# Population dynamics of islet-infiltrating cells in autoimmune diabetes

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Type-1 diabetes in the nonobese diabetic (NOD) mouse starts with an insulitis stage, wherein a mixed population of leukocytes invades the pancreas, followed by overt diabetes once enough insulin-producing β-cells are destroyed by invading immunocytes. Little is known of the dynamics of lymphocyte movement into the pancreas during disease progression. We used the Kaede transgenic mouse, whose photoconvertible fluorescent reporter permits noninvasive labeling and subsequent tracking of immunocytes, to investigate pancreatic infiltrate dynamics and the requirement for antigen specificity during progression of autoimmune diabetes in the unmanipulated NOD mouse. Our results indicate that the insulitic lesion is very open with constant cell influx and active turnover, predominantly of B and T lymphocytes, but also CD11b$^+$c$^+$ myeloid cells. Both naïve- and memory-phenotype lymphocytes trafficked to the insulitis, but Foxp3$^+$ regulatory T cells circulated less than their conventional CD4$^+$ counterparts. Receptor specificity for pancreatic antigens seemed irrelevant for this homing, because similar kinetics were observed in polyclonal and antigen-specific transgenic contexts. This "open" configuration was also observed after reversal of overt diabetes by anti-CD3 treatment. These results portray insulitis as a dynamic lesion at all stages of disease, continuously fed by a mixed influx of immunocytes, and thus susceptible to evolve over time in response to immunologic or environmental influences.

Treg | cell tracer | reporter

Type-1 diabetes (T1D) is an organ-specific autoimmune disease initiated by a breakdown in T lymphocyte tolerance to islet-cell antigens; it comprises two stages: an occult phase of pancreatic inflammation, which reduces the number and function of insulin-producing β-cells and eventually provokes sufficient damage to result in the overt phase of diabetes, when insulin production is insufficient for proper glucose homeostasis. The genetics of T1D in mice and humans point primarily to a dysfunction of CD4$^+$ T cells, because class II genes of the MHC, and several other loci that modify T-cell activation and regulation, are linked to T1D susceptibility (1).

In nonobese diabetic (NOD) mice and other animal models (2), this initial inflammatory phase takes the form of insulitis, wherein a mixed population of leukocytes invades the islets of Langerhans. Insulitis starts around 3–4 wk of age and amplifies progressively until the onset of clinically overt diabetes (predominantly between 14 and 25 wk of age); it involves a wide array of cell types—T and B lymphocytes as well as myeloid cells—macrophages and dendritic cells (3), which can take on the organization of typical tertiary lymphoid structures (4). Importantly, insulitis variably affects different islets in a given animal, heavily infiltrated islets coexisting with fully intact and functional ones, even in advanced prediabetic mice.

Inflammation in islets of human patients has been harder to evaluate, because access to material is obviously more difficult. As cogently reviewed by In't Veld (5), few individuals have actually been analyzed, often in conditions of uncertain diagnosis, with missing genetic or biochemical data, and the histological evaluation complicated by ketoacidosis and variable handling of

cadaveric or surgical samples. Insulitis seems more frequent in individuals presenting with T1D at a young age and assessed soon after onset (6, 7); further, it has been observed in two of three high-risk prediabetic individuals analyzed (8). The proportion of infiltrated islets and the extent of infiltration appear generally lower in human patients than in NOD mice, and a dominance of CD8$^+$ over CD4$^+$ T cells seems frequent (7, 9, 10), with a variable frequency of B lymphocytes (10). These differences may reflect fluctuations over time, or merely the much faster progression of diabetes in the NOD mouse (weeks rather than years).

Two related questions concerning the progression of insulitis arise. First, what are its population dynamics? Is insulitis isolated from the remainder of the immune system and growing locally in response to autoantigens presented there, or is it continuously replenished, and thus possibly modified, by a regular influx of freshly recruited cells? Second, are all of the lymphocytes in the pancreatic infiltrate antigen-specific, or is there also a significant bystander population? The latter point has been debated: using double-retrogenic mice, in which specificity is controlled, Lennon et al. (11) showed that only antigen-specific cells accumulate in the insulitis after transfer into NOD.scid mice, and only T cells specific for β-cell antigens localized to islets in the first hours after transfer (12, 13) in other studies. However, antigen-specific cells identified by tetramer staining are only modestly enriched in the pancreas relative to irrelevant lymphoid organs, and there is still ample participation by other cells (14, 15). Polyclonal populations, unlikely to be antigen specific, accompanied cells from specific clones injected into NOD mice (16). Calderon et al. (17) showed that, in transgenic models, the initial entry of antigen-specific cells modifies local conditions, allowing the recruitment of nonspecific T cells. It should also be noted that

**Significance**

In many autoimmune diseases, self-reactive lymphocytes lead to immunocyte infiltration in the target tissue, whose evolution over time is regulated. Such lesions can be well-tolerated and lead to minimal damage, or have more drastic consequences. We show here, for the insulitis of the nonobese diabetic (NOD) mouse model of autoimmune diabetes, that the autoimmune infiltrate is an open and dynamic cell population, with a high turnover and constant reseeding with fresh cells coming from the general lymphoid circulation; this implies that such a lesion is susceptible to extraneous events and influences, which could either provoke overt disease or instill stronger immunosuppression.

chemokine expression resulting from inflammation has the potential to attract T cells to the islets (17, 18).

To avoid the potential caveats of transfer systems, we set out to investigate population dynamics of the pancreatic infiltrate during disease progression in NOD mice, using a noninvasive labeling and tracking approach. We used the Kaede/NOD transgenic (tg) mouse, which expresses ubiquitously the photoconvertible fluorescent protein Kaede (19–22). We tracked cell movement from a known point of "labeling"—namely, s.c. lymph nodes (LNs), into the pancreas tissue itself, which allowed us to define the kinetics of entry for lymphoid and myeloid cells, much of which turned out to be independent of antigenic specificity.

## Results

### Dynamic Profile of the Pancreatic Infiltrate During Disease Progression.
Insulitis in the NOD mouse comprises a variety of lymphocytes and myeloid cells, whose numbers generally increase over time (23). To set the stage for our studies of immunocyte cell trafficking, we revisited the NOD insulitic lesion to ascertain more precisely the composition of the infiltrate over the course of disease progression. Immunocyte profiles in the pancreas were examined from 4 to 14 wk of age. Similar to previous reports (23–25), a sizable population of leukocytes (CD45$^+$) cells was already present in the NOD pancreas at 4 wk of age, which expanded markedly between 8 and 12–14 wk (Fig. 1$A$). These cells did not correspond to normal resident cells in a healthy pancreas, because much lower numbers of CD45$^+$ cells were observed in the pancreata of insulitis-free NOD.Eα16 (26) and C57BL/6 mice (Table S1). The increase was generally true of all cell populations analyzed, lymphoid or myeloid. CD4$^+$ T cells and B cells were the major components, in roughly equal proportion (Fig. 1 $B$ and $D$). Also in accordance with previous reports (23, 25, 27, 28), myeloid cells constituted the majority of hematopoietic cells in the pancreas at 4 wk (Fig. 1 $C$ and $E$), in

particular with a strong representation of CD11b$^+$ F4/80$^+$ macrophages. However, by the time insulitis was well established (12–14 wk), lymphocytes had taken over as the majority cell type in the lesion (Fig. 1 $D$ and $E$), although diverse types of mononuclear phagocytes (dendritic cells and macrophages) remained present.

Given this increase in cell numbers, we determined the rate of cell division via BrdU labeling experiments. Marked proliferation was observed for T lymphocytes in the pancreas compared with pancreatic lymph nodes (PLNs) and spleen (Fig. 1$F$). CD8$^+$ T cells proliferated most actively, contrasting somewhat with their relatively low numbers. Such cycling rates, if extrapolated over several weeks and assuming no cell death, could potentially lead to a greater increase in cell numbers than what was observed: e.g., from 10 to 14 wk, one would expect the CD8$^+$ population to quadruple in size when, instead, it merely doubled. This observation suggests a rapid turnover as well, either through cell exit or, more likely, via cell death. In contrast, B cells cycled very little, no more than their counterparts in lymphoid organs, consistent with the perception of their role as secondary (albeit important) players in the process.

### Tracking Cells Migrating into the Pancreas.
To analyze the flux of cells into the insulitic lesion, we used a NOD backcross (>13 generations) of the Kaede tg mouse line, which allows noninvasive labeling and tracking of immunocytes in vivo (19–22). In these mice, all cell types express the green fluorescent form of the Kaede protein, which can be irreversibly photoconverted into a red fluorescent form by violet light (29). Cells converted to red fluorescence in one location can then be tracked through a mouse's body at later times. Cervical lymph nodes (CLNs), which have no anatomic or physiologic connection to the pancreas or to diabetes, were the labeling site for all of our experiments. No Kaede-red cells were detected anywhere before excitation (Fig. 2$A$, $Top$). Immediately after photoconversion, most cells in the CLNs had red-shifted, but they had not in other locations (Fig. 2$A$, $Middle$). By 24 h, however, distinct populations of red CD45$^+$ cells were present in lymphoid organs, such as the spleen and in the pancreas (Fig. 2$A$, $Bottom$). The proportion of pancreatic Kaede-red cells continued to increase over the next few days (Fig. 2$B$). Thus, ~0.5–1% of the CD45$^+$ cells present in the pancreatic infiltrate at the day 3 time-point resided in the CLNs 3 d earlier.

Next, we established where these migrating cells were going. Explanted pancreata were imaged 36 h after photoconversion in the CLNs to visualize the recent immigrant Kaede-red cells in situ. Regions containing intact β-cells were identified using reflected light, as described previously (30), and regions of insulitic infiltrate were delineated by their low reflectance, high density of nuclei, and low Kaede-green fluorescence (which happens to be very bright in exocrine pancreas; Fig. S1$A$). The majority of Kaede-red recent immigrants was found in the insulitis region (Fig. 2$C$), although some such cells were also detected inside the β-cell area itself. The latter represented 12.6 ± 10.1% of the immigrants, a proportion consistent with the frequency of T lymphocytes in the insulitis and β-cell mass in NOD pancreata. No recent immigrants were detected in islets devoid of insulitis or in the exocrine pancreas (Fig. S1$B$). Note that we could not identify the actual type of cell present in these images, because the Kaede-red color does not resist fixation for immunofluorescence. However, the estimated number of immigrants detected by microscopy (up to ~9,000 cells per pancreas in 10-wk-old NOD mice) is consistent with the total number of Kaede-red CD45$^+$ cells observed by flow cytometry (Fig. 2$A$ and below).

### Which Cells Migrate into the Pancreas?
The leukocyte subsets migrating into the pancreas were identified by flow cytometric profiling (gating strategy described in Fig. S2). At 24 h after photoconversion, all resident major lymphocyte subsets (CD4$^+$ and CD8$^+$ T cells, B cells) exhibited similar proportions of recent immigrants (photoconverted cells; Fig. 3$A$). Over the following days, these numbers increased slightly and stabilized. In contrast, few or no myeloid cells traveled from the CLNs to the pancreas,



Fig. 1. Dynamic profile of the pancreatic infiltrate during disease progression. (A) Background level of total CD45$^+$ cells in pancreas was measured using flow cytometry. (B–E) The immune cell infiltrate in pancreata from mice at the different indicated ages was enumerated and profiled by flow cytometry. B, Inset shows 4- to 8-wk data on expanded scale. (F) Lymphocyte proliferation during disease progression in NOD. BrdU was administered (1.2 mg BrdU in 200 mL, two injections 10 h apart) to female NOD mice from 4 to 14 wk of age. At 22 h after the first injection of BrdU, BrdU incorporation into CD4$^+$, CD8$^+$ T cells, and CD45R$^+$ B cells was measured by flow cytometry for the pancreas, PLNs, and spleen.
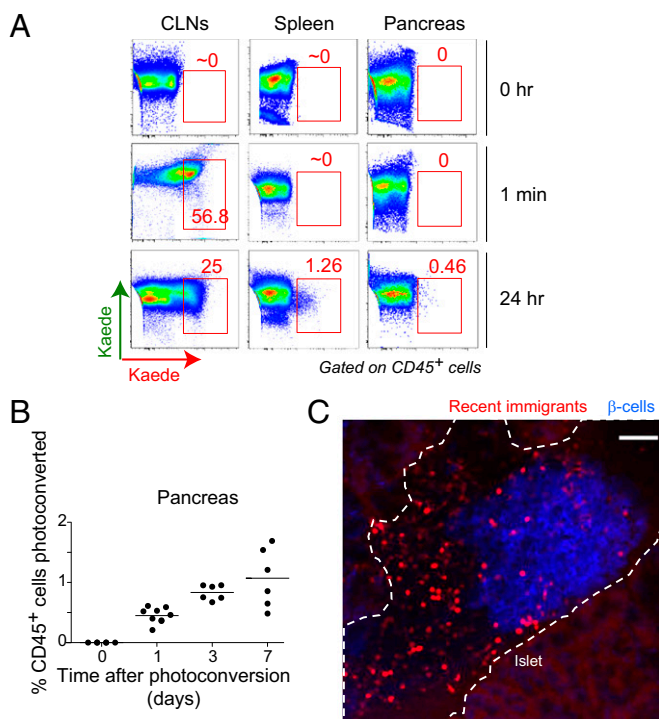
**Fig. 2.** Monitoring cell trafficking to the pancreas. (*A*) Sample flow cytometry data for CD45+ cells from nonphotoconverted CLNs and 0-h, 1-min, and 24-h time points. (*B*) The proportion of CD45+ cells comprised of recent immigrants was measured by flow cytometry data generated at 0, 1, 3, or 7 d after photoconversion of the CLNs in 12- to 14-wk-old mice. (*C*) Islets in explanted pancreata from 10- to 12-wk-old mice were imaged by confocal microscopy 36 h after photoconversion of the CLNs. Kaede-red+ recent immigrants (red), β-cells (blue), and islet periphery (dashed white line). Representative z-stack projection shown here. Six pancreata per group were imaged.

with the exception of CD11b+CD11c+ cells, most likely monocytes (Fig. 3*C*); this was despite evident migration of other myeloid cells between lymph nodes (Fig. 3*D*), indicating that the pancreas is not an open house to all cell types.

The measured proportion of Kaede-red immigrants at a given time-point reflects their rates of migration into the pancreas, but also integrates the relative sizes of the pancreatic and circulating pools, and the existing proportion of labeled cells in both. Generating a true quantitative model of cell dynamics was hindered by the fact that each mouse could be sampled only once and that there was some variability in the rate of red conversion in the irradiated CLN (ranging from 38% to 70% over our experiments). For a numeric estimate of migration rates, we computed a "migration ratio" (proportion of Kaede-labeled cells in the pancreas over that in irrelevant LNs), based on the reasonable assumptions that Kaede-red cells found in LNs and spleen reflect the normal circulation of lymphocytes between lymphoid organs (31), and that the cells entering the pancreas originate from this pool (Fig. 3*E*). At 24 h after photoconversion, a time when the bulk of labeled cells have already exited the CLN, this ratio should adequately reflect limitations in traffic into the pancreas. Indeed, the migration ratios of ~0.5 (Fig. 3*F*) for CD4+ and CD8+ T cells indicated that lymphocytes do circulate extensively to the pancreas, albeit not quite as freely as they do between lymphoid organs. (For B cells, the pancreas migration ratio seemed even higher, close to that of spleen.) In contrast and as expected, migration into PLNs showed the same dynamics as into other LNs (Fig. S3).

We then asked whether effector or memory CD4+ T cells trafficked into the inflamed pancreas preferentially over that of naïve CD4+ T cells; this was not the case. Perhaps surprisingly,

the majority of recent immigrants had a naïve phenotype (CD62+/hiCD44−/low; Fig. 3*G*); cells with effector/memory phenotypes (CD62loCD44hi) were a minority, at a frequency intermediate between spleen and irrelevant lymph nodes, and at lower frequency than among resident cells. Once present, only a small proportion of recent immigrants became activated, as evidenced by CD69 expression (Fig. 3*H*).

FoxP3+CD4+ regulatory T cells (Tregs) play a major role in the control of T1D progression; their proportion of the CD4+ T-cell compartment in the inflammatory infiltrate are roughly similar to those observed in most lymphoid locations (5–15%) (32). We asked how migration of Tregs to the pancreas compared with that of FoxP3− conventional (Tconv) CD4+ T cells at different disease stages. As previously reported (22), Tregs exited more slowly than did Tconv cells from the labeled CLN (Fig. 4*A*); accordingly, a greater proportion of pancreas-infiltrating Tconv than Treg cells consisted of recent immigrants (Fig. 4*C*). Even when the lower labeling of the circulating pool was taken into account by calculating the migration ratio, Treg cells entered the pancreas less effectively than did Tconv cells (Fig. 4*D*), and the pancreatic Treg pool never equilibrated to reach the proportions of Kaede-red cells of the circulation. Thus, Treg cells have far less active migration dynamics than do Tconv cells.

**Is Anti–β-Cell Antigen Specificity Necessary for Lymphocyte Entry into the Insulitic Lesion?** Specificity for pancreatic antigens is believed to be a prerequisite for T cells to infiltrate the islets (11, 33). However, our results point to a very substantial rate of T-cell entry, predominantly of naïve, antigen-inexperienced cells. Polyclonal CD4+ and CD8+ T cells and B cells previously in the irrelevant CLNs entered the pancreas on a continuous basis, and in proportions that were only twofold reduced from those characteristic of cell trafficking between secondary lymphoid organs. (Extrapolation from the proportion of labeled cells in the spleen/LN circulation, we estimate that 0.0135% of the circulating CD4+ T-cell pool entered the insulitic lesion on a daily basis, and that 0.33% of the CD4+ T-cell content in the insulitis entered per day.) Because it seems intuitively unlikely that half of the T cells in the CLNs would be reactive against pancreatic autoantigens, these numbers are difficult to reconcile with a strict requirement for an anti–β-cell antigen specificity of pancreas-infiltrating T cells. To directly address this point, we compared pancreas migration ratios in standard NOD mice and in BDC2.5 T-cell receptor (TCR) tg mice, in which most CD4+ T cells express a TCR specific for an islet antigen presented by the MHC class II (MHC II) molecule I-A$^{g7}$, resulting in florid insulitis. After crossing the Kaede and BDC2.5 tg lines, we compared migration into the pancreas of cells photoconverted 72 h earlier in the CLN; mice of 4–6 and 12–14 wk of age were analyzed, to cover both early and advanced stages of insulitis. There was very little difference in the pancreas migration profiles for BDC2.5 and NOD mice, whether in the proportion of Kaede-red recent immigrants (Fig. 5*A*) or in the calculated migration ratios (Fig. 5*B*). This similarity held for CD4+ T cells, and for CD8+ T and B cells for which the MHC II-restricted BDC2.5 TCR would be irrelevant. However, when expressed as total numbers of Kaede-red recent immigrants, there was much greater entry into the BDC2.5 than the NOD insulitis (Fig. 5*C*), the difference waning somewhat in older mice. Insulitis starts at the same time (~15–18 d of age) in NOD and BDC2.5 mice, but progresses much faster in the latter (34). Thus, the higher numbers but equal proportion of recent immigrants in the BDC2.5 vs. NOD pancreas reflects the difference in total cell numbers already present (Fig. 5*D*). Hence, on the 72-h time-scale probed here, migration into the autoimmune pancreas correlated in a nonspecific manner to the total insulitis size, rather than to a particular drive from antigen-specific receptors.

**Lymphocyte Traffic to the Anti-CD3 "Cured" Pancreas.** Treatment with anti-CD3 is a promising avenue of therapy for T1D. When administered early after disease onset, it has been shown
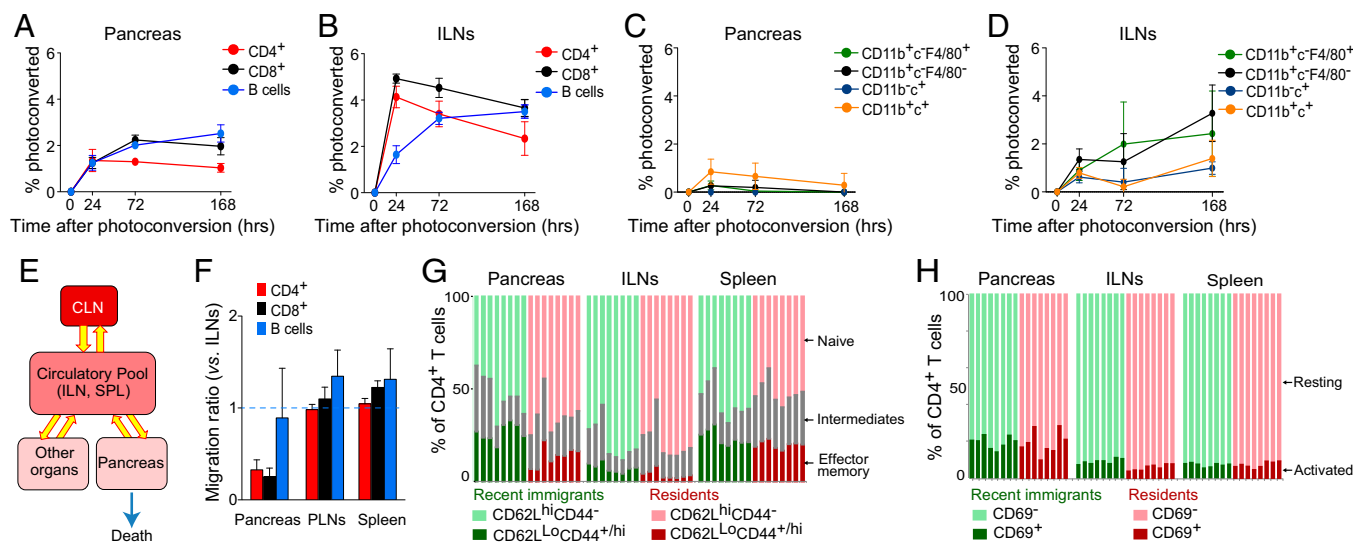
IMMUNOLOGY AND INFLAMMATION

**Fig. 3.** Monitoring lymphoid and myeloid cell trafficking to the pancreas. The proportion of lymphocyte (*A* and *B*) or myeloid (*C* and *D*) populations that was comprised of recent immigrants in pancreas and inguinal LNs (ILNs) was measured by flow cytometry data generated at 0, 24, 72, or 168 h after photoconversion of the CLNs. (*E*) Schematic depiction of mathematical modeling of lymphocyte migration into the pancreas. (*F*) Migration ratios for lymphocyte subsets trafficking to pancreas, PLNs, or spleen vs. control ILNs were calculated from 24-h flow cytometry data. (*G* and *H*) Effector memory phenotype (*G*; CD62L, CD44) and T-cell activation (*H*; CD69) were assessed in resident and recent immigrant CD4+ T cells in pancreas, ILNs, and spleen. Each bar represents an individual mouse.

repeatedly to reverse diabetes in NOD mice and preserve residual β-cell function in T1D patients for 12–24 mo (35–38). Although most diabetic NOD mice treated with anti-CD3 revert to normoglycemia, the treatment does not permanently abolish the autoimmune lesion in the pancreas; rather, a stable state of insulitis is established, with a demarcation between the inflammatory infiltrate and β-cell mass (39, 40) (Fig. 6*A*). We asked whether diabetes reversal with anti-CD3 affected population dynamics of the infiltrate. Recently diabetic Kaede/NOD mice (less than 3 d since confirmed hyperglycemia) were treated with anti-CD3 as described (41). CLN photoconversion was performed on mice that had reverted to normoglycemia for 30 d (a reliable indicator of long-lasting reversion of diabetes), and migration into the pancreas was assessed after 72 h. Migration into the insulitic lesion of anti–CD3-cured mice proved comparable with that typical of 12-wk-old prediabetic mice (Fig. 6 *B* and *C*) for all lymphocyte subsets examined. Thus, diabetes reversal by anti-CD3 does not turn the autoimmune lesion into a "closed shop," but rather one that remains open to traffic and reseeding, which could explain why the effects of anti-CD3 treatment last only for 1–2 y in humans (42).

## Discussion

We have used the Kaede tg mouse system (19), coupled with cytometric and microscopic detection, to investigate immunocyte dynamics during the progression of autoimmune diabetes in the NOD mouse. A key strength of this system is that it allows the tracking of cells in an unmanipulated animal, avoiding the perturbations and artificially high cell numbers associated with transfer systems (11, 33, 43–45), or the nonphysiologic nature of surgical grafts (46, 47). Our results indicate that the insulitic lesion is very open, with high cell influx and turnover, but with evident specificity in the balance of entering cells. These results extend the observations of Calderon et al. (17) in transgenic systems, which showed that an initial onslaught by diabetogenic transgenic T cells opened the door for further nonspecific influx.

Recent immigrants were almost exclusively lymphocytes (B, CD4+ T, and CD8+ T cells), suggesting some level of discretion in who can gain entry to the pancreas. Most myeloid cells, even those that seemed to migrate between secondary lymphoid organs, showed little immigration into the pancreas, except for CD11b+c+ cells, presumably migrating monocytes. Moreover,

entry of Treg cells was disfavored relative to Tconv cells. Tregs strongly impact T1D pathogenesis in a number of mouse models, and there is a gradual decrease in the Treg:Tconv cell ratio in inflamed islets (48). Tang et al. (48) previously suggested that the decline in pancreatic Tregs reflected apoptosis; we now propose that a reduced influx of Treg vs. Tconv cells may also be at play.

This openness is somewhat at odds with the notion that autoreactive pancreas-infiltrating T cells require prior activation in the draining lymph node before entry into the islet (43, 44); this is clearly not the case here, because transit time from the CLNs to the pancreas is far too short to allow for activation and, indeed, such recent immigrants are predominantly naïve and antigen inexperienced. One possible explanation rests in the nature of the transfer models used in earlier experiments. In these earlier studies, small numbers of labeled cells may have
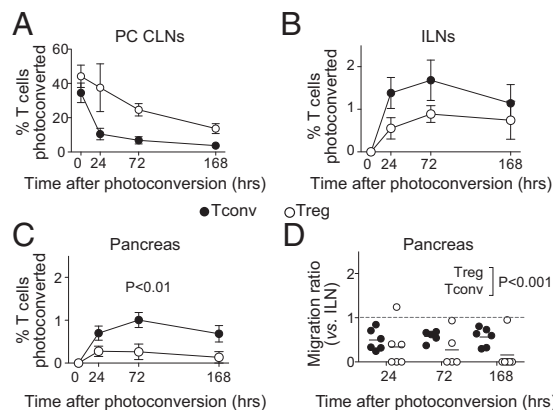


**Fig. 4.** Monitoring Tconv and Treg cell trafficking to the pancreas. (*A*) The proportion of Tconv and Treg populations that was comprised of photoconverted cells at the site of photoconversion (CLNs). (*B* and *C*) Recent immigrants in the pancreas and ILNs were measured by flow cytometry. Data were generated at 0, 24, 72, or 168 h after photoconversion of the CLNs. (*D*) Migration of Tconv and Treg cells to the pancreas vs. the control ILNs was calculated from same flow cytometry data. ***$P < 0.001$ (aggregate *P* value).
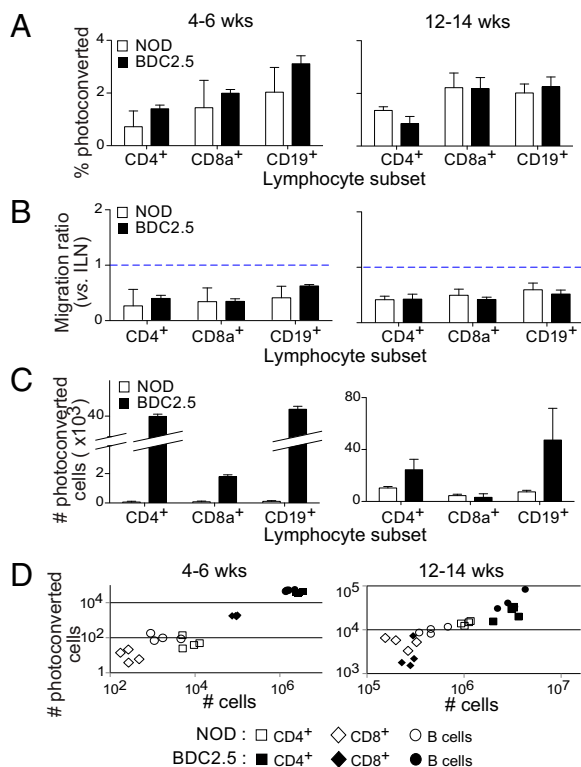
**Fig. 5.** Does antigen specificity affect migration into the lesions? The stages of disease initiation (4–6 wk) and established insulitis (12–14 wk) were examined in both NOD.Kaede (NOD) and BDC2.5.NOD.Kaede (BDC2.5) mice. Migration of lymphocytes (CD4$^+$ T cells, CD8$^+$ T cells and B cells) into the pancreas was measured by flow cytometry 72 h after photoconversion of the CLNs. (A) Proportion of cell population comprised of recent immigrants. (B) Migration of lymphocytes to pancreas, vs. control ILNs was calculated. (C) Number of recent lymphocyte immigrants in pancreas. (D) Number of photoconverted vs. total cells. Four mice per group, two separate experiments.

entered the islets but then continued to recirculate, effective colonization and amplification of LN-activated T cells only occurring by secondary recognition of antigen presented in the islets a few days later (13, 45).

It has been proposed that antigen specificity is a prerequisite for T cells to infiltrate the islets throughout the pathogenesis of T1D (11, 33). How then can one explain the polyclonal and mainly naïve nature of the lymphocyte populations that we detected migrating into the pancreas at the several stages of disease examined? The lack of CD69 induction on most recent immigrant also indicates that most do not respond actively to local antigens. It would seem that, after β-cell antigen-specific cells have penetrated the pancreas and started to establish an insulitic lesion, the door is open to all. This event likely occurs very early on, because we detected the polyclonal influx as early as 4 wk of age in NOD mice. As for the circulation of lymphocytes between lymph nodes, this traffic may be driven by vascular expression of peripheral and mucosal vascular addressins PNAd and MAdCAM-1, which have been demonstrated in infiltrated islets (49). This notion of an initial and specific "seeding event" followed by less-specific amplification may explain the heterogeneous nature of the insulitis in NOD mice and humans, completely unaffected islets coexisting with heavily infiltrated ones.

Whether all cells that enter stay there is another matter, although the substantial number of photoconverted cells detected in the pancreas after 1 wk indicates that this is not a transient phenomenon. We found the recent immigrants to reside predominantly in the inflammatory infiltrate, with only a minor proportion in the β-cell area. This localization contrasts with prior studies reporting that transferred pancreas antigen-reactive

T cells primarily attach to, and extravasate from, the islet microvasculature localized to islets (12, 13). The difference is plausibly linked to the time frame (4 h vs. 24–72 h) and antigen specificity (cognate vs. polyclonal): rapid antigen-driven adherence and extravasation, in one case, more progressive and likely chemokine driven in the other. It is unclear whether antigen-specific T cells that enter the islet through the vasculature inside the β-cell mass remain there, or whether they also end up in the main inflammatory infiltrate outside the β-cell mass.

The "openness" we observed at all stages of progression and even after reversal of T1D by anti-CD3 begs the question of whether it matters; it implies that the composition and regulatory balance of the infiltrate may be constantly altered, in response to newly generated T cells or to environmental challenges that bias the phenotypes of autoreactive T lymphocytes or of antigen-presenting cells. Differentiation cues imparted to lymphocytes by interaction with the microbiota in frontline tissues may thus be ferried and affect pancreatic autoimmunity. Epitope spreading may also be facilitated. In addition, one might speculate that this constant rejuvenation of the autoimmune infiltrate may be an element that contributes to the loss of therapeutic effect of anti-CD3 beyond a year in patients (this length of follow-up is not available in mice).

In summary, we have demonstrated that the insulitic lesion is dynamic at all stages of disease, continuously fed by a mixed influx of immunocytes, and thus susceptible to evolve over time in response to immunologic or environmental influences.
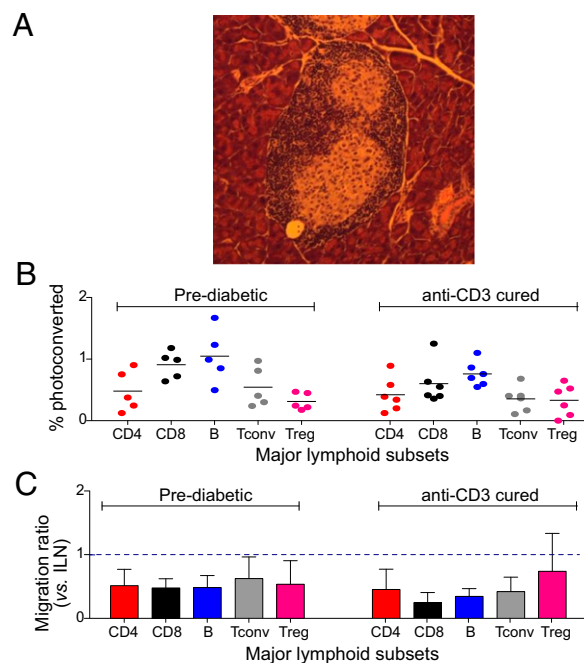
## Materials and Methods

**Fig. 6.** Tracing lymphocyte traffic to the pancreas after therapeutic anti-CD3 treatment. NOD.Kaede mice were treated with anti-CD3 at the onset of diabetes. (A) Histology showing insulitis in an anti–CD3-treated mouse at day 30 after first treatment. Image courtesy of J. Nishio. (B and C) On day 30 after diabetes onset, (B) migration, and (C) migration ratio (vs. ILN) of lymphocytes (CD4$^+$ T, CD8$^+$ T, B cells, Tconv, and Treg cells) into the pancreas was measured by flow cytometry, 72 h after photoconversion of the CLNs. Total of 5–6 mice per group.

diabetic mice (<2 d from diagnosis) were treated, along with insulin pellet supplementation (41), and mice considered cured if normoglycemic beyond 30 d. For BrdU incorporation, 1.2 mg BrdU in 200 μL PBS was administered i.p., two injections 10 h apart. CLN photoconversion was performed (3.5-min transcutaneous illumination with a defocused 405-nm laser, peak power <5 mW; sustained power 0.5–4.9 mW) (22).

**Detection of Cells.** For flow cytometry, the pancreas was harvested (PLNs carefully removed), chopped into 1-mm fragments, and incubated in 1 mg/mL Collagenase Type IV [Life Technologies/Gibco 17104-109], 10 U/mL DNaseI, 1% FCS in DMEM for 18 min at 37 °C in a shaking water bath. This solution was filtered through 40-μm nylon membrane and washed. For detection of BrdU and Foxp3, a staining-buffer set [eBioscience; catalog no. 00-5523-00] was used per manufacturer's instructions. For joint detection of Kaede and Foxp3, cells were fixed (2% paraformaldehyde, 20 min at room temperature) to partially preserve the Kaede signal, before permeabilization for intracellular staining.

For confocal microscopy, images were collected using a custom-built Olympus FV1000 confocal system (Olympus America) with a XLUMPLFLN 20×

water-immersion objective (N.A. 1.0; Olympus America). Islets in explanted pancreata were imaged 36 h after photoconversion of CLNs. Signals were excited using 405-nm, 473-nm, 559-nm, and 633-nm diode lasers. Emitted fluorescence was separated using dichroic beam splitters SDM473, SDM560, and SDM640 in combination with bandpass filters BA430-455, BA490-540, and BA575-620. Confocal imaging settings were optimized for each fluorophore to prevent photobleaching, phototoxicity, and cross-talk between channels. Six pancreata per group were imaged.

**Statistical Analyses.** Statistical significance, as indicated by asterisks, was determined by the Student's $t$ test (two-tailed, unpaired) or two-way ANOVA. $P < 0.05$ was considered significant. $**P < 0.01$; $***P < 0.001$.

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# Supporting Information

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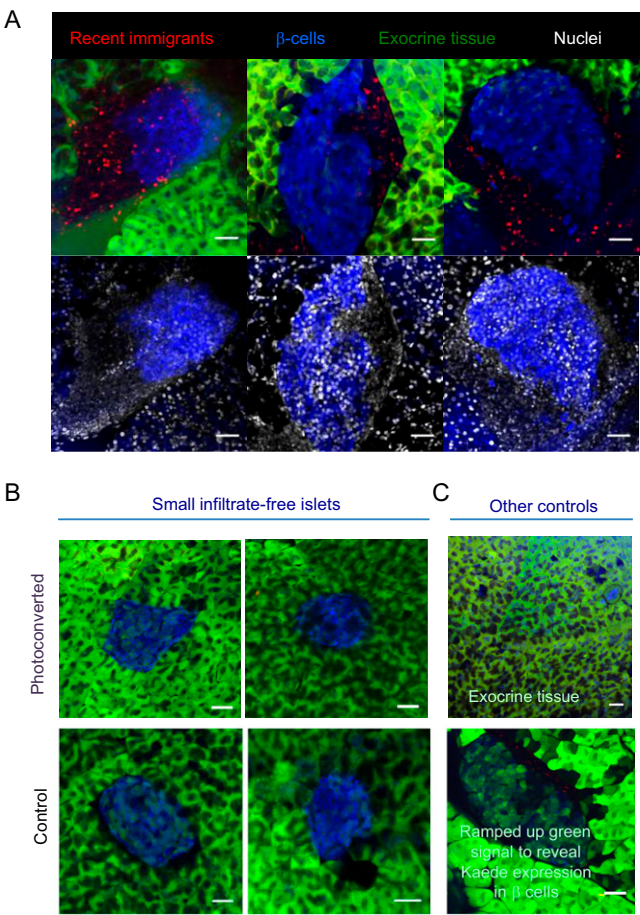


**Fig. S1.** Monitoring cell trafficking to the pancreas. (*A* and *B*) Islets in explanted pancreata from 10- to 12-wk-old mice were imaged by confocal microscopy 36 h after photoconversion of the CLNs. Representative z-stack projections shown here. Six pancreata per group were imaged. (*C*) Other control images depicting lack of recent immigrant cells in exocrine tissue and reduced expression of Kaede in islet compared with exocrine tissue.

**Fig. S2.** Flow cytometry gating strategy. Sample flow gates of (*A*) lymphoid and (*B*) myeloid populations in ILNs. All gated on singlets.

**Fig. S3.** Monitoring cell trafficking to the PLNs. The proportion of each lymphocyte subset population that was comprised of recent immigrants was measured by flow cytometry data generated at 0, 24, 72, or 168 h after photoconversion of the CLNs. Migration of (*A*) CD4$^+$ T and (*B*) B cells to PLNs vs. the control ILNs was plotted.

**Table S1.   Analyzing immune cell infiltrate in the pancreas of disease-prone (NOD) and control mice (C57BL/6, NOD.Eα16)**

| | Age of mice, wk | |
| --- | --- | --- |
| Strain | 4 | 8 |
| C57BL/6 | 134 ± 47 | 20 ± 19 |
| NOD.Eα16 | 33 ± 8 | 120 ± 52 |
| NOD | 8,801 ± 5,500 | 307,000 ± 46,941 |

The background level of total CD45$^+$ cells in pancreata was measured ex vivo using flow cytometry. Mean no. CD45$^+$ cells ± SD is shown.