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

Jinguo Wang1, Sue Tsai1,2, Afshin Shameli2, Jun Yamanouchi3, Gonnie Alkemade1,2, and Pere Santamaria1,2

1Julia McFarlane Diabetes Research Centre and Department of Microbiology and Infectious Diseases, Institute of Infection, Immunity and Inflammation, Faculty of Medicine, University of Calgary, Calgary, AB, Canada T2N 4N1; and 2Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, 2300 RC, Leiden, The Netherlands

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” IGRP206-214 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 IGRP206-214-reactive CD8+ T cells. Conversely, IGRP206-214-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.

Results and Discussion

Knock-in NOD Mice Expressing a T cell Invisible IGRP206-214 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 IGRP206-214 (25) are replaced with alanines (K209A and F213A) (Fig. L4). The IGRP(K209A/F213A) peptide cannot trigger the activation or elicit the cytotoxicity of 8.3-CD8+ T cells (25), which express a transgenic IGRP206-214-reactive TCR (22), and does not impair, either in vitro or in vivo, their responsiveness to a subsequent challenge with IGRP206-214 (Fig. S1). As expected, IGRP(K209A/F213A)-homozygous knock-in NOD mice (NOD.IGRPK209A/F213A-KI/KI) and wild-type NOD mice displayed indistinguishable thymic and splenic T cell profiles (Fig. 1B) and exported similar numbers of IGRP206-214-reactive CD8+ cells to the circulation (Fig. 1C). Analyses of pancreata from prediabetic animals indicated that both types of mice developed insulitis 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.IGRPK209A/F213A-KI/KI mice did not contain IGRP206-214-reactive CD8+ T cells, as determined by NRP-V7/K4 tetramer staining (Fig. 1F), and did not produce IFNγ in response to NRP-V7 peptide-pulsed APCs (Fig. 1G). Impaired recruitment of IGRP206-214-reactive CD8+ T cells was associated with a significant increase in recruitment of other autoreactive T cell specificities that


The authors declare no conflict of interest.

This article is a PNAS Direct Submission. Freely available online through the PNAS open access option.

1J.W. and S.T. contributed equally to this work.

2To whom correspondence should be addressed. E-mail: psantama@ucalgary.ca.

This article contains supporting information online at www.pnas.org/cgi/doi/10.1073/pnas.0913835107/DCSupplemental.
CD8
250
100
200

Fig. 1. NOD.IGRP209A/F213A KI/KI mice develop insulin 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 thymi and spleens from NOD and NOD.IGRP209A/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; CD4-SP, CD4 single positive; CD4-SP, CD4 single positive. (C) Frequency of NRP-V7/214-reactive CD8+ T cells in peripheral blood. Peripheral blood mononuclear cells (PBMCs) from 10-week-old mice (NOD, n = 7; NOD.IGRP209A/F213A KI/KI, n = 8) were stained with NRP-V7/214-K4 tetramers and anti-CD8 mAb. Data correspond to 4 independent experiments using one to five mice/experiment. (D) Insulin secretion. Pancreata from non-diabetic 32-week-old mice (NOD, n = 9; NOD.IGRP209A/F213A KI/KI, n = 5) were examined for islet inflammation. Pancreata were from one cohort of NOD mice and two different cohorts of NOD.IGRP209A/F213A KI/KI mice. (E and F) CD4+ and CD8+ T cell (E) and NRP-V7/K4 tetramer+ CD8+ T cell content (F) in freshly isolated islets of NOD (n = 6; 3 independent experiments) vs. NOD.IGRP209A/F213A KI/KI mice (n = 7; 4 independent experiments). (G) Absence of IGRP206-214-reactive CD8+ T cells in the islet infiltrates of NOD versus NOD.IGRP209A/F213A KI/KI mice (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 μM) irradiated NOD spleenocytes. The IFNγ content in the supernatants (at 48 h) was measured by ELISA. Data correspond to the means ±SEM. (H) Diabetes incidence in female NOD (n = 56) and NOD.IGRP209A/F213A KI/KI (n = 27) mice. The average blood glucose levels in newly diagnosed diabetic NOD and NOD.IGRP209A/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.IGRP209A/F213A KI/KI, n = 20; 50% diabetic at 149 ± 36 days). Data in B–F correspond to the means ±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.IGRP209A/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 insulin 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.IGRP209A/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-precultured 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.IGRP209A/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 insulitic NOD mice within a week after adoptive transfer (Fig. 2 A and B). Analysis of the islet infiltrates of these insulitic 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. 2 C–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. 2 C 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. 2 C and E and Fig. S2) and total 8.3-CD8+ T cells (Fig. 2 D and F). By the third week, there was a further increase in the extent of cell division in islets (Fig. 2 C 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. 2 D–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 (intraislet) proliferation.

A remarkably different outcome was obtained when these experiments were done in age-matched, insulitic NOD.IGRP209A/F213A KI/KI hosts. Whereas the transfused 8.3-CD8+ T cells readily homed to the spleen, PLNs, and MLNs of insulotic NOD.IGRP209A/F213A KI/KI hosts (Fig. 2 C and D), they did not proliferate in the PLNs (Fig. 2 A–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.IGRP209A/F213A KI vs. NOD mice (Fig. 2 B 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 insulotic NOD.IGRP209A/F213A KI/KI hosts, where they could not be found throughout the 3-week study period (Fig. 2 C–F), despite the presence of severe local inflammation (Fig. 1D). In fact, up to more than 6% (~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.IGRP209A/F213A KI/KI hosts (Fig. 2F). Thus, nonspecific inflammatory cues emanating from insulin 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. Naïve 8.3-CD8+ T cells are not recruited to the inflamed pancreatic islets of NOD.IGRPK209A/F213A(KI) mice. (A) Proliferation of naïve 8.3-CD8+ T cells in the PLN. CFSE-labeled naïve 8.3-CD8+ T cells were transfused into 10- to 12-week-old NOD (n = 3) or NOD.IGRPK209A/F213A(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 ± SEM. (B) Summary of data described in A. Values were obtained with Mann-Whitney U-test. (C) Recruitment and proliferation of naïve 8.3-CD8+ T cells from 8.3-NOD.Thy1.1 donor mice to lymphoid organs and islets of 10- to 12-week-old insulitic NOD and NOD.IGRPK209A/F213A(KI) hosts 1, 2, and 3 weeks after transfer. CFSE histograms correspond to Thy1.1"CD8+ cells. (D) Mean ± 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 ± SEM of percentages of proliferated cells (for PLN) 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 PLN) or recruited (for islet T cell isolates) 8.3-CD8+ T cells (mean ± 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.

Wang et al.

PNAS | May 18, 2010 | vol. 107 | no. 20 | 9319

Preactivated IGRP206-214-Specific Cytotoxic T Lymphocytes Also Fail to Home to the Insulitic Lesions of NOD.IGRPK209A/F213A(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 × 10⁴) into nonsensitizing (3-week-old) or insulitic (10- to 12-week-old) NOD and NOD.IGRPK209A/F213A(KI) hosts. Whereas CFSE+ 8.3-CTLs were rapidly recruited into nonsensitizing 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 nonsensitizing NOD.IGRPK209A/F213A(KI) hosts for up to 6 weeks after transfer (Fig. 4A and B), suggesting that activated
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+K1/K1 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 into 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 autoimmune pancreatitis, that bystander CD8⁺ T cells, once activated, are effectively retained in the target tissue, even after activation, in a model of highly polyclonal spontaneous autoimmunity, such as pancreatic islets during diabetogenesis, are autoautoreactive. Autoautoreactive CD8⁺ T cells now do 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-transgenic NOD (NOD.IGRP206-214) mice, we transfected an adenoviral targeting construct carrying a mutated exon 5 (encoding an IGRP206-214 epitope in which the two TCR contact residues were replaced by Ala: (VLYLATNVAL/K209AF213AKCOX12 5N-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 cells were screened by Southern blot analysis using Avril or Apal-digested DNA and 5′ - and 3′-specific probes differentiating wild-type and mutated (11.6 and 11.1 kb for 5′ - and 3′-probes) restriction 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.IGRP206-214K1/K1 mice, we backcrossed the targeted IGRP206-214K1 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.IGRP206-214K1/K1 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 (VLYLATNVAL), ILSB15-23 (LYLVCGERG), NRP-V7 (KYNKANVFL), TUM (KYQAVTTTL), and LCMV GP33 (KAVYNFATM) and the corresponding tetramers (PE labeled) were prepared as described (27, 28). Briefly, the peptides were folded with recombinant human β2m 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 μg/mL) in FACS buffer (0.1% sodium azide and 1% FBS in PBS) for 1 h at 4 °C, washed, and incubated with FITC-conjugated anti-CD6 (5 μg/mL) for 30 min at 4 °C. For other stains, thymi, spleens, and lymph node cell suspensions were analyzed by 3-color flow cytometry using anti-CD8-PerCP (53-3.67), anti-CD4-FITC (IM7), and tetramer-PE, or with anti-CD8-PerCP (53-6.7), tetramer-PE, and FITC-conjugated anti-Thy-1.2 mAb, or with anti-CD8-PerCP (53-6.7) and PE-conjugated anti-Thy-1.1 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.IGRP206-214K1/K1 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 μM) irradiated NOD splenocytes for 48 h. The IFNγ-con-
in different organs. Data correspond to three to four independent experiments/time point and strain (one mouse/time point/strain in each experiment). Representative images are shown. An adjacent tissue section was stained with H&E (Left). (White scale bars, 20 μm.) 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<sub>K209A/F213A</sub><sup>KI/KI</sup> 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+ T cells were injected i.v. into 10- to 12-week-old NOD or NOD.IGRP<sub>K209A/F213A</sub><sup>KI/KI</sup> 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 ± SEM. (D) Data from C presented as absolute numbers of cells per million lymphocytes (mean ± SEM). P values in C and D were obtained with two-way ANOVA. (E) Analysis of pancreatic 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 insulitis.

Adoptive Transfer. Splenic CD8+ T cells were purified using IMAG CD8 beads (BD Bioscience) following the manufacturer's protocols, labeled with CFSE (2.5 μM), and injected i.v. (10<sup>7</sup> CD8+ T cells). In cotransfer experiments employing P14 CD8+ T cells, splenocytes from 8.3-NOD or 8.3-NOD.Thyl.1 donor mice were cultured in the presence of NRP-V7 peptide (1 μM) 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 μM CFSE and transfused i.v. (15 × 10<sup>6</sup>) 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<sup>+</sup> and thy1.2+) or for dilution of CFSE in the CD8+ gate.

Immunopathology. Formalin-fixed, paraffin-embedded pancreas sections were stained with H&E and scored for insulitis (see below). To examine pancreatic islets for infiltration by transfused CFSE<sup>+</sup> T cells, pancreata were embedded in OCT medium and frozen in a dry ice/acetone bath. Cryosections (5 μm) 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 ~20 islets per mouse.

Insulitis Scores. Scoring of insulitis lesions was performed as described (26). The degree of mononuclear cell infiltration was scored as: 0, none; 1, peri-insulitis; 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<sup>2</sup>, or two-way ANOVA tests. Statistical significance was assumed at P < 0.05.

Online Supplemental Material. Fig. S1 shows that the IGRP<sub>K209A/F213A</sub> epitope is not recognized by, and does not alter the functional responsiveness of IGRP<sub>val.214-reactive</sub> 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<sub>K209A/F213A</sub><sup>KI/KI</sup> hosts.
ACKNOWLEDGMENTS. We thank N. Ghyselinck for the pFlEx vector; S. Bou, M. DeCrom, M. Foote, B. Han, T. Irvine, H. Metselaar, and S. Thiesen for technical assistance; L. Kennedy and L. Robertson for FACS; and Y. Yang and J. Clemente-Casares for feedback on the manuscript. This work was supported by the Canadian Institutes of Health Research (CIHR), The Natural Sciences and Engineering Research Council of Canada, and the Juvenile Diabetes Research Foundation (JDRF). J.W. was supported by a fellowship from the Canadian Diabetes Association. S.T. and A.S. were supported by a CIHR-training grant (S.T.) and the Alberta Heritage Foundation for Medical Research (AHFMR) (S.T. and A.S.). G.A. was supported by the Dutch Diabetes Research Foundation. P.S. is a scientist of the AHFMR and a JDRF scholar. The authors have no conflicting financial interests.


Supporting Information

Wang et al. 10.1073/pnas.0913835107

**A** Response of 8.3-CTL against TUM, IGRP$_{206-214}$, or IGRP$_{K209A/F213A}$ in the presence of TUM or IGRP$_{K209A/F213A}$

**B** Cytotoxicity of 8.3-CTL pre-challenged with TUM or IGRP$_{K209A/F213A}$ against peptide-pulsed targets

**C** Responsiveness of naive 8.3-CD8+ T-cells pre-treated with TUM or IGRP$_{K209A/F213A}$ to peptide-pulsed APCs

**D** Response of 8.3-CTL against IGRP$_{206-214}$ in the presence of TUM or IGRP$_{K209A/F213A}$

**E** Response of adoptively transferred 8.3-CD8+ T-cells recovered from NOD or NOD.IGRP$_{K209A/F213A}$ KI/KI hosts to IGRP$_{206-214}$, TUM or IGRP$_{K209A/F213A}$

Fig. 5I. The IGRP$_{K209A/F213A}$ epitope is not recognized by, and does not alter the functional responsiveness of IGRP$_{206-214}$-reactive 8.3-CD8$^+$ T cells either in vitro or in vivo. (A) Differentiated 8.3-CD8$^+$ T cells secrete IFNγ in response to splenocytes pulsed with IGRP$_{206-214}$ but not IGRP$_{K209A/F213A}$. IFNγ content in the supernatants was measured at 24 h of culture. (B) IGRP$_{K209A/F213A}$ does not inhibit the responsiveness of differentiated 8.3-CD8$^+$ T cells to IGRP$_{206-214}$. Differentiated 8.3-CD8$^+$ T cells were cultured with IGRP$_{206-214}$-pulsed (0.1 μg/mL) splenocytes in the presence of various concentrations of TUM or IGRP$_{K209A/F213A}$ for 24 h and the supernatants collected to measure the IFNγ concentration. (C) Pretreatment of naive 8.3-CD8$^+$ T cells with IGRP$_{K209A/F213A}$ (or TUM) peptide does not alter their subsequent responsiveness to IGRP$_{206-214}$. Naive splenic 8.3-CD8$^+$ T cells were preincubated with 10 μg/mL IGRP$_{K209A/F213A}$ or TUM for 2 days. CD8$^+$ T cells were then purified and tested for their ability to proliferate (Left) and secrete IFNγ (Right) in response to bone marrow-derived dendritic cells pulsed with 0.1 μg/mL IGRP$_{206-214}$, TUM, or IGRP$_{K209A/F213A}$. (D) Differentiated 8.3-CD8$^+$ T cells cannot kill targets pulsed with IGRP$_{K209A/F213A}$, and preincubation of 8.3-CD8$^+$ CTL with IGRP$_{K209A/F213A}$ does not inhibit their cytotoxic activity against RMA-SK$^4$ targets pulsed with IGRP$_{206-214}$. The 8.3-CD8$^+$ CTLs were preincubated with bone marrow-derived dendritic cells pulsed with 1 or 10 μg/mL of either TUM or IGRP$_{K209A/F213A}$ for 24 h, purified away from DCs using mAb-coated magnetic beads, and used as effectors in a standard $^{51}$Cr-release assay using RMA-SK$^4$ cells pulsed with 10 μg/mL of IGRP$_{206-214}$, TUM or IGRP$_{K209A/F213A}$ at an 8:1 effector:target ratio in triplicate wells. Percentage of killing was calculated as $^{51}$Cr in test well – spontaneous release(maximum release – spontaneous release) x 100. (E) Encounter of IGRP$_{K209A/F213A}$ by 8.3-CD8$^+$ T cells in vivo does not impair their functional responsiveness to IGRP$_{206-214}$ ex vivo. Ten million 8.3-CD8$^+$ T cells (Thy1.2$^+$) were adoptively transferred into NOD.Thy1.1 or NOD.IGRP$_{K209A/F213A}$ KI/KI hosts (8- to 12-weeks old). Seven days posttransfer, Thy1.2$^+$ 8.3-CD8$^+$ T cells were isolated from the spleen and lymph nodes of the hosts using antibody-coated magnetic beads and tested for proliferation and IFNγ against irradiated NOD splenocytes pulsed with TUM, IGRP$_{206-214}$, or IGRP$_{K209A/F213A}$. Data correspond to mean ± SE of triplicate cultures.
**Fig. S2.** Recruitment of naive 8.3-CD8\(^+\) T cells into the pancreatic islets of NOD mice is preceded by antigen-induced proliferation in the PLN. Recruitment and proliferation of adoptively transferred naive 8.3-CD8\(^+\) T cells (10\(^7\)) from 8.3-NOD.Thy1.1 donor mice in the lymphoid organs and islets of insulitic NOD and NOD.\(\text{IGRP}_{K209A/F213A}^{K2/KI}\) hosts 1, 2, and 3 weeks after transfer. Histograms correspond to percentages of cells within each CFSE peak (i.e., from Fig. 2C) (mean ± SEM). Data correspond to three to six experiments for each time point and host type.

**Fig. S3.** Naive 8.3-CD8\(^+\) T cells fail to proliferate in the PLN of NOD.\(\text{IGRP}_{K209A/F213A}^{K2/KI}\) hosts. Proliferation of adoptively transferred naive 8.3-CD8\(^+\) T cells (10\(^7\)) from 8.3-NOD.Thy1.1 donor mice in the lymphoid organs of insulitic NOD and NOD.\(\text{IGRP}_{K209A/F213A}^{K2/KI}\) hosts 1, 2, and 3 weeks after transfer. Histograms correspond to percentages of nonproliferating CFSE\(^+\) cells (mean ± SEM). Data correspond to three to six experiments for each time point and host type. \(P\) values shown were obtained with two-way ANOVA. Values corresponding to the 1-week and 3-week time points in the spleen and MLNs were also statistically different as measured with Mann-Whitney U-test (spleen: \(P = 0.0011\) and 0.0029, respectively; and MLN: \(P = 0.002\) and 0.0343, respectively).