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Effector-Memory T Cells Develop in Islets and Report Islet Pathology in Type 1 Diabetes

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CD8+ T cells are critical in human type 1 diabetes and in the NOD mouse. In this study, we elucidated the natural history of islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)-specific CD8+ T cells in NOD diabetes using MHC-tetramer technology. IGRP206-214-specific T cells in the peripheral lymphoid tissue increased with age, and their numbers correlated with insulitis progression. IGRP206-214-specific T cells in the peripheral lymphoid tissue expressed markers of chronic Ag stimulation, and their numbers were stable after diagnosis of diabetes, consistent with their memory phenotype. IGRP206-214-specific T cells in NOD mice expand, acquire the phenotype of effector-memory T cells in the islets, and emigrate to the peripheral lymphoid tissue. Our observations suggest that enumeration of effector-memory T cells of multiple autoantigen specificities in the periphery of type 1 diabetic subjects could be a reliable reporter for progression of islet pathology. The Journal of Immunology, 2014, 192: 572–580.

The pancreas is inaccessible for study in type 1 diabetes (T1D). Therefore, parameters in the blood have been investigated to study the natural history of islet immunopathology. However, the detection of Ag-specific T cells in peripheral blood has been difficult because of their low frequency and affinity. Sensitive assays have been developed to measure the number and function of islet-specific T cells from the blood of patients, including MHC tetramers and functional assays, such as ELISPOT (1). Although T cell responses to islet Ags, such as insulin, preproinsulin (2), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), protein tyrosine phosphatase, receptor type, N (3), and glutamic acid decarboxylase (4), have been detected in the blood of recent-onset T1D patients, these tests are not yet used in routine clinical practice. Although progress continues to be made, some studies reported a significant difference in the frequency of autoreactive T cells between recent-onset T1D subjects and healthy controls (3), whereas other studies reported no difference (5, 6). Moreover, some functional assays require intensive in vitro stimulation before responses in recent-onset patients are detected; in such scenarios, responses are often found in healthy humans, as well.

The NOD mouse model is commonly deemed as the best model of T1D (7). In contrast to humans, it is possible to compare peripheral blood parameters and islet pathology in mice. MHC class I tetramers that detect Ag-specific T cells have been used to detect CD8+ T cells specific for the islet Ag IGRP206-214 (referred to as “IGRP-specific CD8+ T cells” in this article). Their numbers in peripheral blood predicted development of diabetes but correlated poorly with islet pathology (8). This result was interpreted as indicating that T cells activated in the draining lymph node can be detected in the blood en route to the islets. Recently, our work (9) highlighted that changes to CD8+ T cells occur in the islet itself rather than in the node, including upregulation of granzyme B and γ-IFN. These findings raised the possibility that effector T cells in the peripheral blood are not on their way to the islet, but the reverse is true: they have exited from the islet. Therefore, their numbers reflect proliferation and expansion in the islet rather than cells generated in the peripheral lymphoid tissue or elsewhere. This is significant for immunopathogenesis, as well as for diagnostic testing. For example, if Ag-specific effector-memory T cells in the blood were causally linked to insulitis, they may be a better distinguishing biomarker for T1D subjects than just Ag-specific T cells.

Using tetramer-enrichment technology to enumerate naive and memory IGRP-specific CD8+ T cell frequencies, we studied the natural history of islet-specific T cells in the NOD mouse. We show that IGRP-specific CD8+ T cells in the peripheral lymphoid tissue (pooled spleen and nonpancreatic lymph nodes) and blood of NOD mice correlate with severity of insulitis. These cells are Ag-experienced cells with an effector-memory phenotype that previously acquired cytotoxic and memory function in the islets. Thus, islet-specific T cells in the peripheral lymphoid tissue emigrate from infiltrated islets, and their increased numbers is contributed to by intraislet proliferation.
Materials and Methods

Mice

All animal studies conducted at St. Vincent’s Institute or at the Walter and Eliza Hall Institute followed the guidelines approved by the respective institutional animal ethics committee. NOD.TNFr1−/− and NOD.RIP-SCOS1 mice were described previously (10, 11). NODPl mice (transgenic for proinsulin under the MHC class II promoter) are protected from diabetes, and IGRP-specific CD8⁺ T cells are not expanded (12, 13). NOD.Pl mice express the TCRβ rearrangements of the H-2Kd-restricted, β-cell-reactive, CD8⁺ T cell clone NY.83. NOD.41 mice express the TCRβ rearrangements of the I-Aβ-restricted CD4⁺ T cell clone NY.4.1 (14)

Flow cytometry

Abs used were anti-CD4 (RM4-5), anti-CD3 (145-2C11), anti-CD44 (1M7), anti-CD69 (H1.2F3), anti-2L2 (MEL-14) (BD Biosciences), anti-CD11c (N418), anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-F4/80 (BMS8), anti-CD127 (AT834), anti-KLRL1 (2F1/KLRL1) (BioLegend), and anti-CD8α (5H10) (Invitrogen). Analysis was performed on a FACSFortessa (BD) using FlowJo software (TreeStar). Intracellular staining was performed using a Cytofix/Cytoperm kit (BD Biosciences). For the gating strategy, single cells were gated on forward and side scatter, and we excluded dead cells using propidium iodide. From the live cell population, CD11c⁺ CD11b⁺ B220⁻ F4/80⁻ CD3⁺ cells were gated as the T cell population for analysis. The CD8⁺ T cells were then gated, and the IGRP tetramer–positive population was analyzed.

Tetramer and magnetic bead–based enrichment

A tetramer and magnetic bead–based enrichment method was described (15). Single-cell suspensions (10⁷ cells) were stained with 8 nM IGRP206–214 (NY.LKTWFL) H-2Kd tetramer (ImmunoID, Parkville, VIC, Australia), washed, and stained with anti-PE MicroBeads (Miltenyi Biotec). Magnetic separation was performed using an AutoMACSpro system (Miltenyi Biotec).

Whole pancreas digestion

Whole pancreas were cut into small pieces and digested mechanically for 25 min in 0.35 mg/ml collagenase and DNase. The digested sample was filtered through a 70-μm mesh, and T cells were separated from acinar tissue by a Ficoll gradient.

Histological analysis

Immunohistochemical staining and scoring of frozen pancreas sections were performed as described (16). Pancreas sections were scored for insulitis. Islets were scored and given a grade of 0 to 4: 0 = no infiltrate; 1 = peri-islet infiltrate (<25% islet destruction); 2 = intraislet infiltrate (<50% islet destruction); 3 = intraislet infiltrate (50–75% islet destruction); and 4 = complete islet destruction. The insulitis score was calculated as follows: [0.25 × number of grade-1 islets] + [0.5 × number of grade-2 islets] + [0.75 × number of grade-3 islets] + number of grade-4 islets/total number of estimated islets. For staining of CD4⁺ and CD8⁺ T cells, frozen sections were stained with biotinylated anti-CD4 and anti-CD8 (BD Pharmingen) using the avidin–biotin system for immunoperoxidase staining (Vector Laboratories, Burlingame, CA).

Islet grafting

Islets of Langerhans were isolated from mice according to methods described previously (17). Islets were grafted under the kidney capsule of NOD.RAG-1−/− mice. Recipient mice were monitored for blood glucose levels weekly. Mice with blood glucose > 15 mM on two consecutive days were considered diabetic.

In vivo CTL assay

The in vivo killer assay was modified from Oehlen and Brudusch–Riem (18). Single-cell suspensions of NOD/Lt splenocytes (10⁵ cells/ml) were used as targets. We used NOD/Lt splenocytes because IGRP-specific CD8⁺ T cells in NOD/Lt splenocytes were undetectable (data not shown). Targets were pulsed with 2.5 μg/ml IGRP206–214 peptide. Peptide-pulsed cells were labeled with 5 μM CFSE (Molecular Probes; CFSE™). Control unpulsed cells were labeled with 0.5 μM CFSE (CFSE™). A total of 5 × 10⁶ cells of each was pooled and injected i.v. into recipients. Single-cell suspensions of pancreatic lymph nodes and spleen were collected and analyzed 18–20 h later. The percentage specific lysis was calculated as (1 − CFSEhi/NOD) × 100, where r = CFSEhi population/CFSElo population.

Results

Ag-experienced IGRP-specific CD8⁺ T cells increase in the peripheral lymphoid tissue as mice age

To accurately estimate the low frequency of autoreactive T cells, we used the recently described method of combined use of fluorochrome-labeled tetramers and magnetic particle–based cell enrichment. We stained cells in the peripheral lymphoid tissue (pooled spleen and nonpancreatic lymph nodes, ~2 × 10⁵ total cells) with PE-conjugated IGRP tetramer and then with magnetic beads coupled to Abs specific for PE. We pooled the spleen and the nonpancreatic lymph nodes because the frequency of IGRP-specific CD8⁺ T cells in individual lymph nodes is too low to give a useful readout. Moon et al. (15), who described this technique, also pooled these cells. The sample was passed through a magnetic column to capture all of the tetramer-bound cells plus ~10⁶ contaminants. Therefore, the total number of cells in this bound fraction was small enough that it could be analyzed in its entirety by flow cytometry. The tetramer-bound cells were distinguished from contaminants by staining with a mixture of fluorochrome-labeled Abs specific for T cell– and non–T cell–specific surface proteins (dump Abs). In this way, we could estimate the absolute number of Ag-specific T cells in the peripheral lymphoid tissue in a whole NOD mouse (Fig. 1A). Hereafter, the number of cells in peripheral lymphoid tissue indicates the absolute number of cells from the pooled spleen and inguinal and mesenteric lymph nodes in a whole mouse.

The absolute number of IGRP-specific CD8⁺ T cells in the peripheral lymphoid tissue of young NOD mice was low (6–8 wk old = 49 ± 13) but increased after 10 wk (12–15 wk old = 5056 ± 1247) (Fig. 1B, 1C). The percentage of CD8⁺ T cells that are IGRP-specific also increased after 10 wk (6–8 wk old = 0.002 ± 0.001%, 12–15 wk old = 0.148 ± 0.06, p = 0.005) (Fig. 1D). The number of IGRP-specific CD8⁺ T cells was maintained at high levels (>2000) in the peripheral lymphoid tissue of old NOD mice. In diabetic NOD mice maintained on insulin for 6 wk, there were very few islets remaining in the pancreas, but IGRP-specific CD8⁺ T cells remained at high levels in the peripheral lymphoid tissue. The majority of IGRP-specific CD8⁺ T cells detected in the peripheral lymphoid tissue of old NOD mice (12–15 wk old) and diabetic NOD mice were CD44hi Ag-experienced cells compared with those in young NOD mice (6–8 wk old) (Fig. 1A, 1B). These observations led us to hypothesize that the increased population of IGRP-specific CD8⁺ T cells seen in the peripheral lymphoid tissue as insulitis progresses have already been to the islets and migrated back into the peripheral lymphoid tissue.

The frequency of IGRP-specific CD8⁺ T cells in the peripheral lymphoid tissue correlates with severity of insulitis

A previous study (8) did not find a significant correlation between the number of IGRP-specific CD8⁺ T cells in the pancreas and peripheral blood, and it was interpreted that T cells were appearing in blood after priming from the pancreatic lymph nodes rather than from islets. Because the sensitivity of detecting rare Ag-specific T cells is increased using the tetramer-enrichment method, we tested whether there was correlation between insulitis and the abundance of IGRP-specific CD8⁺ T cells in the peripheral lymphoid tissue. We first compared insulitis scores with T cell numbers. The insulitis scores in 15-wk-old NOD mice strongly correlated with the number of IGRP-specific CD8⁺ T cells in the peripheral lymphoid tissue (r = 0.66, p < 0.0001) (Fig. 2A). There was also good correlation between the number of IGRP-specific CD8⁺ T cells in the pancreas and peripheral lymphoid tissue of 8–12-wk-old NOD mice (r = 0.41, p = 0.003) (Fig. 2B).
When we examined the peripheral blood, the number of IGRP-specific CD8+ T cells also correlated with the number of IGRP-specific CD8+ T cells in the pancreas (n = 20, $r^2 = +0.2773$, $p = 0.0171$) (Fig. 2C).

We wished to further explore whether IGRP-specific CD8+ T cells found in the peripheral lymphoid tissue and blood came from the pancreatic lymph nodes after initial priming (i.e., recently differentiated effector cells that may have never been in the islets). To explore this, we made use of NOD mutants that have normal T cell activation and proliferation in the pancreatic lymph nodes but reduced islet infiltration. If peripheral IGRP-specific CD8+ T cells have come from pancreatic lymph nodes, they should be equal in number in mutant mice and in NOD mice. NOD.TNFR1$^{-/-}$ mice are deficient in TNFR1, and NOD.RIPSOCS1 mice overexpress SOCS1 in $\beta$ cells. The absolute number of IGRP-specific CD8+ T cells in the thymus of NOD, NOD.RIPSOCS1, and NOD.TNFR1$^{-/-}$ mice was similar, indicating that the number of IGRP-specific T cells produced in the thymus of these mice is similar (Supplemental Fig. 2C). These mice have reduced insulitis, but the level of proliferation and cell death of transferred IGRP-specific CD8+ T cells in the pancreatic lymph nodes was similar to NOD mice (Supplemental Fig. 2D) (9, 11); the mechanism that accounts for the reduced insulitis in these strains appears to act postpriming and postinitial proliferation in the pancreatic lymph node. The absolute numbers of IGRP-specific CD8+ T cells in peripheral lymphoid tissue of NOD.TNFR1$^{-/-}$ and NOD.RIPSOCS1 mice were greatly reduced compared with NOD mice (157 ± 158 versus 2987 ± 4073, $p = 0.010$; 854 ± 354 versus 2987 ± 4071, $p = 0.005$) (Fig. 2D, 2E). This would be surprising if T cells in peripheral lymphoid tissue came directly from the pancreatic lymph nodes and suggests that homing to islets and proliferation there may be required for the increased IGRP-specific CD8+ T cells in the peripheral lymphoid tissue of older NOD mice.

IGRP-specific CD8+ T cells in the peripheral lymphoid tissue and peripheral blood express memory markers

Because the IGRP-specific CD8+ T cells in the peripheral lymphoid tissue increase with age, we studied the surface phenotype on IGRP-specific CD8+ T cells in the peripheral lymphoid tissue of young (6–8-wk-old) and old (12–15-wk-old) NOD mice. In the young mice, IGRP-specific CD8+ T cells were CD44$^\text{lo}$, CD62L$^\text{hi}$, and CD69$^\text{lo}$ (Fig. 3A). Compared with the young mice, IGRP-specific CD8+ T cells in the peripheral lymphoid tissue of old NOD mice expressed high levels of CD44 and had downregulated CD62L and CD69. CD44$^\text{hi}$, CD62L$^\text{lo}$, and CD69$^\text{lo}$ is the surface phenotype of memory T cells (Fig. 3B). Interestingly, IGRP-specific CD8+ T cells were present in the peripheral lymphoid tissue and circulation after diabetes, when there was very little insulin left in

FIGURE 1. Absolute number of Ag-experienced IGRP$^{206–214}$-specific CD8+ T cells in the peripheral lymphoid tissue of NOD mice increases with age. IGRP-specific CD8+ T cells were enriched using the tetramer and magnetic bead method and enumerated using flow cytometry. (A) Representative flow cytometry diagrams of IGRP-specific T cells in the peripheral lymphoid tissue comparing young (6–8-wk-old) and old (12–15-wk-old) NOD mice. (B) The absolute number of CD44$^\text{lo}$ and CD44$^\text{hi}$ IGRP-specific CD8+ T cells in peripheral lymphoid tissues of young and old NOD mice. The number (C) and percentage (D) of IGRP-specific CD8+ T cells were determined in the peripheral lymphoid tissue of individual NOD mice at various ages. Each symbol represents the absolute number (B, C) or percentage (D) of IGRP-specific CD8+ T cells from an individual mouse. Horizontal lines indicate mean and error bars represent SEM on the scatter plots. The Mann–Whitney U test was used to compare the data sets.
the pancreas (Fig. 3B, Supplemental Fig. 2A, 2B). This is similar to effector-memory T cells seen after a cleared infection because Ag persistence is not required for the maintenance of memory CD8\(^+\) T cells (19), and the number of memory CD8\(^+\) T cells present at the end of an infection is maintained throughout life (20). Hence, we looked at expression of memory markers killer cell lectin-like receptor subfamily G, member 1 (KLRG1) and IL-7R \(\alpha\) (CD127) (21). IGRP-specific CD8\(^+\) T cells in the peripheral lymphoid tissue of 12–15-wk-old NOD mice expressed high levels of KLRG1 and CD127 compared with CD4\(^+\) and CD8\(^+\) T cells (not specific for IGRP) (Fig. 3B). We found that IGRP-specific CD8\(^+\) T cells in the blood, pancreatic lymph nodes, peripheral lymphoid tissue, and bone marrow expressed similar levels of memory markers as did cells from the pancreas (Supplemental Fig. 1). High levels of KLRG1 and CD127 were described as being expressed on subsets of effector-memory T cells that develop in chronic viral infection (e.g., HSV), whereas memory T cells from an acute viral infection (e.g., influenza) would be KLRG1\(^{lo}\) and CD127\(^{hi}\) (22). In T1D, chronic inflammation and repeated exposure of T cells to Ag in the pancreas may explain why IGRP-specific CD8\(^+\) T cells express high levels of memory markers CD127 and KLRG1.

**FIGURE 2.** IGRP\(_{206-214}\) CD8\(^+\) T cells in the peripheral lymphoid tissue correlate with insulitis score in the pancreas. (A) Linear regression between IGRP-specific CD8\(^+\) T cells in the peripheral lymphoid tissue and insulitis score in the pancreas \((n = 18, r^2 = +0.6642, p < 0.0001)\). (B) IGRP-specific CD8\(^+\) T cells from the pancreas and peripheral lymphoid tissue of NOD (8–12-wk-old) were enumerated and plotted on a linear regression graph \((n = 30, r^2 = +0.4064, p = 0.003)\). (C) IGRP-specific CD8\(^+\) T cells from the pancreas and peripheral blood of NOD mice (8–12-wk-old) were enumerated and plotted on a linear regression graph \((n = 20, r^2 = +0.2773, p = 0.0171)\). A two-tailed \(t\) test was used to calculate the \(p\) value of each linear regression. (D) Representative flow cytometry plots of tetramer-enriched IGRP-specific CD8\(^+\) T cells in the peripheral lymphoid tissue of NOD mice compared with NOD. RIPSOCS1 and NOD.TNFR1\(^{-/-}\) mice \((n > 5\) each). (E) Number of IGRP-specific CD8\(^+\) T cells in the peripheral lymphoid tissues of 12–15-wk-old NOD, NOD.RIPSOCS1, and NOD.TNFR1\(^{-/-}\) mice. Horizontal lines indicate the mean, and error bars represent SEM. The Mann–Whitney \(U\) test was used to compare the data sets.

**IGRP-specific CD8\(^+\) T cells acquire memory cell phenotype in the islets**

The timing or intensity of multiple input signals, such as Ag, co-stimulation, and proinflammatory cytokines, can regulate key characteristics of memory T cell development. Because these signals are highest in the islets, we wanted to test whether effector IGRP-specific CD8\(^+\) T cells have to enter the pancreas before they can differentiate into effector-memory T cells. For this, we transferred CFSE-labeled naive IGRP-specific CD8\(^+\) T cells from NOD8.3 TCR-transgenic mice into NOD mice and examined KLRG1 and CD127 expression on the proliferating cells. After 7 d, KLRG1 and CD127 were upregulated on CFSE-labeled cells in the islets but not in the inguinal or pancreatic lymph nodes (Fig. 3C, 3D). This experiment suggests that IGRP-specific CD8\(^+\) T cells acquire effector-memory phenotype in the islets, not in the pancreatic lymph node. In contrast to the transferred naive cells, it is interesting that most endogenous IGRP-specific CD8\(^+\) T cells in the pancreatic lymph nodes displayed an effector-memory phenotype (Supplemental Fig. 1). This suggests they are not naive cells on their way to the islets after priming but are more likely to be T cells that have exited from the islets (i.e., that the IGRP-specific T cells in the pancreatic lymph node of NOD mice are mainly islet emigrants).
FIGURE 3. IGRP_{206-214}-specific CD8^+ T cells in NOD mice display surface markers of effector-memory T cells. Representative plots of surface staining of CD8^+ T cells from the tetramer-enriched peripheral lymphoid tissue comparing NOD mice aged 6–8 wk (A) or 12–15 wk (B) (Figure legend continues)
**IGRP-specific CD8+ T cells in the peripheral lymphoid tissue are functional killers**

We studied the cytotoxic function of IGRP-specific CD8+ T cells in the peripheral lymphoid tissue of young and old NOD mice. For this, functional in vivo CTLs against IGRP peptide were analyzed in 6–8-wk-old and 15-wk-old NOD mice (prediabetic) (Fig. 4A, 4B). NODPI mice served as nonresponder controls, because we showed previously that these mice have a very low frequency of IGRP-specific CD8+ T cells (13). The IGRP-specific CD8+ T cells from 6–8-wk-old mice showed minimal specific lysis in the peripheral lymphoid tissue (0.5 ± 0.4%) and pancreatic lymph nodes (3.8 ± 2.5%). Fifteen-week-old NOD mice showed increased specific lysis in peripheral lymphoid tissue (22.1 ± 4.7%) and pancreatic lymph nodes (30.7 ± 6.3%) \((p = 0.0013\) and 0.0016, respectively, versus NODPI mice; \(p = 0.0006\) and 0.0136, respectively, versus 6–8-wk-old NOD mice; Fig. 4B). We also looked in islets, but unfortunately there was insufficient homing of target cells into the islets to have a useful read-out.

The cells were analyzed for cytokine and CD107a (a marker for degranulation and cytotoxic activity) expression. We showed previously that CD8+ T cells only acquire greater effector function, such as CD107a expression, after being in the islets (9). Some IGRP-specific CD8+ T cells in the peripheral lymphoid tissue of 15-wk-old NOD mice expressed CD107a and TNF-\(\alpha\) without any stimulation (Fig. 4C). When 9-wk-old NOD mice were studied, CD107a was detected in the few CD44hi IGRP-specific cells but not in the CD44lo IGRP-specific CD8+ T cells or CD44hi CD8+ T cells (Fig. 4D). Because we noted that in vivo cytotoxicity of IGRP-specific T cells increases as the mice age, we determined the proportion of CD44lo, IGRP-specific T cells that expressed CD107a, granzyme B, and TNF-\(\alpha\) in older mice (Supplemental Fig. 2E). A small proportion of CD44lo, IGRP-specific T cells (that were not restimulated ex vivo) express CD107a, granzyme B, and TNF-\(\alpha\) (12-wk-old NOD mice: 20.98 ± 3.55%, 3.96 ± 3.97%, and 7.21 ± 1.60%, respectively; diabetic NOD mice: 5.32 ± 0.97%, 15.7 ± 1.50%, and 6.21 ± 1.038%, respectively).

**T cells are able to migrate from islet grafts into the peripheral lymphoid tissue and mediate T1D**

The fact that T cells in peripheral lymphoid tissue have come from islets suggests that at least some effector T cells do not die in the target tissue but exit and recirculate. To determine whether T cells from the islets were able to migrate into the peripheral blood and peripheral lymphoid tissue, 150 islets infiltrated with T cells from NOD (15-wk-old) mice were placed under the kidney capsule of NOD.RAG1-/- mice. Three of five mice with NOD islet grafts developed diabetes in 4–6 wk (Fig. 5A). We could not detect T cells in the peripheral blood of the grafted recipients 1 wk after the procedure, suggesting that T cells did not enter the circulation because of the surgical manipulation (Fig. 5B). Both CD8+ and CD4+ T cells were detected in the spleen and the pancreas of recipient mice that had been grafted with NOD islets (Fig. 5B, 5C). These findings indicate that CD8+ and CD4+ T cells were able to migrate from the transplanted islets into the peripheral lymphoid tissue, invade the endogenous islets, and mediate T1D (Supplemental Fig. 2B). As a control, we also transplanted islets from NOD4.1 (4-wk-old) mice under the kidney capsule of NOD.RAG1-/- mice. NOD4.1 is a CD4-transgenic model that develops accelerated diabetes and was used so that recipients would develop diabetes rapidly. We did not use NOD8.3 CD8-TCR transgenic cells because they do not home into the uninnflamed islets (as in NOD.RAG1-/- mice) to transfer diabetes efficiently. Not surprisingly, three of four NOD.RAG1-/- recipients developed diabetes in 4 wk following transplantation with NOD4.1 islets.

**Discussion**

We present several pieces of experimental evidence that IGRP-specific effector-memory CD8+ T cells in the peripheral lymphoid tissue of NOD mice have emigrated from islets. First, the T cell number in the periphery correlated with the severity of islet pathology and was reduced in mutant strains of NOD mice with reduced islet pathology but normal pancreatic lymph node T cell priming. Second, transferred naive T cells acquired effector memory phenotype in the islets, after exit from the pancreatic lymph node, and the peripheral T cells displayed a phenotype similar to that acquired in islets. Finally, islet-infiltrating T cells emigrated from islets transplanted into syngeneic, but immunodeficient NOD mice, were found in the peripheral lymphoid organs and invaded the native pancreas and caused diabetes, indicating that this chain of events can occur. We suggest that this also can occur from native, untransplanted islets. In T1D, we suggest that islet pathology results in proliferation and differentiation of autoreactive T cells that become significantly greater in number than in unaffected mice or humans.

The persistence in the peripheral lymphoid tissue, even after the diagnosis of diabetes, and the ability to kill Ag-loaded target cells in vivo indicate that IGRP-specific CD8+ T cells have differentiated to a stable effector-memory state. Transfer of naive cells showed that initial Ag contact and activation took place in the pancreatic lymph node. However, differentiation to the subsequently stable effector-memory phenotype was only seen in the pancreatic islets. Effector T cells further proliferate and gain cytotoxic function with high levels of granzyme B and IFN-\(\gamma\) expression in the islets (9). Expansion in the presence of high Ag concentration, help from CD4+ T cells, activated dendritic cells, and high local concentrations of proinflammatory cytokines are likely to be responsible for T cell differentiation and contribute significantly to insulitis progression.

In T1D, CD8+ T cells encounter their cognate Ag many times and undergo multiple rounds of Ag stimulation. It was shown in other settings that every additional Ag stimulation leads to an increase in the number of differentially regulated genes and, thus, to further differentiation of memory CD8+ T cells. Surface expression of CD62L decreased and KLRG1 increased with repeated Ag exposure in memory CD8+ T cells (23). Thus, repeated Ag challenges alter lineage commitment in favor of effector-memory T cell populations (24, 25).
FIGURE 4. IGRP206–214-specific CD8+ T cells in the peripheral lymphoid tissue are functional killers. A total of $10^7$ NOR/Lt splenocytes was pulsed with 2.5 $\mu$g/ml IGRP206–214 peptide. These target cells (IGRP-pulsed cells) were labeled with 5 $\mu$m CFSE and coinjected with control cells (NOR/Lt splenocytes not pulsed with IGRP peptide) labeled with 0.5 $\mu$m CFSE into 6–8-wk-old and 15-wk-old NOD and NODPI recipient mice. Peripheral lymphoid tissue and pancreatic lymph nodes were removed from the recipients 18–20 h after injection of target cells and analyzed by flow cytometry. Specific lysis is reflected by a decrease in the ratio between the target and control populations in 6–8-wk-old and 15-wk-old NOD mice compared with the ratio in NODPI mice. (A) Representative graphs exemplifying IGRP-specific in vivo CTLs in NOD mice showing a reduction in peptide-pulsed CFSEhi cells versus unpulsed CFSElo cells compared with the NODPI nonresponder control. (B) Data show mean IGRP-specific lysis, and each symbol represents an individual mouse. The Mann–Whitney U test was used for statistical analysis. (C) Representative dot plots showing TNF-α and CD107a expression in unstimulated IGRP-specific CD8+ T cells isolated from peripheral lymphoid tissues of 15-wk-old NOD mice. For comparison, TNF-α in naive IGRP-specific CD8+ T cells from 9-wk-old NOD mice is also shown. (D) Ag-experienced CD44hi IGRP-specific CD8+ T cells from 9-wk-old NOD mice express CD107a. The dot plot shows the level of CD44 expression on IGRP-specific CD8+ T cells in 9-wk-old mice, and the graph shows the level of CD107a expression in the indicated cells. The plots are representative of three independent experiments.
KLRG1 and CD127 also provide mechanistic information. KLRG1 is a receptor expressed by NK cells and T cells that binds to E-cadherin (26). KLRG1 is upregulated on T cells only when there is inflammation (27), and it has been used as a blood T cell marker for subjects undergoing chronic viral infections (22). KLRG1 and CD127 expressed together on CD8+ memory T cells represent cells that have experienced persistent viral infections (28), where there is persistent Ag stimulation. However, the significance of the similarity between patterns of KLRG1 and CD127 expression in autoimmune diabetes and certain chronic viral infections, but not in others, is uncertain. Memory CD8+ T cells are maintained by survival and proliferative signals through receptors that contain the common cytokine receptor γ-chain, such as receptors for IL-7 and IL-15 (29). In T1D, recent studies (30, 31) showed that blocking IL-7R restored normoglycemia in diabetic NOD mice, probably by upregulation of PD-1 on memory T cells.

Islet T cells with the surface phenotype of effector-memory T cells subsequently emigrate, most likely via the pancreatic lymph node, to the bloodstream and lymphoid tissue (Supplemental Fig. 3), from there they can potentially recirculate to other islets. Emigration of effector-memory T cells may be linked to islet destruction and loss of cognate Ag, because it did not occur to a significant extent in young mice, even after the onset of insulitis. It was previously thought that T cells might remain in islets and die when Ag is depleted. In our study, T cells were able to migrate from transplanted islets into the native pancreas, possibly via the pancreatic lymph node, and destroy the endogenous islets. We used this experiment as a model for what may happen to T cells in the native pancreatic islets and recognize that the situations are not identical. Islet destruction is a highly asynchronous event and occurs for many weeks before and after diabetes onset in mice and for years before and after diagnosis in humans. Using in vivo microscopy methods, Coppieters et al. (32) visualized local T cells leaving the islets and entering nearby blood vessels. Trudeau et al. reported waves of T cells appearing in the blood, which they interpreted as being due to T cells from the pancreatic lymph node (8); however, our data suggest that this may be due to islets being destroyed. This is important for islet transplantation because effector-memory T cells that have emigrated from the islets into the circulation might be crucial in causing recurrent autoimmune attack of islet grafts.

Understanding the origin of Ag-specific memory T cells is likely to be clinically useful in human diabetes. Isoforms of CD45—CD45RA, found preferentially on naive T cells, and CD45RO, found preferentially on memory T cells—were used to distinguish between the T cell responses in patients with or without T1D. T cell responses to glutamic acid decarboxylase were preferentially detected on CD45RA- cells compared with CD45RA+ (naive) cells in recent-onset T1D subjects (6). In another study (33), T1D patients showed enrichment for CD8+ T cells recognizing the preproinsulin15–24 epitope, with an effector-memory phenotype. It is likely that the data in humans will be more complex than those in the NOD mouse strain, which all have the same genotype. Studying a large patient cohort, grouping of patients based on autoantibody response, and measuring a combination of specific effector-memory T cell responses might be a useful reporter for islet pathology in humans.

In conclusion, these studies indicate that measuring effector-memory Ag-specific T cells may be a useful biomarker for insulitis. Furthermore, they refine established ideas about the relative...
roles of the pancreatic lymph node and the inflamed islet in the development of effector-memory T cells and progression of insulitis. The cells measured in the blood have arrived there after spending time as part of the islet lesion where they expanded and differentiated.

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**A)**

Peripheral blood  | Pancreas  | Peripheral Lymphoid Tissue  | Pancreatic Lymph Nodes  | Bone Marrow

CD44  
CD62L  
KLRG1  
CD127

**B)**

IGRP<sub>206-214</sub> tetramer positive cells

- Peripheral blood
- Pancreas
- Peripheral lymphoid tissue
- Pancreatic lymph node
- Isotype controls

**C)**

- KLRG1 MFI on IGRP<sub>206-214</sub>-specific T cells
- CD127 MFI on IGRP<sub>206-214</sub>-specific T cells

**SUPPLEMENTARY FIGURE 1**
Pancreatic Islets

Naive islet-specific CD8+ T cells
Activated islet-specific CD8+ T cells
Islet-specific effector-memory CD8+ T cells

Pancreatic Lymph Node

Circulation & peripheral lymphoid tissue

Thymus

NOD 12-15-WEEK-OLD

Migration of naive CD8+ T cells
Migration of activated CD8+ T cells
Migration of effector-memory CD8+ T cells

Differentiation into effector memory T cells
SUPPLEMENTARY FIGURE 1. A) Representative FACS plots showing expression of indicated surface markers on IGRP\textsubscript{206-214}-specific CD8\textsuperscript{+} T cells enriched from the blood, pancreas, peripheral lymphoid tissue, pancreatic lymph nodes and bone marrow cells of 12-15-week-old NOD mice (n=7). B) Representative histograms comparing different surface markers on IGRP-specific CD8\textsuperscript{+} T cells from the blood, pancreas, peripheral lymphoid tissue and pancreatic lymph node of 12-15-week-old NOD mice (n=7). C) Scatter plot of MFI of KLRG1 and CD127 on IGRP-specific CD8\textsuperscript{+} T cells in blood, pancreas, peripheral lymphoid tissue and pancreatic lymph node. Error bars represent mean and SEM. One-way ANOVA with a Bonferroni’s test was used to compare all the sets of data, and in both graphs the columns are not statistically significant.

SUPPLEMENTARY FIGURE 2. A) Representative histological sections comparing insulin content in the pancreas between diabetic and non-diabetic NOD mice (n=5). B) Representative histological analysis of insulin, CD4 and CD8 in a diabetic NOD pancreas. C) Representative flow cytometry plots of the absolute number of IGRP-specific CD8\textsuperscript{+} T cells in the thymus of NOD, NOD.RIPSOCS1 and NOD.TNFR1-/- mice. (n > 5 for each strain) D) CFSE labeled 8.3 T cells were injected into 15-week-old NOD and NOD.RIPSOCS1 mice. The percentage of transferred T cells that were annexin V positive was analysed in the pancreatic lymph nodes of these mice. Each plot is representative of 3 experiments. E) Representative dot plots showing CD107a, TNF\alpha and granzyme B expression in unstimulated IGRP-specific CD8\textsuperscript{+} T cells isolated from peripheral lymphoid tissues of 12-week-old NOD mice and diabetic NOD mice. For comparison, CD107a, TNF\alpha and granzyme B in naïve IGRP-specific CD8\textsuperscript{+} T cells from peripheral lymphoid tissues of NOD8.3 mice is also
SUPPLEMENTARY FIGURE 3. Proposed schematic diagram depicting the development of islet-specific CD8\(^+\) T cells in a 12-15 week-old NOD mouse. Naïve islet-specific T cells (blue) escape thymic selection and enter the pancreatic lymph nodes and get activated (green) and proliferate. Activated T cells then enter the islets where they proliferate further and differentiate into effector-memory T cells (red). Colored arrows represent the migration of islet-specific T cells between the different organs.