

In situ recognition of autoantigen as an essential gatekeeper in autoimmune CD8⁺ T cell inflammation

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A current paradigm states that non-antigen-specific inflammatory cues attract noncognate, bystander T cell specificities to sites of infection and autoimmune inflammation. Here we show that cues emanating from a tissue undergoing spontaneous autoimmune inflammation cannot recruit naive or activated bystander T cell specificities in the absence of local expression of cognate antigen. We monitored the recruitment of CD8⁺ T cells specific for the prevalent diabetogenic epitope islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)_{206–214} in gene-targeted nonobese diabetic (NOD) mice expressing a T cell “invisible” IGRP_{206–214} sequence. These mice developed islet inflammation and diabetes with normal incidence and kinetics, but their inflammatory lesions could recruit neither naive (endogenous or exogenous) nor ex vivo-activated IGRP_{206–214}-reactive CD8⁺ T cells. Conversely, IGRP_{206–214}-reactive, but not nonautoreactive CD8⁺ T cells rapidly homed to and accumulated in the inflamed islets of wild-type NOD mice. Our results indicate that CD8⁺ T cell recruitment to a site of autoimmune inflammation results from an active process that is strictly dependent on local display of cognate pMHC and suggest that CD8⁺ T cells contained in extralymphoid autoimmune lesions are largely autoreactive.

diabetes | lymphocyte | islet-specific glucose-6-phosphatase catalytic subunit-related protein | islet inflammation | lymphocyte recruitment

Recognition of cognate peptide-major histocompatibility complexes (pMHC) on the surface of dendritic cells (DC) by naive T lymphocytes in lymph nodes draining a site of infection or autoimmune inflammation elicits the lymphocytes' activation, proliferation, and differentiation into cytolytic effectors. Upon activation, lymphocytes also acquire the ability to survey nonlymphoid tissues for presence of their cognate target antigens, with a preference for inflamed tissues as well as tissues drained by the lymph nodes where activation took place (1–3). Studies in a number of infection and autoimmune disease models have suggested that recruitment of T lymphocytes into a site of extralymphoid inflammation does not require local expression of cognate (foreign or self) pMHC on tissue cells or tissue-associated antigen-presenting cells (APCs) (4–7). Accordingly, it is generally thought that non-antigen-specific inflammatory cues such as cytokines and chemokines emanating from the local microenvironment can recruit noncognate (i.e., bystander) T cells to a site of foreign or self antigen-triggered tissue inflammation (8–19). Notwithstanding that in vitro-activated bystander T cell clones can transiently comigrate with their cognate counterparts into noninflamed tissue in adoptive T cell transfer experiments and that tissue-specific expression of cytokine and/or chemokine transgenes in normal tissues can trigger bystander T cell inflammation, these models do not faithfully mimic the events that take place in spontaneous autoimmune inflammation. Specifically, it is unclear that bystander T cell specificities can effectively compete with their cognate polyclonal counterparts, recognizing pMHC in situ, for occupation of the inflammatory space.

Type 1 diabetes (T1D) in both humans and nonobese diabetic (NOD) mice is a chronic autoimmune disease that results from inflammation of pancreatic islets and destruction of pancreatic β cells by T cells targeting numerous β cell autoantigens (20, 21). A significant fraction of the islet-associated CD8⁺ T cells in NOD

mice recognize the mimotope NRP-V7 in the context of the MHC molecule K^d (22–25). These CD8⁺ T cells are diabetogenic (22, 26), target a peptide from islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP_{206–214}, similar to NRP-V7) (27), and circulate in the peripheral blood at a relative high frequency (>1/200 CD8⁺ T cells), particularly as clinical disease nears (27, 28).

To ascertain whether local expression of cognate pMHC is a sine-qua-non condition for recruitment and/or accumulation of CD8⁺ T cells to the pancreas during spontaneous autoimmune diabetes, we generated a gene-targeted NOD strain capable of developing islet inflammation but expressing a T cell “invisible” IGRP_{206–214} epitope. We find that these mice develop insulinitis and diabetes essentially like wild-type NOD mice but cannot recruit endogenous or exogenous IGRP_{206–214}-specific CD8⁺ T cells, regardless of their activation state or degree of islet inflammation.

Results and Discussion

Knock-in NOD Mice Expressing a T cell Invisible IGRP_{206–214} Epitope. We generated a gene-targeted NOD strain expressing a mutant form of IGRP in which the two T cell receptor (TCR)-contact residues of IGRP_{206–214} (25) are replaced with alanines (K209A and F213A) (Fig. 1A). The IGRP_{K209A/F213A} peptide cannot trigger the activation or elicit the cytotoxicity of 8.3-CD8⁺ T cells (25), which express a transgenic IGRP_{206–214}-reactive TCR (22), and does not impair, either in vitro or in vivo, their responsiveness to a subsequent challenge with IGRP_{206–214} (Fig. S1). As expected, IGRP_{K209A/F213A}-homozygous knock-in NOD mice (NOD.IGRP_{K209A/F213A}^{KI/KI}) and wild-type NOD mice displayed indistinguishable thymic and splenic T cell profiles (Fig. 1B) and exported similar numbers of IGRP_{206–214}-reactive CD8⁺ cells to the circulation (Fig. 1C). Analyses of pancreata from prediabetic animals indicated that both types of mice developed insulinitis lesions of similar severity (Fig. 1D), similar CD4⁺ T cell content, and slightly different (but not statistically different) CD8⁺ T cell content (Fig. 1E).

Notably, however, the islet-associated T cells of prediabetic NOD.IGRP_{K209A/F213A}^{KI/KI} mice did not contain IGRP_{206–214}-reactive CD8⁺ T cells, as determined by NRP-V7/K^d tetramer staining (Fig. 1F), and did not produce IFN γ in response to NRP-V7 peptide-pulsed APCs (Fig. 1G). Impaired recruitment of IGRP_{206–214}-reactive CD8⁺ T cells was associated with a significant increase in recruitment of other autoreactive T cell specificities that

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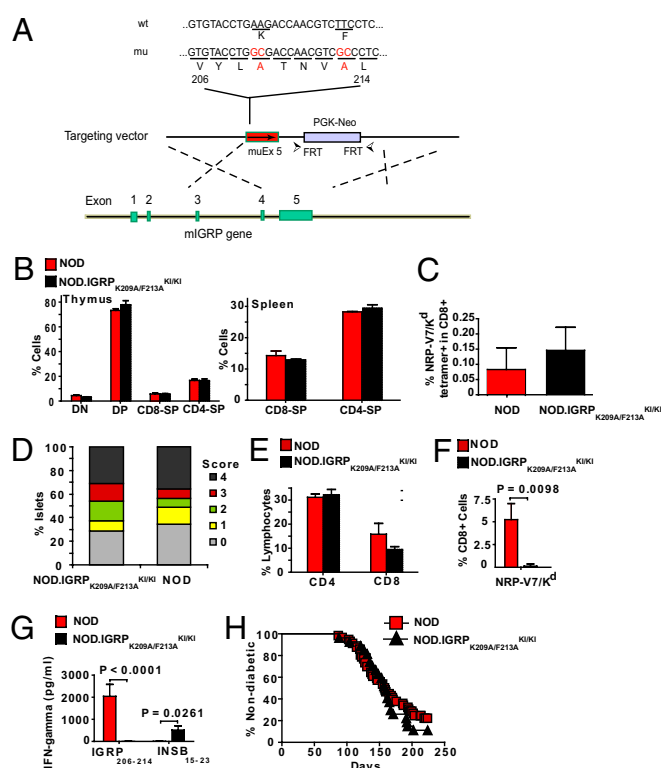


Fig. 1. NOD.IGRP_{K209A/F213A}^{KI/KI} mice develop insulinitis and diabetes without recruiting IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T cells into pancreatic islets. (A) Targeting strategy. The FRT-flanked PGK-neo cassette was removed from targeted ES cells by transient transfection of Flp recombinase-encoding cDNA. (B) Distribution of lymphocyte subsets in thymus and spleen of NOD and NOD.IGRP_{K209A/F213A}^{KI/KI} mice ($n = 3$ and 5 , respectively; 3 independent experiments). DN, double negative; DP, double positive; CD8-SP, CD8 single positive; CD4-SP, CD4 single positive. (C) Frequency of NRP-V7-reactive CD8⁺ T cells in peripheral blood. Peripheral blood mononuclear cells (PBMCs) from 10-week-old mice (NOD, $n = 7$; NOD.IGRP_{K209A/F213A}^{KI/KI}, $n = 8$) were stained with NRP-V7/K^d tetramers and anti-CD8 mAb. Data correspond to 4 independent experiments using one to five mice/experiment. (D) Insulinitis scores. Pancreata from nondiabetic 32-week-old mice (NOD, $n = 3$; NOD.IGRP_{K209A/F213A}^{KI/KI}, $n = 5$) were examined for islet inflammation. Pancreata were from one cohort of NOD mice and two different cohorts of NOD.IGRP_{K209A/F213A}^{KI/KI} mice. (E and F) CD4⁺ and CD8⁺ T cell (E) and NRP-V7/K^d tetramer⁺ CD8⁺ T cell content (F) in freshly isolated islets of NOD ($n = 6$; 3 independent experiments) vs. NOD.IGRP_{K209A/F213A}^{KI/KI} mice ($n = 7$; 4 independent experiments). (G) Absence of IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T cells in the islet infiltrates of NOD.IGRP_{K209A/F213A}^{KI/KI} ($n = 10$; 10 independent experiments) vs. NOD mice ($n = 3$; 3 independent experiments). Islet-associated CD8⁺ T cells were cultured in IL-2 for 7 days and challenged with peptide-pulsed (10 μ M) irradiated NOD splenocytes. The IFN γ content in the supernatants (at 48 h) was measured by ELISA. Data correspond to the means \pm SEM. (H) Diabetes incidence in female NOD ($n = 56$) and NOD.IGRP_{K209A/F213A}^{KI/KI} ($n = 27$) mice. The average blood glucose levels in newly diagnosed diabetic NOD and NOD.IGRP_{K209A/F213A}^{KI/KI} mice are: 22.3 ± 2.5 vs. 23.7 ± 3.3 mM, respectively. In males, the incidence and average age at onset of disease were also similar in both strains (NOD: $n = 15$; 40% diabetic at 133 ± 33 days; and NOD.IGRP_{K209A/F213A}^{KI/KI}: $n = 20$; 50% diabetic at 149 ± 36 days). Data in B–F correspond to the means \pm SEM. *P* values in F and G were obtained with Mann-Whitney *U*-test.

are present at very low precursor frequencies in the islets of prediabetic mice (20, 21, 28), such as insulin-B₁₅₋₂₃-reactive CD8⁺ T cells (29) (Fig. 1G). As a result, NOD and NOD.IGRP_{K209A/F213A}^{KI/KI} mice developed T1D with virtually identical incidence curves (Fig. 1H). These data indicated that (i) IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T cells are completely excluded from insulinitic lesions in the absence of local expression of IGRP₂₀₆₋₂₁₄ and that (ii) initiation and progression of spontaneous T1D in NOD mice does not

require the accumulation of IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T cells into pancreatic islets.

Severely Impaired Recruitment of Naive IGRP₂₀₆₋₂₁₄-Reactive CD8⁺ T Cells to the Inflamed Islets of NOD.IGRP_{K209A/F213A}^{KI/KI} Mice. To further investigate the role of local cognate pMHC vs. non-antigen-specific inflammatory cues in the recruitment of CD8⁺ T cells to pancreatic islets, we ascertained whether naive and in vitro-preactivated IGRP₂₀₆₋₂₁₄-reactive 8.3-CD8⁺ T cells could undergo activation in the pancreatic lymph nodes (PLN) and/or home to the inflamed islets of prediabetic 10- to 12-week-old NOD.IGRP_{K209A/F213A}^{KI/KI} hosts (i.e., in response to preexisting local inflammatory cues). Adoptively transferred naive CFSE-labeled 8.3-CD8⁺ T cells (10^7) proliferated in the PLNs [and, to a much lesser extent, in the mesenteric lymph nodes (MLNs) and spleen] of insulinitic NOD mice within a week after adoptive transfer (Fig. 2A and B). Analysis of the islet infiltrates of these insulinitic NOD hosts 1, 2, and 3 weeks after T cell transfer revealed rapid recruitment (within 1 week) of actively proliferating 8.3-CD8⁺ T cells (Figs. 2C–F). Notably, almost all of the 8.3-CD8⁺ T cells found within islets at this stage had undergone more than two cell divisions, and most of the cells that had only divided fewer than three times were found exclusively in the PLNs (Fig. 2C and Fig. S2), suggesting that recruitment of autoreactive CD8⁺ T cells into the pancreas is invariably preceded by antigen-induced activation in the PLNs. The islets (but not the PLNs) of hosts analyzed 2 weeks after T cell transfer contained higher percentages of proliferating cells (Fig. 2C and E and Fig. S2) and total 8.3-CD8⁺ T cells (Fig. 2D and F). By the third week, there was a further increase in the extent of cell division in islets (Fig. 2C and Fig. S2) in association with reductions in the percentages and total number of proliferated 8.3-CD8⁺ T cells, presumably due to attrition by activation-induced cell death (i.e., in response to repetitive stimulation of differentiated CD8⁺ T cells by cognate pMHC) (Fig. 2D–F). Thus, accumulation of autoreactive CD8⁺ T cells in the inflamed islets of prediabetic NOD mice is associated with (i) T cell activation and proliferation in the PLNs, (ii) recruitment of actively proliferating cells into pancreatic islets, and (iii) additional rounds of local (intra-islet) proliferation.

A remarkably different outcome was obtained when these experiments were done in age-matched, insulinitic NOD.IGRP_{K209A/F213A}^{KI/KI} hosts. Whereas the transfused 8.3-CD8⁺ T cells readily homed to the spleen, PLNs, and MLNs of insulinitic NOD.IGRP_{K209A/F213A}^{KI/KI} hosts (Fig. 2C and D), they did not proliferate in the PLNs (Fig. 2A–C and E and F and Figs. S2 and S3), confirming that this event requires cross-presentation of β cell-derived IGRP₂₀₆₋₂₁₄. There was also a reduction in the proliferation of cognate 8.3-CD8⁺ T cells in the MLNs and spleens of NOD.IGRP_{K209A/F213A}^{KI/KI} vs. NOD mice (Fig. 2B and C), suggesting that some of the T cells that are activated in the PLNs and/or islets (or the activating IGRP₂₀₆₋₂₁₄-loaded APCs) of wild-type NOD mice migrate to distant secondary lymphoid organs during disease progression. Most notably, the adoptively transferred cells failed to home to pancreatic islets of insulinitic NOD.IGRP_{K209A/F213A}^{KI/KI} hosts, where they could not be found throughout the 3-week study period (Fig. 2C–F), despite the presence of severe local inflammation (Fig. 1D). In fact, up to more than 6% ($\sim 10^4$) of all of the islet-associated lymphocytes of NOD hosts were donor derived, compared to virtually none of those isolated from the NOD.IGRP_{K209A/F213A}^{KI/KI} hosts (Fig. 2F). Thus, nonspecific inflammatory cues emanating from insulinitic lesions cannot single-handedly (in the absence of local cognate pMHC) recruit naive bystander IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T cells to the site.

T1D-Irrelevant CD8⁺ T Cell Specificities Are Not Recruited to the Inflamed Pancreatic Islets of Wild-Type NOD Mice. To rule out the possibility that this outcome was a peculiarity of the IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T cell population, we tracked the recruitment of adoptively transferred naive Thy1.2⁺ lymphocytic choriomeningitis

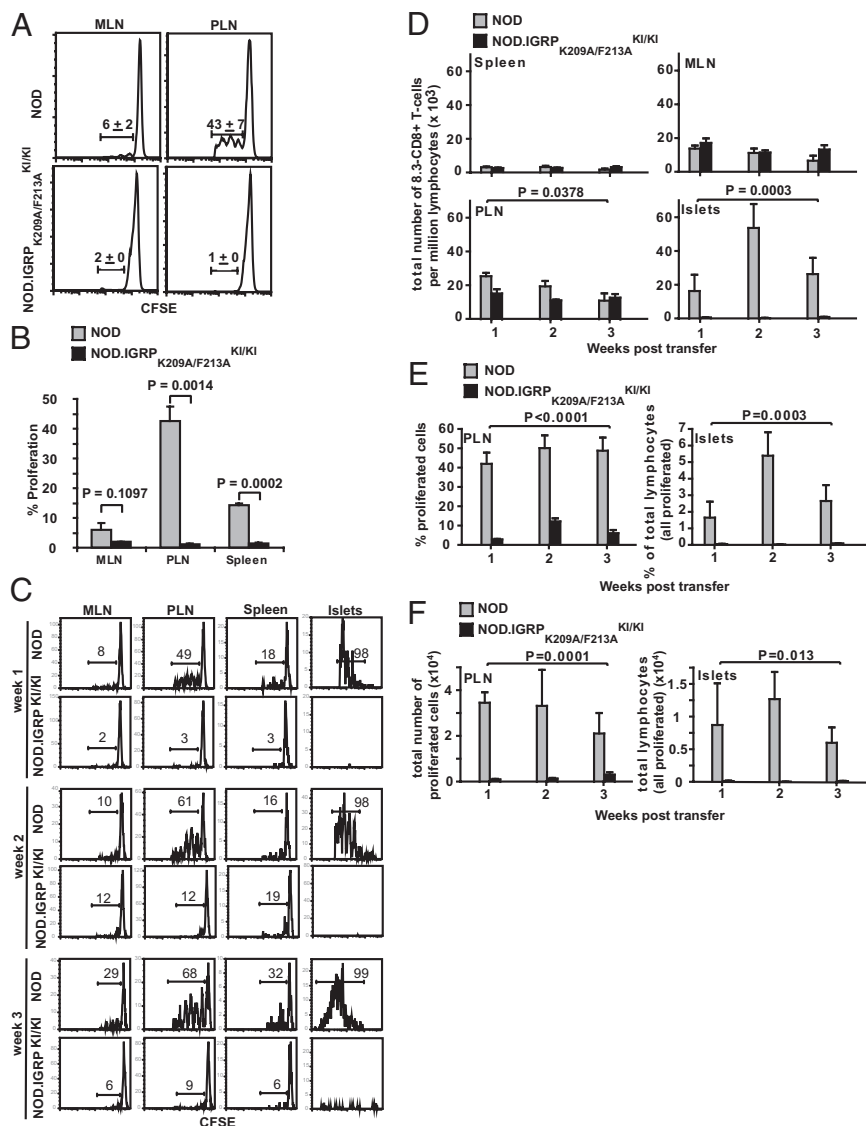


Fig. 2. Naive 8.3-CD8⁺ T cells are not recruited into the inflamed pancreatic islets of NOD.IGRP_{K209A/F213A}^{KI/KI} mice. (A) Proliferation of naive 8.3-CD8⁺ T cells in the PLN. CFSE-labeled naive 8.3-CD8⁺ T cells were transfused into 10- to 12-week-old NOD ($n = 3$) or NOD.IGRP_{K209A/F213A}^{KI/KI} ($n = 3$) recipients (three independent experiments, each using both host types). Dilution of CFSE was measured by flow cytometry 7 days posttransfer. Values correspond to the average percentage of proliferated CD8⁺ T cells \pm SEM. (B) Summary of data described in A. P values were obtained with Mann-Whitney U -test. (C) Recruitment and proliferation of naive 8.3-CD8⁺ T cells from 8.3-NOD.Thy1.1 donor mice in the lymphoid organs and islets of 10- to 12-week-old insulinitic NOD and NOD.IGRP_{K209A/F213A}^{KI/KI} hosts 1, 2, and 3 weeks after transfer. CFSE histograms correspond to Thy1.1⁺CD8⁺ cells. (D) Mean \pm SEM of total numbers of 8.3-CD8⁺ T cells per million lymphocytes. Data in C and D correspond to three to six experiments/time point and host type (one mouse/time point/host in each experiment). (E) Mean \pm SEM of percentages of proliferated cells (for PLNs) or total number of donor lymphocytes (for islet T cell isolates, where all donor-derived T cells were proliferating) (three to six experiments/time point and host type; one mouse/time point/host in each experiment). (F) Absolute numbers of proliferated (for PLNs) or recruited (for islet T cell isolates) 8.3-CD8⁺ T cells (mean \pm SEM) (three to six experiments/time point and host type; one mouse/time point/host in each experiment). P values in D–F were obtained by two-way ANOVA.

virus (LCMV) gp33-specific CD8⁺ T cells [a nonautoreactive, T1D-irrelevant T cell population from LCMV gp33-TCR-transgenic (P14) NOD donor mice] in insulinitic NOD.Thy1.1 hosts. This was done by cotransfusing these cells with an equal number of naive Thy1.2⁺ 8.3-CD8⁺ T cells and by analyzing the hosts' lymphoid organs and pancreatic islets for presence of both T cell pools 1, 7, 14, and 21 days after transfer. Whereas Thy1.2⁺ NRP-V7/K^d-tetramer⁺ and gp33/D^b-tetramer⁺ CD8⁺ T cells were rapidly recruited to the spleen, PLNs, and MLNs within 1 day of transfer, only the former homed to a significant degree to pancreatic islets (Fig. 3A and B). Accordingly, naive IGRP_{206–214}-reactive CD8⁺ T cells need to engage cognate pMHC on local APCs and/or on β cells to access and/or accumulate (owing to retention and/or proliferation) in inflamed tissue.

Preactivated IGRP_{206–214}-Specific Cytotoxic T Lymphocytes Also Fail to Home to the Insulinitic Lesions of NOD.IGRP_{K209A/F213A}^{KI/KI} Hosts. To investigate the role of T cell activation in the recruitment and/or accumulation of bystander T cells to inflamed and noninflamed islets, we transfused CFSE-labeled, in vitro-differentiated Thy1.1⁺ 8.3-cytotoxic T lymphocytes (CTLs) (1.5×10^7) into noninsulinitic (3-week-old) or insulinitic (10- to 12-week-old) NOD and NOD.IGRP_{K209A/F213A}^{KI/KI} hosts. Whereas CFSE⁺ 8.3-CTLs were rapidly recruited into noninsulinitic NOD islets (Fig. 4A), leading to rapid loss of insulin-producing β cells and rampant development of diabetes in all hosts within 5 days (Fig. 4B), they were neither recruited to islets nor caused any obvious β cell loss or diabetes in any noninsulinitic NOD.IGRP_{K209A/F213A}^{KI/KI} hosts for up to 6 weeks after transfer (Fig. 4A and B), suggesting that activated

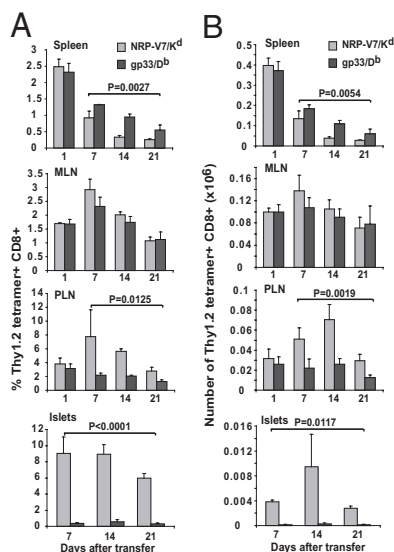


Fig. 3. Selective recruitment of cognate autoreactive T cells into pancreatic islets of NOD mice. (A) Naive 8.3-CD8⁺/Thy1.2⁺ and P14-CD8⁺/Thy1.2⁺ T cells were mixed at 1:1 (8×10^6 cells each) and injected i.v. into 10- to 12-week-old NOD.Thy1.1 hosts. Hosts were killed at various time points after transfer and their peripheral lymphoid organs and islet cell suspensions analyzed for presence of NRP-V7/K^d and GP33/D^b tetramer⁺ Thy1.2⁺ CD8⁺ T cells. Data correspond to three mice/time point ($n = 12$ mice) and three independent experiments (one mouse/time point in each experiment) and are shown as mean \pm SEM. (B) Data from A presented as absolute numbers of cells (mean \pm SEM). *P* values were obtained with two-way ANOVA.

CD8⁺ T cells cannot home to noninflamed tissue in the absence of local cognate pMHC. Similar results were obtained when 8.3-CTLs were transfused into insulitic hosts. The 8.3-CTLs were progressively recruited to, and accumulated in the PLNs and MLNs of both types of mice during the first 3 days after transfer (~2- to 3-fold on day 3 vs. day 1; Fig. 4 C and D). In contrast, whereas CFSE⁺ 8.3-CTL accumulated in the islets of insulitic NOD mice (~7- to 9-fold on day 3 vs. day 1), they did not do so in the insulitic islets of NOD.IGRP_{K209A/F213A}^{KI/KI} hosts (Fig. 4 C and D), confirming a critical role for cognate pMHC on retention and accumulation of IGRP₂₀₆₋₂₁₄-specific CTL in nonlymphoid tissue. Taken together, these data suggest that T cell occupation of the inflamed islet space in spontaneous autoimmune diabetes is not due to “diffusion” from the periphery in response to inflammatory and chemotactic cues, but rather to an active process that involves local recognition of cognate pMHC.

Our observations challenge the generally held assumption that T cell infiltrates in inflamed extralymphoid tissues, such as pancreatic islets in diabetes, contain a mixture of both cognate and noncognate (i.e., bystander) T cell specificities. Our results demonstrate, in a model of highly polyclonal spontaneous autoimmunity, that bystander CD8⁺ T cells, even after activation, are strongly selected against for retention and accumulation in the target organ. This contention does not argue against the idea that bystander T cells can transiently migrate to a site of inflammation nonspecifically, such as in response to cytokine-induced chemokine receptor ligands like CXCL9, CXCL10, and CXCL11 (30, 31). Rather, our data strongly argue that, in the absence of cognate pMHC, nonspecifically recruited CD8⁺ T cells cannot effectively compete with cognate T cell specificities for occupation of space. Recent studies in dual TCR retrogenic, sublethally irradiated bone marrow chimeras coexpressing T1D-relevant and irrelevant MHC class II-restricted autoreactive CD4⁺ T cell specificities in NOD.scid hosts suggest that naive CD4⁺ T cells may also require local engagement of cognate pMHC for recruitment to noninflamed islets (32). However, whether bystander

naive and/or activated CD4⁺ T cells can home to inflamed islets, particularly in a model of spontaneous polyclonal inflammation such as the one described herein, remains to be determined.

Whichever the case for CD4⁺ T cells might be, and assuming that this paradigm can be generalized to other antigenic specificities, our data suggest that the majority of all CD8⁺ T cells that are recruited to sites of autoimmune inflammation, such as pancreatic islets during diabetogenesis, are autoreactive. Autoreactive CD8⁺ T cells need not have to engage cognate pMHC directly on the β cell surface to be effectively retained at the target site; recognition of pMHC on vascular endothelial cells (2, 33) or on a tissue-resident professional APC population might be sufficient. This would explain why NOD mice expressing a RIP-driven adenoviral E19 transgene, whose β cells express significantly reduced levels of pMHC class I (34), and NOD mice with a β cell-specific disruption of β -2 microglobulin, which cannot display pMHC class I complexes on the surface (35), recruit CD8⁺ T cells to pancreatic islets.

Materials and Methods

Mice. The 8.3-TCR-transgenic NOD mice (Thy1.2⁺) have been described (22). Thy1.1-congenic NOD mice (NOD.Thy1.1) and LCMV-Gp33-specific TCR-transgenic (P14) NOD mice were obtained from the T1D repository (The Jackson Laboratory). To generate IGRP_{K209A/F213A}^{KI/KI} mice, we transfected an *igrp* targeting construct carrying a mutated exon 5 (encoding an IGRP₂₀₆₋₂₁₄ epitope in which the two TCR contact residues were replaced by Ala: (VYLA)TNV(A); K209A/F213A) into CK35 129/Sv-derived murine embryonic stem (ES) cells (Fig. 1). The FRT-flanked PGK-neo cassette was removed from targeted ES cells by transient transfection of Flp recombinase-encoding cDNA. Transfected ES clones were screened by Southern blot analysis using AvrII or Apal-digested DNA and 5'- and 3'-specific probes differentiating wild-type (11.6 and 11.1 kb for 5' and 3' arms, respectively) vs. targeted bands (7.7 and 6.7 kb, respectively). Type II recombinants arising from Flp-mediated deletion of the PGK-neo cassette were identified by Southern blotting using the 3' probe described above. To produce NOD.IGRP_{K209A/F213A}^{KI/WT} mice, we backcrossed the targeted IGRP_{K209A/F213A} allele from germline-competent 129/Sv chimeras onto the NOD background for at least six generations. Genomewide SNP analyses at the N6 backcross were done to confirm homozygosity for all known NOD diabetes-susceptibility alleles. Mice were intercrossed at the N6 or N7 generations to produce NOD.IGRP_{K209A/F213A}^{KI/KI} homozygotes. These studies were approved by the Faculty of Medicine's Animal Care Committee and followed the guidelines of the Canadian Council of Animal Care.

Peptides and Tetramers. The peptides IGRP₂₀₆₋₂₁₄ (VYLKTNVFL), INS_{B15-23} (LYLVCGERG), NRP-V7 (KYNKANVFL), TUM (KYQAVTTTL), and LCMV GP33 (KAVYFNATM) and the corresponding tetramers (PE labeled) were prepared as described (27, 28). Briefly, the peptides were folded with recombinant human β 2m and respective mouse heavy chains and subjected to gel filtration purification, biotinylation, and ion exchange purification using an AKTA FPLC system (GE Healthcare). The final product was verified by both denaturing SDS/PAGE and native PAGE analysis.

Islet Isolation. Pancreatic islets were isolated by hand-picking after collagenase P digestion of the pancreas, cultured overnight in IL-2-containing media [to avoid additional enzymatic digestion steps and thus enhance T cell recovery and viability, as described (36)], disrupted into single cells, stained, and analyzed by flow cytometry.

Flow Cytometry. Peripheral blood and islet cell suspensions were stained with tetramers (5 μ g/mL) in FACS buffer (0.1% sodium azide and 1% FBS in PBS) for 1 h at 4 $^{\circ}$ C, washed, and incubated with FITC-conjugated anti-CD8 α (5 μ g/mL) for 30 min at 4 $^{\circ}$ C. For other stains, thymi, spleens, and lymph node cell suspensions were analyzed by 3-color flow cytometry using anti-CD8-PerCP (53-6.7), anti-CD4-FITC (IM7), and tetramer-PE, or with anti-CD8-PerCP (53-6.7), tetramer-PE, and FITC-conjugated anti-Thy1.2 mAb, or with anti-CD8-PerCP (53-6.7) and PE-conjugated anti-Thy1.1 mAb. Cells were washed, fixed in 1% PFA/PBS, and analyzed by FACS. All mAbs were from BD Pharmingen. Data were analyzed by FlowJo (Tree Star).

Specificity of Islet-Associated CD8⁺ T Cells. Islet-infiltrating cells from NOD or NOD.IGRP_{K209A/F213A}^{KI/KI} mice were cultured for 7 days in the presence of 0.5 units/mL of rIL-2 to expand in vivo-activated islet-associated T cells. Upon washing, CD8⁺ T cells were cultured, in the absence of exogenous IL-2, with peptide-pulsed (10 μ M) irradiated NOD splenocytes for 48 h. The IFN γ con-

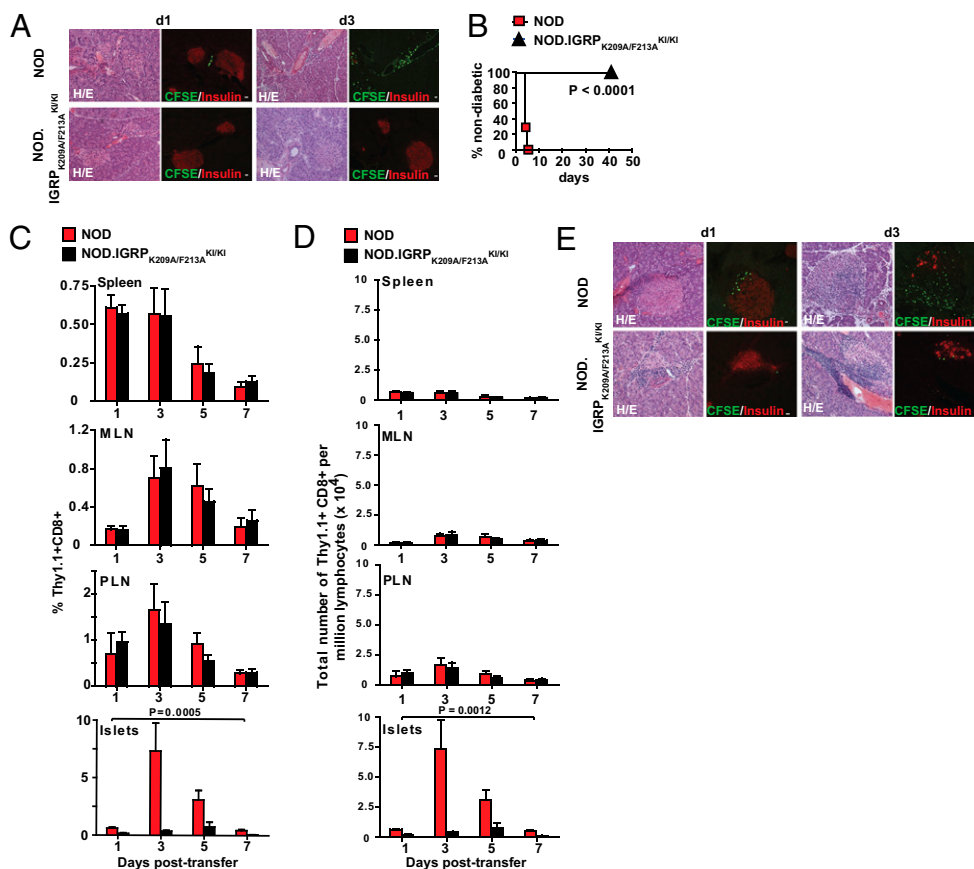


Fig. 4. The pancreatic islets of NOD.IGRP^{K209A/F213A}^{K1/K1} mice also fail to recruit activated 8.3-CD8⁺ T cells, even when inflamed. (A) In vitro-activated, CFSE-labeled 8.3-CD8⁺ T cells were injected i.v. into 3-week-old NOD or NOD.IGRP^{K209A/F213A}^{K1/K1} hosts. Pancreatic sections were stained with anti-insulin antibodies and examined for presence of CFSE⁺ T cells by confocal microscopy on days 1 and 3 after transfer (three mice/time point; 10–20 islets/mouse; three independent experiments). Representative images are shown. An adjacent tissue section was stained with H&E (Left). (White scale bars, 20 μ m.) On NOD hosts' day 1 samples, CFSE⁺ T cells were predominantly found in the peri-insular space. Note the near complete depletion of insulin⁺ cells in the CFSE⁺ T cell-containing areas of NOD hosts' day 3 samples. (B) Incidence of diabetes in 3-week-old NOD ($n = 7$) or NOD.IGRP^{K209A/F213A}^{K1/K1} recipients ($n = 6$) of 8.3-CTL (two independent experiments, each including three to four mice/strain type). P values were obtained with log rank test. (C) In vitro-activated, CFSE-labeled 8.3-CD8⁺ Thy1.1⁺ T cells were injected i.v. into 10- to 12-week-old NOD or NOD.IGRP^{K209A/F213A}^{K1/K1} hosts. Hosts were analyzed for presence of Thy1.1⁺ CD8⁺ T cells in different organs. Data correspond to three to four independent experiments/time point and strain (one mouse/time point/strain in each experiment) and are shown as mean \pm SEM. (D) Data from C presented as absolute numbers of cells per million lymphocytes (mean \pm SEM). P values in C and D were obtained with two-way ANOVA. (E) Analysis of pancreas sections from 10- to 12-week-old mice transfused with CFSE-labeled in vitro-activated 8.3-CD8⁺ T cells on days 1 and 3 after transfer, for presence of CFSE⁺ T cells (three independent experiments; one mouse/time point/strain in each experiment). Images of islets are representative of severe insulinitis.

tent in the supernatants was measured by ELISA (Duoset, R&D Systems). Values obtained with the negative control peptide TUM were subtracted.

Adoptive Transfer. Splenic CD8⁺ T cells were purified using iMAG CD8 beads (BD Bioscience) following the manufacturer's protocols, labeled with CFSE (2.5 μ M), and injected i.v. (10^7 CD8⁺ T cells). In cotransfer experiments employing P14 CD8⁺ T cells, each mouse received a mixture of 8×10^6 8.3-CD8⁺ and 8×10^6 P14-CD8⁺ T cells. To generate in vitro-activated 8.3-CD8⁺ T cells, splenocytes from 8.3-NOD or 8.3-NOD.*Thy1.1* donor mice were cultured in the presence of NRP-V7 peptide (1 μ M) for 3 days in the absence of exogenous IL-2. These conditions generate highly diabetogenic CTL (Fig. 4B). The proliferating CD8⁺ T cells (>95% of the cells) were then labeled with 2.5 μ M CFSE and transfused i.v. (15×10^6) into unmanipulated 3-week- or 10- to 12-week-old hosts. Mice were killed 1, 3, 5, 7, 14, or 21 days later and their spleens, PLNs, and MLNs examined for presence of donor CD8⁺ T cells (Tetramer⁺ and thyl.2⁺) or for dilution of CFSE in the CD8⁺ gate.

Immunopathology. Formalin-fixed, paraffin-embedded pancreas sections were stained with H&E and scored for insulinitis (see below). To examine pancreatic islets for infiltration by transfused CFSE⁺ T cells, pancreata were embedded in OCT medium and frozen in a dry ice/acetone bath. Cryosections (5 μ m) were fixed with 3% paraformaldehyde, stained with guinea pig anti-insulin antibodies and Cy3-conjugated rabbit anti-guinea pig antibodies (Invitrogen). The sections were then mounted with prolong-Gold (Invitrogen) and analyzed

with an Olympus FV1000 confocal microscopy system. Immediately adjacent sections were stained with H&E. We analyzed \sim 20 islets per mouse.

Insulinitis Scores. Scoring of insulinitis lesions was performed as described (26). The degree of mononuclear cell infiltration was scored as: 0, none; 1, peri-insulinitis; 2, infiltration covering <25% of the islet; 3, covering 25–50% of the islet; and 4, covering >50% of the islet.

Diabetes. Diabetes was monitored by measuring urine glucose levels twice weekly. Animals were considered diabetic after two consecutive readings greater than or equal to 3⁺. The average blood glucose levels in mice diagnosed as diabetic using this criteria are 21.96 ± 3.8 mM, and none of these mice have blood glucose levels below 16 mM.

Statistical Analyses. Data were compared by two-tailed Mann-Whitney U -test, χ^2 , or two-way ANOVA tests. Statistical significance was assumed at $P < 0.05$.

Online Supplemental Material. Fig. S1 shows that the IGRP^{K209A/F213A} epitope is not recognized by, and does not alter the functional responsiveness of IGRP_{206–214}-reactive CD8⁺ T cells either in vitro or in vivo. Fig. S2 shows that recruitment of naive 8.3-CD8⁺ T cells into the pancreatic islets of NOD mice is preceded by antigen-induced proliferation in the PLN. Fig. S3 shows that naive 8.3-CD8⁺ T cells do not proliferate in the PLN of NOD.IGRP^{K1/K1} hosts.

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