

# Initiation of Autoimmune Diabetes by Developmentally Regulated Presentation of Islet Cell Antigens in the Pancreatic Lymph Nodes

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## Summary

Little is known about the events triggering lymphocyte invasion of the pancreatic islets in prelude to autoimmune diabetes. For example, where islet-reactive T cells first encounter antigen has not been identified. We addressed this issue using BDC2.5 T cell receptor transgenic mice, which express a receptor recognizing a natural islet beta cell antigen. In BDC2.5 animals, activated T cells were found only in the islets and the lymph nodes draining them, and there was a close temporal correlation between lymph node T cell activation and islet infiltration. When naive BDC2.5 T cells were transferred into nontransgenic recipients, proliferating cells were observed only in pancreatic lymph nodes, and this occurred significantly before insulinitis was detectable. Surprisingly, proliferation was not seen in 10-day-old recipients. This age-dependent dichotomy was reproduced in a second transfer system based on an unrelated antigen artificially expressed on beta cells. We conclude that beta cell antigens are transported specifically to pancreatic lymph nodes, where they trigger reactive T cells to invade the islets. Systemic or extrapancreatic T cell priming, indicative of activation via molecular mimicry or superantigens, was not seen. Compromised presentation of beta cell antigens in the pancreatic lymph nodes of juvenile animals may be the root of a first “checkpoint” in diabetes progression.

Key words: autoimmunity • antigen presentation • T lymphocytes • transgenic mice • nonobese diabetic

Insulin-dependent diabetes mellitus (IDDM)<sup>1</sup> is caused by autoimmune destruction of the insulin-producing beta cells of the pancreatic islets of Langerhans (for reviews, see references 1 and 2). Much of our current knowledge about the complex pathogenesis of IDDM derives from studies on the nonobese diabetic (NOD) mouse, which succumbs to a disease closely resembling that of human patients (for a review, see reference 3). These studies have revealed an influx of inflammatory cells into the islets, termed insulinitis, starting at ~4–5 wk after birth; however, significant beta cell destruction, and consequently diabetes, is not evident until ~12 wk of age. Of the cells that constitute the insulinitic lesion, T cells appear to play a primary role. Nonethe-

less, other cell types—such as B cells, macrophages, and dendritic cells (DCs)—also contribute to disease progression.

The events responsible for triggering IDDM in a susceptible individual are clouded in uncertainty. The role of environmental factors (4–6), the nature of the initiating inflammatory cell (7, 8), and the identity of the inciting antigen(s) (9–11) have all been vigorously debated. Among the important unresolved issues is where the initial stimulation of naive, islet-reactive T cells takes place. One possibility is that it occurs within the islets, where autoantigens are presented either by the beta cells that synthesize them or by APCs in the vicinity. This scenario might seem unlikely given the body of data establishing that naive T cells circulate through peripheral lymphoid organs rather than through tissues (12), but it could be that either the pancreatic islets or islet-reactive T cells are special in this respect. Another possible site of initial stimulation is in the LNs draining the pancreas. This is an attractive option because of its analogy to what happens when foreign proteins are

<sup>1</sup>Abbreviations used in this paper: CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; IDDM, insulin-dependent diabetes mellitus; IIL, intraislet lymphocyte; ILN, inguinal lymph node; MLN, mesenteric lymph node; NOD, nonobese diabetic; OT-I, OVA-specific CD8<sup>+</sup> T cells; PLN, pancreatic lymph node; RAG, recombination activating gene; RIP, rat insulin promoter; RLN, renal lymph node; tg, transgenic.

introduced through the skin: antigen is captured by immature Langerhans cells, ferried to the draining LNs, and is presented there to recirculating naive T cells by the now-mature Langerhans cells (13). However, to date there is little experimental evidence of such a sequence of events in the progression of diabetes or, indeed, of any autoimmune disease. A third possibility is that potentially diabetogenic T cells are first stimulated systemically or at a distant site, either in a tissue or in one of the peripheral lymphoid organs. This proposed scenario reflects arguments that islet-reactive T cells are “awakened” upon activation by cross-reactive environmental antigens or microbial superantigens (5, 6).

To distinguish between these different possibilities, we took advantage of the BDC2.5 TCR transgenic (tg) model of diabetes (14). BDC2.5 tg mice carry the rearranged TCR  $\alpha$  ( $V\alpha 1$ ) and  $\beta$  ( $V\beta 4$ ) chain genes from a diabetogenic, beta cell-specific,  $CD4^+$  T cell clone isolated from a diabetic NOD mouse (15, 16). T cells expressing the transgene-encoded receptor are efficiently selected on the NOD background and contribute substantially to the peripheral T cell repertoire. Nevertheless, the autoreactive T cells appear to ignore the beta cells for the first 2 wk of life. At that time, they abruptly invade the islets and insulinitis progresses rapidly, such that at 3–4 wk of age almost all islets are heavily infiltrated. Thus, the initiation of autoimmune attack is especially clear and synchronous in BDC2.5 mice, suggesting that this model might prove highly advantageous for studying the triggering of IDDM.

Here we present results from two types of experiments—monitoring T cell activity at different sites in prediabetic BDC2.5/NOD tg mice, and tracking the behavior of naive BDC2.5 T cells adoptively transferred into nontransgenic, NOD-background hosts. The data prompt us to conclude that diabetogenic T cells are first stimulated in the LNs immediately draining the pancreas, and that antigen availability at this site is a critical determinant of disease initiation.

## Materials and Methods

**Mice.** The generation of BDC2.5/NOD TCR tg mice has been described previously (14, 17).  $C\alpha^0$ /NOD and recombination activating gene (RAG) $^0$ /NOD animals were obtained by repetitively backcrossing null mutations in either the TCR- $\alpha$  (18) or RAG-1 (19) genes onto the NOD/Lt genetic background followed by intercross to obtain homozygous mutant mice (mice used in the present experiment were at the sixth and eighth backcross generation, respectively). BDC2.5 animals were typed by flow cytometry using mAbs against CD4 and  $V\beta 4$ . The  $C\alpha$  and RAG null mutations were identified by Southern blotting or flow cytometric analysis of PBLs. These animals were maintained in the Institut de Génétique et de Biologie Moléculaire et Cellulaire animal facility in Strasbourg, under Ministère de l’Agriculture (Agrément 67227) and European Economic Community guidelines. Generation of rat insulin promoter (RIP)-mOVA mice and mice expressing a transgenic CD8-restricted OVA-specific TCR (OT-I) has also been described (20), and these lines were maintained in the Walter and Eliza Hall Institute animal facility in Melbourne.

**Isolation of Intraislet Lymphocytes.** The pancreatic LNs (PLNs) were removed, and the remaining pancreas tissue was carefully

cut into small pieces using fine scissors. 5–10 islet infiltrates (easily identified in BDC2.5 pancreata as “bubbles” surrounding normal islet tissue) were hand-picked under a dissecting microscope, and converted into a single-cell suspension for flow cytometry analysis.

**Insulinitis.** Thin sections from Bouin’s solution-fixed, paraffin-embedded pancreas specimens were stained with hematoxylin-eosin and scored for insulinitis—a minimum of 40 independent islets per animal on multiple sections.

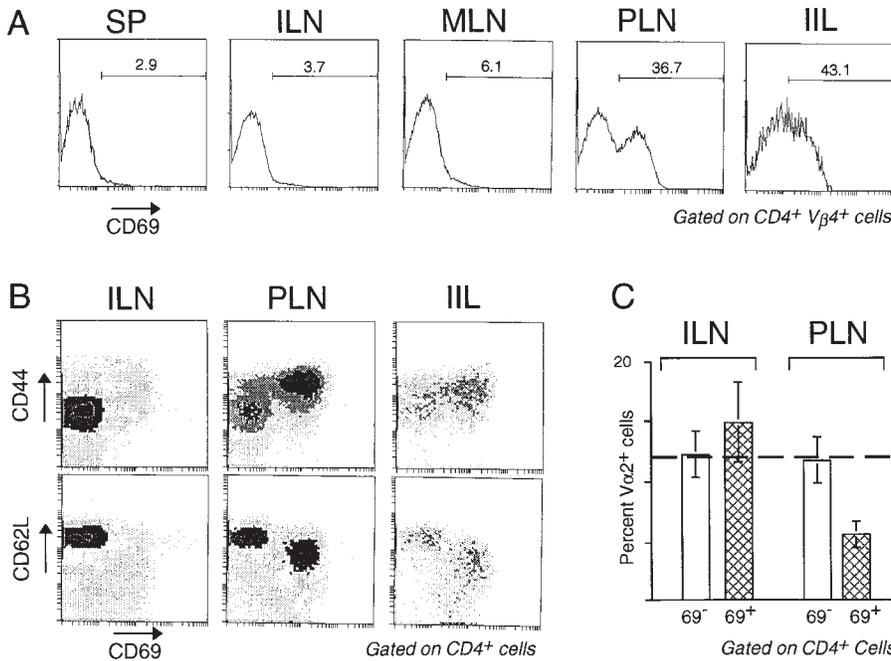
**Antibodies and Flow Cytometry.** T cell analysis was performed as described previously (21). The following mAbs were used: PE-conjugated CD4 (Caltag); biotin- or allophycocyanin-conjugated CD8 (Caltag); FITC-conjugated CD69 (PharMingen); IM7, specific for CD44 (22); Mel14, specific for CD62L (23); and B20.1, specific for  $V\alpha 2$  (24). Biotinylated Ab was revealed by aminomethylcoumarin-conjugated streptavidin and the other Abs by Texas red-conjugated goat anti-rat IgG (Jackson ImmunoResearch Labs).

**CFSE Labeling and Adoptive Transfer.** For the BDC2.5 system, single-cell suspensions from pooled spleens were depleted of erythrocytes in 0.83%  $NH_4Cl$ , adjusted to  $10^7$ /ml in PBS, and incubated for 10 min at  $37^\circ C$  in the presence of  $2.5 \mu M$  5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) prepared as a 5 mM stock solution in DMSO. After incubation, the cells were washed twice with RPMI supplemented with 5% FCS, and adjusted to  $10^8$ /ml. In each experiment,  $200 \mu l$  ( $2 \times 10^7$  cells) were injected, intravenously for adult and intraperitoneally for 10-d-old recipients. These two routes of inoculation were comparable, since homing to the PLNs followed identical kinetics (data not shown). At various times after inoculation, LNs were removed and analyzed for the presence of CFSE-labeled cells.

For the OT-I system (20, 25), single-cell suspensions from OT-I/RAG $^0$  mice were depleted of  $CD24^+$  and  $CD4^+$  cells by treatment with J11d and RL-172 mAbs (26, 27) and complement. The proportion of OT-I cells was then determined by flow cytometry, staining for CD8 and  $V\alpha 2$ .  $2 \times 10^6$  OT-I cells were injected intravenously into adult or 10-d-old RIP-mOVA mice. After 67 h, the LNs were removed and assessed for CFSE-labeled cells as described (20, 25).

## Results

**Activated BDC2.5 Tg T Cells Reside Specifically in the Pancreatic Islets and Their Draining LNs.** As a first step, we examined the activation status of T cells located at different sites in the periphery of BDC2.5 TCR tg mice (Fig. 1). Very few  $CD4^+V\beta 4^+$  cells in the spleen and most LNs, including the inguinal (ILN) and mesenteric (MLN), expressed the activation marker CD69; in contrast, a large proportion of cells in the PLNs and within the pancreatic islets (intraislet lymphocytes, IILs) had an activated phenotype (Fig. 1 A). This activation was linked to the presence of the transgene, as these large populations of  $CD69^+$  cells were not found in PLNs of nontransgenic littermates (data not shown). The activated PLN and IIL cells were similar to each other and typical of other activated T cells in their augmented expression of CD44 and diminished display of CD62L (Fig. 1 B). These findings suggest that the antigen that triggers BDC2.5 tg T cells is present specifically in the pancreas and the immediately draining LNs. This notion was supported by an analysis of TCR  $\alpha$  chain usage by T cells at the different sites: in the PLNs, cells expressing the en-



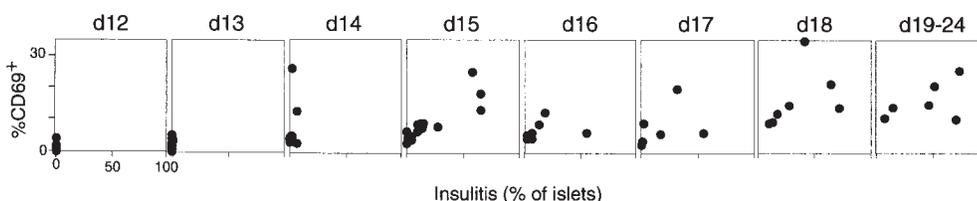
**Figure 1.** Activated lymphocytes in BDC2.5 mice. (A) CD69 expression on CD4<sup>+</sup>Vβ4<sup>+</sup> T cells from spleen (SP), ILNs, MLNs, PLNs, and IILs from a young BDC2.5 mouse. The percentage of positive cells included within the indicated region is shown for each histogram. (B) Representative biparametric CD69/CD44 and CD69/CD62L histograms of gated CD4<sup>+</sup> T cells from the same sources. (C) Underrepresentation of nontransgenic Vα2<sup>+</sup> TCRs among CD69<sup>+</sup>CD4<sup>+</sup> lymphocytes in PLNs compared with ILNs from BDC2.5 mice. Dashed line, an approximation of the percentage of Vα2<sup>+</sup> cells within CD4<sup>+</sup> T cells in the whole lymphoid system of the analyzed mouse (estimated from pooled data of spleen and LN T cells).

dogenously encoded Vα2 chain were underrepresented (and presumably those displaying the transgene-encoded chain overrepresented), whereas the opposite was true in other nodes, such as the ILNs (Fig. 1 C).

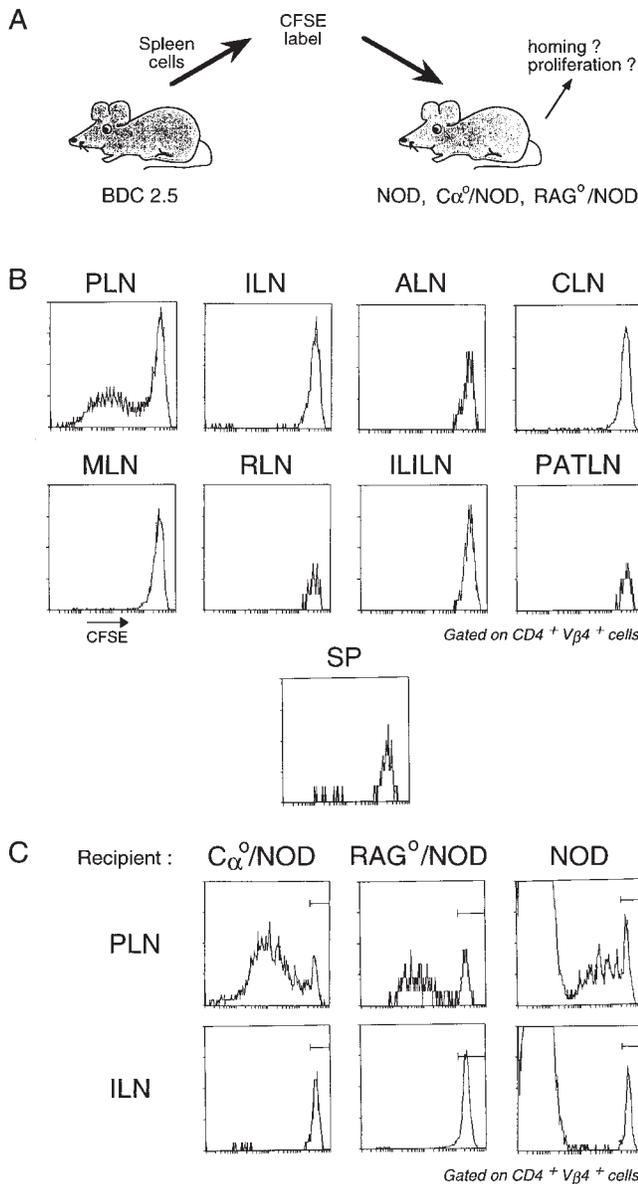
*There Is a Close Temporal Correlation between the Appearance of Activated BDC2.5 T cells in the PLNs and of Infiltrated Islets.* The presence of activated T cells in both the islets and draining LNs of BDC2.5 tg mice raised the question of whether they were first stimulated at the former site or at the latter. Priming may be quite synchronous in individual BDC2.5 animals as insulinitis begins abruptly between days 15 and 18 after birth. Therefore, we performed a careful kinetic analysis, day by day beginning on day 12, scoring the percentage of CD69<sup>+</sup> T cells in the PLNs versus the proportion of islets containing invading lymphocytes. As illustrated in Fig. 2, there was generally a good correlation between the fraction of activated PLN cells and infiltrated islets. On day 14, a few mice had CD69<sup>+</sup> cells in the PLNs in the absence of detectable insulinitis, but this was not a general observation. On the basis of these data, we concluded that activation of T cells in the PLNs and islet infiltration had a close temporal correlation, but could not establish conclusively which of the two was the primary event.

*Proliferating Cells Are Localized Specifically in the PLNs Shortly after Transfer of Naive BDC2.5 T Cells.* A possible reason for the inconclusive results in the preceding experiment is that, although it is considerably more synchronous than in standard NOD mice, the initiating event in BDC2.5 transgenics is still scattered over a window of 3 or 4 d in different individuals, rendering it difficult to precisely “capture” in a significant number of animals. Therefore, we switched to an adoptive transfer system, hoping that if the BDC2.5 T cells entered the circulation at exactly the same time in different animals, disease initiation would be highly synchronous. The transfer system is depicted in Fig. 3 A: splenocytes are removed from BDC2.5 tg mice, labeled in vitro with the cytoplasmic dye CFSE, and transferred into NOD-background recipients; some days later, different lymphoid organs are removed, and homing and proliferation are assayed by cytofluorimetric assessment of the presence and degree of dilution of CFSE staining. When CFSE-labeled cells proliferate, their fluorescence is distributed equally between the two daughter cells, resulting in a 2<sup>n</sup>-fold dilution in fluorescence intensity, where *n* represents the number of cell divisions.

Fig. 3 B presents histograms of CFSE staining in CD4<sup>+</sup>Vβ4<sup>+</sup> cells from spleen and a variety of LNs 3 d after



**Figure 2.** Temporal correlation between the activation of T cells in PLNs and islet infiltration in young BDC2.5 mice. Percentage of infiltrated islets was plotted against the percentage of CD69<sup>+</sup> among CD4<sup>+</sup> T cells in PLNs in BDC2.5 mice of different ages. Each dot represents an individual mouse.



**Figure 3.** Proliferation of CFSE-labeled transferred BDC2.5 spleen cells occurs selectively in PLNs. (A) The transfer protocol: splenocytes from BDC2.5 mice are labeled with CFSE *in vitro*, then transferred to  $C\alpha^0/NOD$  recipients. Proliferation of the transferred  $CD4^+V\beta4^+$  T cells is visualized, a few days after transfer, as progressive decrease of labeling intensity in individual cells. (B) CFSE label 3 d after transfer to  $C\alpha^0/NOD$  recipients in PLNs, ILNs, axillary LNs (ALN), cervical LNs (CLN), MLNs, RLNs, iliac LNs (ILILN), parathymic LNs (PATLN), and spleen. (C) Proliferation in PLNs occurs in  $C\alpha^0/NOD$ ,  $RAG^0/NOD$ , and in normal NOD recipients.

transfer of splenocytes from a young, adult BDC2.5 mouse into a young, adult  $C\alpha^0/NOD$  host. CFSE-labeled cells were present in the spleen and the many LNs examined, but only in the PLNs was there significant dilution of label, signaling activation followed by proliferation. These data from the transfer system closely mimic those derived from regular BDC2.5 animals: the antigen that triggers BDC2.5 T cells is present in the LNs immediately draining the pan-

creas, but not in the other peripheral lymphoid organs examined.

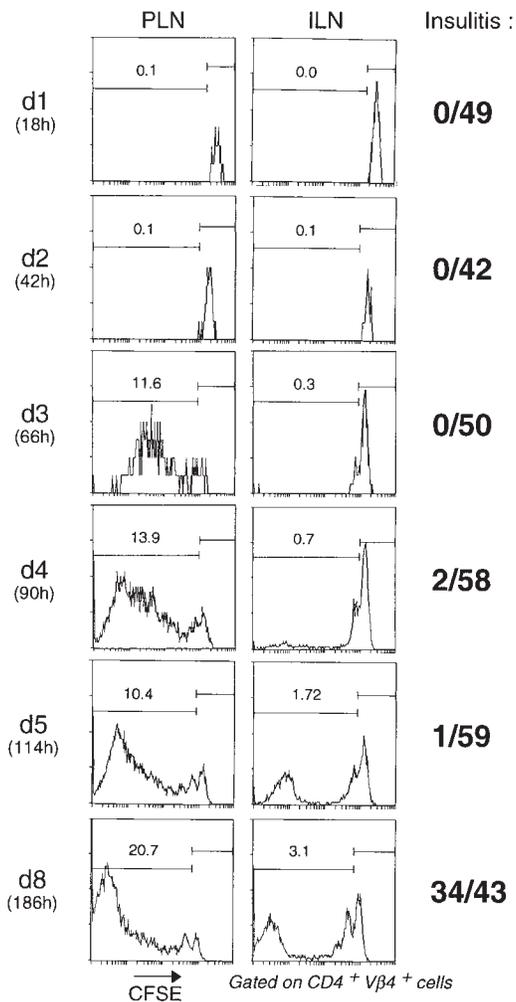
It has been hypothesized that insulinitis in NOD mice, in particular infiltration of  $CD4^+$  T cells, requires initial islet cell damage mediated by  $CD8^+$  T cells (7, 8, 28–34). In our experiments, proliferating BDC2.5 T cells were found in the PLNs of  $C\alpha^0/NOD$  mice, which suggests that transport of the relevant autoantigen to this node does not require prior islet cell destruction by T cells displaying other  $\alpha/\beta$  specificities. In fact, PLN-specific proliferation of BDC2.5 cells was observed after transfer into standard NOD,  $C\alpha^0/NOD$ , and  $RAG^0/NOD$  hosts—to a similar extent (Fig. 3 C) and with similar kinetics (data not shown). Thus, other lymphocytes neither inhibit nor are required for BDC2.5 cells to access their antigen in the PLNs.

*Proliferating BDC2.5 T Cells Appear in the PLNs before Islet Infiltration Can Be Detected.* We then exploited the transfer system to address the question of whether naive BDC2.5 T cells are first stimulated in the islets or in their draining LNs.  $C\alpha^0/NOD$  mice were used as recipients to preclude interference from host  $\alpha/\beta$  T cells and to simplify the detection of transferred cells, particularly in the islets. CFSE-labeled splenocytes from young, adult BDC2.5 transgenics were injected into nontransgenic  $C\alpha^0/NOD$  recipients of similar age and, at various times after injection, the relevant LNs were removed for cytofluorimetric analysis, and pancreas sections were prepared for histological evaluation.

Results from a typical experiment are summarized in Fig. 4. Already on day 1 after transfer, CFSE-labeled donor cells appeared in both the PLNs and ILNs, but without proliferation for the first 2 d. On day 3, PLN cells abruptly exhibited strong dilution of the CFSE label, signifying proliferation, and staining levels gradually diminished over the next 5 d. ILN cells showed no dilution of the CFSE label on day 3, but a few cells with fully reduced staining could be detected 1 d later, their low and uniform level of CFSE indicative of recirculating cells. Substantial numbers of such cells were found on day 5, and even more on day 8. Islet infiltration paralleled the appearance of recirculating cells in the ILNs, though with some delay—not detectable on day 3, rare for the next 2 d, substantial only on day 8.

Clearly, then, proliferating cells emerged in the PLNs before the onset of islet invasion. A similar dissociation between the two events was seen in seven independent experiments.

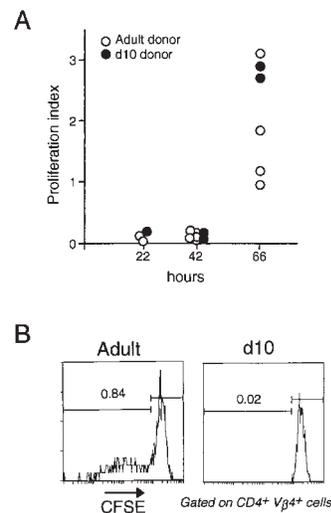
*The BDC2.5 Antigen Cannot Be Detected in the PLNs of Very Young NOD Mice.* Previous studies have highlighted two “checkpoints” in the progression of disease in BDC2.5 TCR tg mice (14, 17, 35), and these seem to be characteristic of standard NOD mice as well (36). Checkpoint 1 concerns the onset of insulinitis: even though BDC2.5 transgenics harbor plenty of T cells expressing an islet beta cell-reactive receptor from shortly after birth, invasion of the islets is not detectable until 15–18 d of age. In theory, this delay could reflect either T cell incompetence or antigen unavailability in the very young mice.



**Figure 4.** Proliferation of transferred BDC2.5 cells in the PLNs occurs before islet cell infiltration. In a representative experiment, a cohort of  $C\alpha^0/NOD$  mice were injected with  $5 \times 10^7$  CFSE-labeled BDC2.5 spleen cells and subsequently analyzed for CFSE dilution in PLNs and ILNs at various times after transfer. The “proliferation index” shown for each histogram is obtained by dividing the fraction of proliferating cells by the fraction of nonproliferating cells (gates as shown). Insulinitis values on right: number of infiltrated islets over the total number of islets scored.

To evaluate the first explanation, we compared the ability of splenic T cells removed from 10-d-old and adult BDC2.5 transgenics to proliferate in the PLNs after transfer into adult  $C\alpha^0/NOD$  hosts (Fig. 5 A). The T cells from 10-d-old mice appeared to be just as active as those from adults, proliferating cells being detectable in the PLNs with similar kinetics and to a similar degree. Thus, T cell incompetence is unlikely to be the explanation for the delayed onset of insulinitis.

To assess the second explanation, antigen unavailability, we performed the reciprocal experiment, comparing the ability of splenic T cells from adult BDC2.5 transgenics to proliferate in the PLNs of 10-d-old and adult  $C\alpha^0/NOD$  recipients (Fig. 5 B). Here, there was a striking difference in behavior: as usual, proliferating T cells appeared in the PLNs 3 d after transfer into adult hosts, but did not when



**Figure 5.** A comparison between adult and 10-d-old BDC2.5 mice as donors and recipients in the transfer model. (A)  $5 \times 10^7$  T cells from 10-d-old or adult BDC2.5 donors were transferred to adult  $C\alpha^0/NOD$  recipients. The graph is a summary of several independent experiments, showing the proliferation index of  $CD4^+V\beta4^+$  T cells in PLNs at different time points, calculated as for Fig. 4; each dot represents an individual mouse. (B) BDC2.5 donor T cells were transferred to adult or 10-d-old  $C\alpha^0/NOD$  mice, as indicated (proliferation index as above).

introduced into 10-d-old mice. These results argue that the delayed onset of insulinitis in BDC2.5 tg mice reflects unavailability of the autoantigen in juvenile animals.

*Another Beta Cell-derived Autoantigen Is Undetectable in the PLNs of Very Young Mice.* We wondered whether reduced availability during the first weeks of life is particular to the BDC2.5 antigen or can be generalized to other islet cell antigens. Relevant data on a few other islet antigen/TCR pairs have been reported—in all cases pertaining to a viral protein artificially expressed on beta cells. In mice displaying influenza hemagglutinin on beta cells, activated hemagglutinin-specific T cells were found throughout the peripheral lymphoid organs, and insulinitis or diabetes could occur before 3 wk of age (37–39). In animals synthesizing SV40 T antigen in beta cells, activated T cells were confined to the PLNs, MLNs, and Peyer’s patches, and could be detected already 1 wk after birth (40). These findings argue that unavailability of islet antigens in juvenile animals may not be a general phenomenon. However, both systems have complications: T cells recognizing hemagglutinin can cross-react with murine self-antigens (41); SV40 T antigen is an oncogene whose expression provokes hyperplasia, tumors, and probably cell damage (42). We sought to reexamine this issue with an antigen/TCR pair not known to be subject to such complications.

RIP-mOVA tg mice express a membrane-bound form of OVA under the dictates of the insulin promoter, although significant amounts of OVA are made by both pancreatic islet beta cells and kidney proximal tubule cells; OT-I transgenics express the TCR genes from an MHC class I-restricted, OVA-specific T cell clone (20). When naive OT-I T cells were transferred into RIP-mOVA mice on the C57Bl/6 genetic background, proliferating  $CD8^+$  cells appeared specifically in the LNs draining the pancreas and kidney, the result of “cross-presentation” of the OVA epitope by bone marrow-derived (presumably dendritic) cells (20, 43). Thus, T cell activation in this system appeared to be nearly as specific as in the BDC2.5 system.

As expected, and as illustrated in Fig. 6, proliferating OT-I T cells were found in both the PLNs and renal LNs (RLNs) on day 3 after transfer into adult hosts. In contrast, no proliferating cells were detected in the PLNs after introduction into 10-d-old recipients, although they were observed in significant numbers in the RLNs of these animals. Thus, the inability of APCs in the PLNs of very young mice to trigger islet-reactive T cells is not antigen specific, does not depend on the class of MHC restricting element, and is not a peculiarity of the NOD genetic background. Interestingly, the defect is not a general property of LN APCs in juvenile animals, as those associated with the RLNs are quite competent.

## Discussion

This study focused on the events that trigger autoimmune attack of the pancreas—in particular where and when islet-reactive T cells first encounter antigen. Tackling these questions was facilitated by the use of BDC2.5 TCR tg mice, as the early events of diabetes pathogenesis are particularly clear and synchronous in these animals.

*The Site of Initial Encounter.* The first indication that BDC2.5 T cells are initially stimulated in the vicinity of the pancreas came from monitoring the activation state of T cells at various sites in the periphery of prediabetic BDC2.5/NOD tg mice. Activated cells (expressing high levels of CD69 and CD44, and low levels of CD62L) were found within the islets and PLNs, but not in the spleen or in the many other LNs examined, including the MLNs which drain the pancreas more distally (Fig. 1, A and B). Results from the transfer system provided support for the notion that BDC2.5 T cells are initially stimulated in the pancreas surroundings (Fig. 3 B), and further established that they encounter antigen first in the PLNs rather than within the islets (Fig. 4). These findings argue strongly against a role for molecular mimicry in the BDC2.5 system—had the initial stimulus been viral or dietary, we

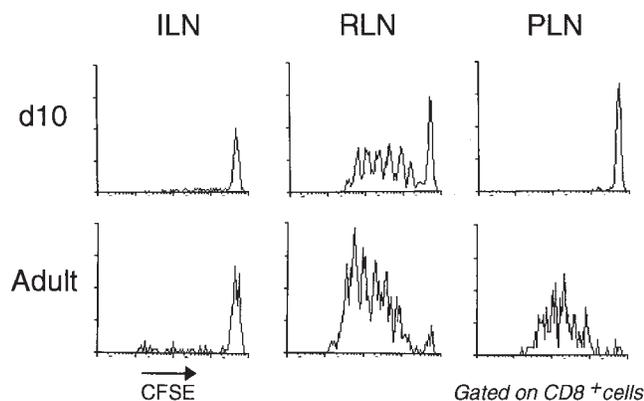
should have seen activated/proliferating T cells at other locations, not strictly confined to the vicinity of the pancreas.

How does the BDC2.5 antigen arrive in the PLNs? A definitive answer to this question will probably not come until this antigen has been identified. At present, we know only that it is associated with the membrane fraction of beta cell vesicular structures (44), and that its presentation may depend on intact vesicles (our unpublished results). A likely possibility is that the antigen is picked up in the islets by immature DCs through phagocytosis or micropinocytosis, and is ferried to the PLNs via afferent lymphatic vessels, coincident with DC maturation. Certainly, DCs are present in early insulinitic lesions, even before the arrival of lymphocytes (45–47). It is also possible that beta cell proteins are, themselves, transported to the PLNs through the afferent lymphatics, and it is there that the BDC2.5 antigen is taken up, processed, and presented. Incorporation of antigens into vesicles seems not to be necessary for effective transport to the PLNs because very diverse beta cell neoantigens appear to be presented in this node, such as membrane-bound OVA (20) and nuclear SV40 T antigen (40).

That beta cell antigens would be picked up by DCs in the islets, be ferried to the draining PLNs, be presented there to circulating naive T cells, and finally provoke those with anti-beta cell reactivity to invade the islets would be a sequence of events nicely analogous to the prevailing paradigm on antigen surveillance (13). As discussed below, this scenario is also consistent with the little existing data on standard NOD mice. It remains to fill in the many missing details.

*The Time of Initial Encounter—Checkpoint 1.* The transfer system permitted us to address another critical issue concerning the events triggering autoimmune diabetes. Insulinitis is not detectable in standard NOD mice before 4–6 wk of age, a delay classically considered to reflect the time it takes to activate islet-reactive T cells and expand them to high numbers. However, BDC2.5 TCR tg mice, with their enormous preformed antiislet repertoire, were found to exhibit a similar lag before the initiation of insulinitis (14), prompting the speculation that diabetes progression is regulated, the transition to insulinitis constituting a first checkpoint (14, 36). In theory, checkpoint 1 could reflect a deficiency in either T cell competence or antigen availability. To distinguish between the two, we performed crisscross transfer experiments, introducing 10-d-old or adult donor cells into adult hosts, as well as adult donors into 10-d-old or adult hosts. The defect appears to be in how antigen is made available, as both types of T cells proliferated in the PLNs after transfer into adult hosts (Fig. 5 A), whereas adult donors proliferated only in adult but not in 10-d-old hosts (Fig. 5 B, and data not shown).

Reduced availability of the BDC2.5 antigen in the PLNs of very young mice could have several roots. One class of explanations invokes the antigen itself—it may not be synthesized yet, may be made at too low a level, or may not be in the appropriate state. The first possibility is ruled out by the finding that preactivated, rather than naive, BDC2.5 T cells can infiltrate the islets within days after transfer into



**Figure 6.** Proliferation of OVA-specific CD8<sup>+</sup> T cells in adult and 10-d-old RIP-mOVA mice. Recipients were injected with  $2 \times 10^7$  splenocytes from OT-I transgenic mice, and loss of CFSE by proliferation was evaluated in gated CD8<sup>+</sup> T cells in ILNs, RLNs, and PLNs (see above) 67 h after transfer.

neonatal NOD recipients (48). However, this result does not exclude the other two possibilities because activated T cells can be stimulated by lower concentrations of antigen than can naive cells (25), and because effective release to the draining LNs could depend on antigen being in a particular form or subcellular compartment, e.g., part of a multiprotein complex or encased within a vesicle. Evaluating these possibilities will require a comparison of the effective concentration of BDC2.5 antigen in the LNs of different-aged animals, possible through *in vitro* presentation assays, but difficult and arduous given the scarcity of islets in very young mice (our unpublished observations).

A second class of explanations for early BDC2.5 antigen unavailability invokes the APCs—it may be that the relevant antigen is perfectly available, but that the appropriate APCs for ferrying it to or presenting it in the PLNs are at fault. This immaturity may affect homing of DCs to the pancreas, their ability to acquire antigens once there, or in their traffic onwards to the PLNs. There is precedent for defective APC function in neonates in other contexts (49, 50). Yet, our results suggest that the APC defect should be a regional one, given that the RLNs of 10-d-old mOVA hosts were quite capable of supporting proliferation of transferred naive OT-I cells (Fig. 6), and the pancreas may thus be somehow special. Perhaps different populations of DC precursors reside in the pancreas and kidney of very young animals, or perhaps there are different cytokine milieus in the two organs, that in the pancreas being suboptimum for DC mobilization (due, for example, to reduced levels of TNF- $\alpha$ , GM-CSF, or IL-4 [13, 51]). The appearance of DCs in the pancreas may require particular metabolic or endocrine changes occurring at that time (45). Refining the transfer system by incorporating mutant or Ab-treated donors or hosts should aid us in distinguishing between these various possibilities.

Whatever the explanation, the events underlying this transition are not specific to NOD mice, as checkpoint 1 is also observed when the BDC2.5 transgene is crossed onto the C57Bl/6 genetic background (17).

*Generality.* It is important to consider whether our

conclusions about BDC2.5 T cells and the antigen they recognize are generalizable. The original BDC2.5 clone was isolated rather late in disease progression, from pooled spleen and LNs of a diabetic NOD female (15), so one might question its relevance to disease initiation. However, it has already been demonstrated that BDC2.5 T cells can engender full-blown diabetes in the absence of other lymphocytes (14), and we show here that no other lymphocytes are required for BDC2.5 cells to access their antigen in the PLNs (Fig. 3 C). It may also be worth noting that the BDC2.5 antigen has properties similar to the antigens recognized by several other mouse (44) and human (52) islet-reactive T cell clones. Nonetheless, it would be useful to compare the behavior of other diabetes-provoking antigens and the corresponding diabetogenic T cells at the time of disease initiation.

The little existing data on standard NOD mice are generally consistent with the observations on BDC2.5 TCR transgenics. Three findings are in line with the notion that initial stimulation of islet-reactive T cells occurs in the PLNs: (a) alloxan-mediated destruction of the pancreas of perinatal NOD animals prevented the emergence of potentially diabetogenic T cells, arguing against primary activation at extrapancreatic sites (53); (b) treating juvenile NOD animals with anti-CD40 ligand or anti- $\beta$ 7 integrin blocked insulinitis, but did not prevent the appearance of islet-reactive T cells, indicating that priming need not occur within the islets (54, 55); and (c) DCs in the PLNs of NOD animals indeed seem to present islet-derived antigens (56). No existing results favor or disfavor the idea that presentation of beta cell antigens is compromised in the PLNs of standard NOD mice at a very young age, although there is clear evidence of a checkpoint 1 at  $\sim$ 3 wk (36).

In conclusion, autoimmune diabetes in BDC2.5 TCR tg mice is initiated by developmentally regulated presentation of islet beta cell antigens in the PLNs, and this is most likely true for standard NOD mice. It will be interesting to see whether this phenomenon occurs in other autoimmune diseases as well.

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