point). *B*, Blood glucose levels of DO11 × RIP-mOVA mice injected with toxin-conjugated anti-CD22 or control conjugate twice per week from 2 wk of age. Data are combined from two separate experiments (five individual mice), and percent diabetes is shown. *C*, CD19 staining of lymphocytes isolated from the indicated tissues of 12-wk-old DO11 × RIP-mOVA mice that had been injected with control-calicheamicin or CD22-calicheamcin as above. *D*, Absolute numbers of CD19^{+} B cells, but not CD4^{+} T cells, were reduced in B cell-depleted animals. Graph shows mean cell number with SD for spleen ($n = 4$). Similar data were obtained for all sites examined. *E*, Representative Foxp3 staining on CD4 T cells isolated from the spleen of B cell-depleted or control animals.

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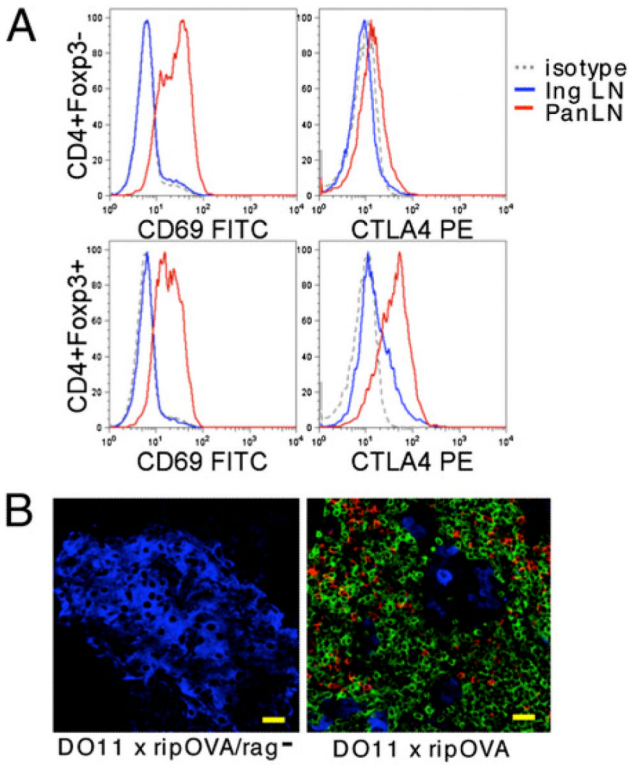

FIGURE 2. T cells respond to pancreas-derived Ag in DO11 × RIP-mOVA/rag^{-} mice but fail to infiltrate the pancreas.

A, PanLN and IngLN from DO11 × RIP-mOVA/rag^{-} mice were stained by FACS. Expression of CD69 or CTLA-4 on CD4^{+}Foxp3^{-} or CD4^{+}Foxp3^{+} T cells is shown. Histograms show pooled samples from four mice. *B*, Frozen pancreas sections from 18-wk-old rag^{+} or rag^{-} DO11 × RIP-mOVA mice were stained for insulin (blue), CD4 (green), and IgM (red). Scale bars, 20 μm; original magnification ×40. *A* and *B* are representative of three and more than six independent experiments, respectively.

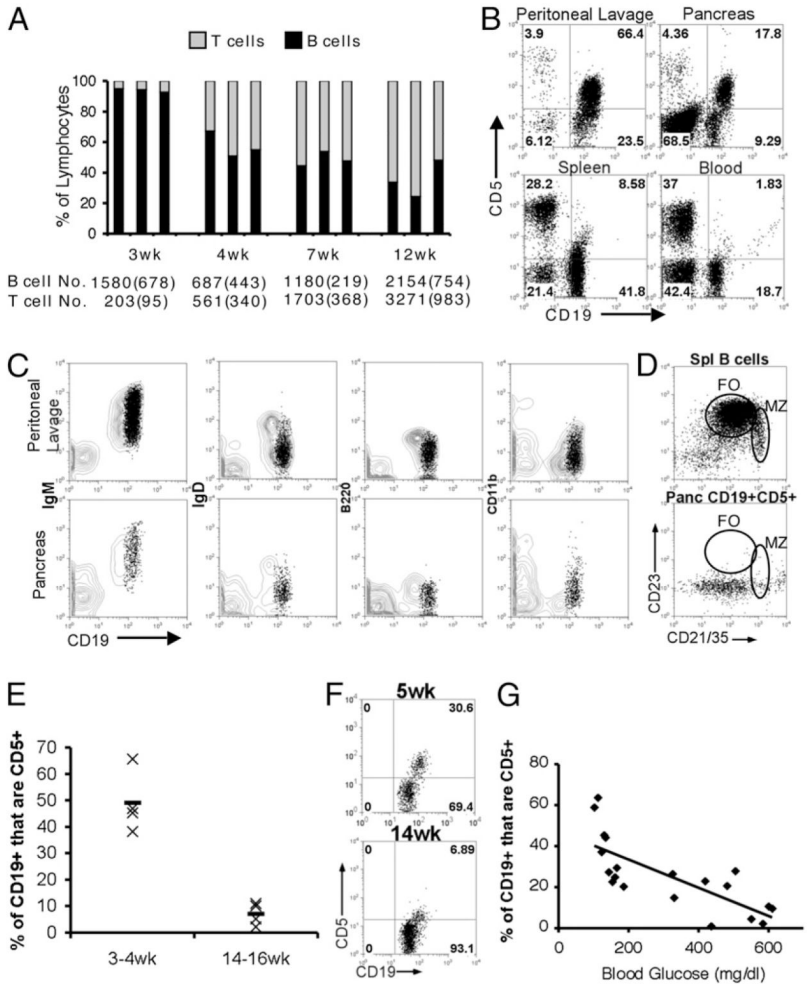

FIGURE 3. B1 cells are early participants in pancreas infiltration.

A, The relative contribution of T cells and B cells to pancreatic infiltration in DO11 × RIP-mOVA mice was monitored by FACS for CD4 and CD19. Each bar shows pooled data from three mice, and mean cell counts (with SD) are shown below. *B*, Lymphocytes isolated from the indicated tissues of 3-wk-old DO11 × RIP-mOVA mice were stained for CD19 and CD5. *C*, The phenotype of gated $CD19^+CD5^+$ cells isolated from the peritoneal cavity or the pancreas of 4-wk-old DO11 × RIP-mOVA mice was compared. Contours represent total cells within the lymphocyte gate, and the overlaid dot plot shows gated $CD19^+CD5^+$ cells. Plots show pooled data from three mice. *D*, $CD19^+CD5^+$ cells from the pancreas of 4-wk-old DO11 × RIP-mOVA mice were compared with splenic B cells for expression of CD21 and CD23. FO and MZ populations are indicated. *E* and *F*, Digested pancreas tissue from DO11 × RIP-mOVA mice of the indicated age was stained with Abs to CD19 and CD5. The percentage of $CD19^+$ cells expressing CD5 ($n = 4$) and representative FACS plots are shown. *G*, Digested pancreas tissue from DO11 × RIP-mOVA mice aged 3–16 wk was stained with Abs to CD19 and CD5. The percentage of $CD19^+$ cells expressing CD5 is plotted against the precull blood glucose reading for each animal. *A* and *C* were repeated twice, and *B* and *D* were repeated three or more times. *E* shows data from two separate experiments, and *F* shows data from more than five separate experiments.

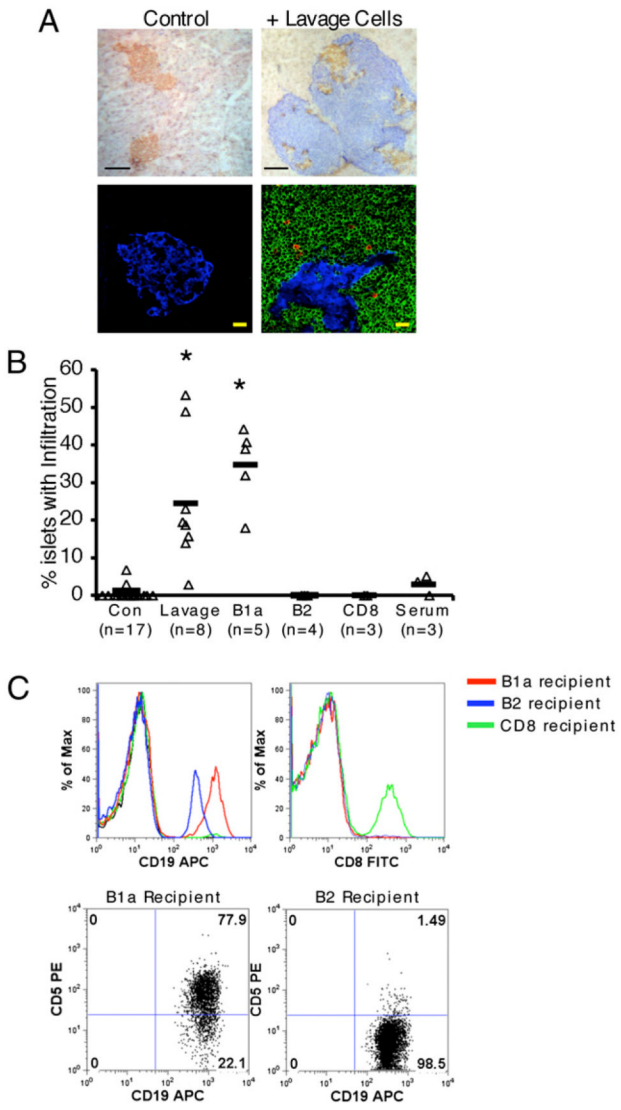

FIGURE 4. Adoptive transfer of B1 cells initiates T cell pancreas infiltration.

A, DO11 × RIP-mOVA/rag$^-$ mice were injected with PBS or 5 × 10^6 BALB/c peritoneal lavage cells. Six weeks later, pancreas sections were analyzed. *Upper panel* shows insulin (brown) and DO11 T cells (blue). Scale bars, 125 μm; original magnification ×10. *Lower panel* shows insulin (blue), CD4 (green), and IgM (red). Scale bars, 20 μm; original magnification ×40. *B*, DO11 × RIP-mOVA/rag$^-$ mice were injected i.p. with the indicated cell populations or sera from diabetic DO11 × RIP-mOVA mice, and 6 wk later, pancreas sections were scored for T cell infiltration. Each data point is one mouse (minimum 15 islets scored per mouse). Pancreas sections from recipients of peritoneal lavage or purified B1a cells were significantly more infiltrated than controls (*p< 0.01). Data are representative of more than four independent experiments. *C*, Staining of peritoneal lavage cells from DO11 × RIP-mOVA/rag$^-$ mice that had been reconstituted i.p. with B1a, B2, or CD8 cells 6 wk previously. Data are representative of more than four independent experiments.

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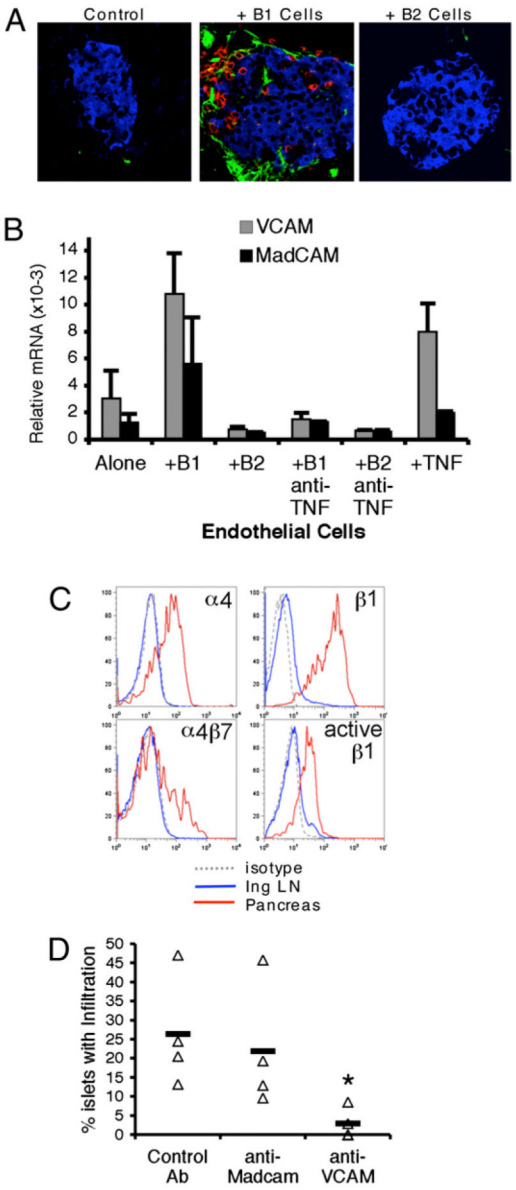

FIGURE 5. The ability of B1 cells to trigger T cell pancreas infiltration depends on VCAM-1.

A, DO11 × RIP-mOVA/rag^{-} mice were injected i.p. with 10^6 BALB/c CD19^{-} cells (control), B1 cells, or B2 cells. Seven days later, pancreas sections were stained for VCAM-1 (green), CD3 (red), and insulin (blue) (original magnification ×40). *B*, A total of 0.7×10^6 purified B1 or B2 cells were added to endothelial cell monolayers in the presence of neutralizing anti–TNF-α Ab or rTNF-α where indicated. Six hours later, endothelial cells were recovered, and mRNA levels of VCAM-1 and MAdCAM-1 were assessed ($n \geq 5$; four independent experiments). *C*, Pancreas-infiltrating and LN-resident CD4^{+} T cells from 4-wk-old DO11 × RIP-mOVA mice were stained for expression of the indicated integrins. Each plot shows pooled data from two mice and is representative of two independent experiments. *D*, DO11 × RIP-mOVA/rag^{-} mice were reconstituted with 10^6 B1 cells, and blocking Abs were injected as detailed in *Materials and Methods*. Six weeks later, T cell pancreas infiltration was scored. Each symbol shows the proportion of pancreatic islets

infiltrated in a single mouse (minimum of 15 islets scored per mouse), and data are combined from two independent experiments. *$p = 0.0212$.

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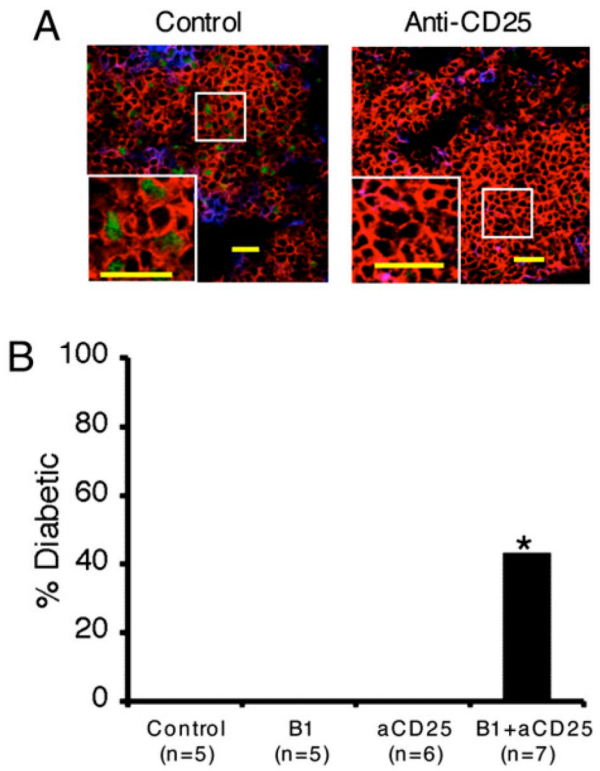

FIGURE 6. Treg depletion triggers loss of blood glucose homeostasis in B1 cell-reconstituted animals.

DO11 × RIP-mOVArag^{-} mice were injected i.p. with 3 × 10^{6} BALB/c B1 cells and anti-CD25 mAb or control mAb. *A*, Pancreas sections were stained for Foxp3 (green), CD4 (red), and CD11c (blue). Scale bars, 20 μm; original magnification ×40. *B*, Percent diabetes is shown 4 wk following the first injection.

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Table I

Proportion of B1 cells, MZ B cells, and FO B cells isolated from the pancreas of DO11 × RIP-mOVA mice at the indicated age

Weeks	B1 Cells	MZ B Cells	FO B Cells
3–4	49.03 (11.74)	0.53 (0.25)	50.44 (11.55)
14–16	7.11 (4.12)	0.42 (0.65)	92.47 (3.94)

The proportion of B1 cells ($CD5^+$), MZ B cells ($CD21^{hi}CD23^{lo}$), and FO B cells ($CD5^-$) within the $CD19^+$ pancreas-infiltrating population was measured. Mean values (with SD) are shown (n = 4 or 5). CD9 and IgM were used as additional markers to verify MZ B cell phenotype.