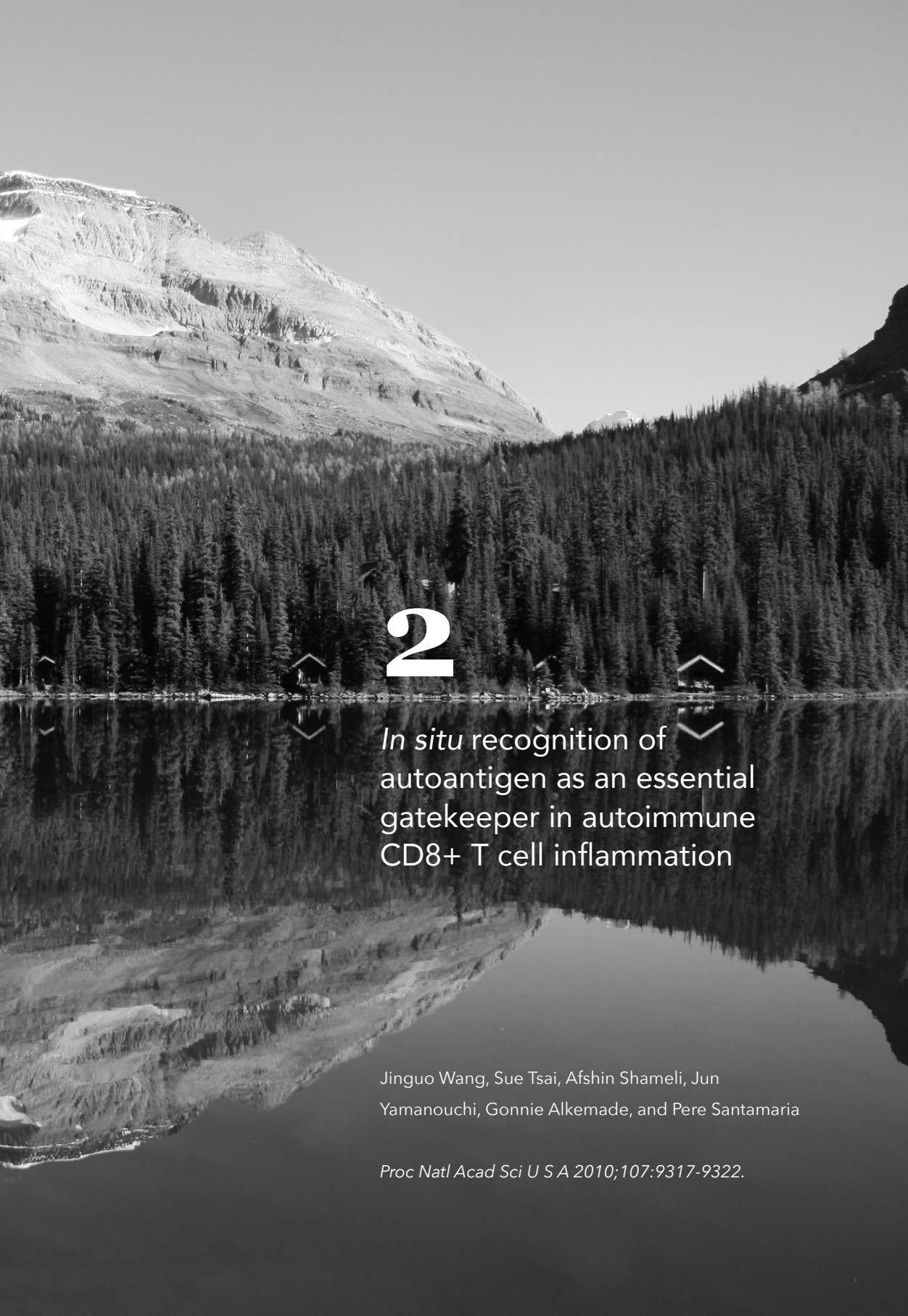


2

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

Jinguo Wang, Sue Tsai, Afshin Shameli, Jun Yamanouchi, Gonnie Alkemade, and Pere Santamaria

Proc Natl Acad Sci U S A 2010;107:9317-9322.

ABSTRACT

A current paradigm states that non-antigen-specific inflammatory cues attract non-cognate, 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 naïve 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 $IGRP_{206-214}$ 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 non-autoreactive 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 extra-lymphoid autoimmune lesions are largely autoreactive.

INTRODUCTION

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 non-lymphoid 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 extra-lymphoid inflammation does not require local expression of cognate (foreign or self) pMHC on tissue cells or tissue-associated antigen-presenting cells (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 non-cognate (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 co-migrate with their cognate counterparts into non-inflamed 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 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+ 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 insulitis 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 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 pre-diabetic 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 pre-diabetic 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 IFNγ 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 are present at very low precursor frequencies in the islets of pre-diabetic mice (20, 21, 28), such as insulin-B$_{15-23}$-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) IGRP$_{206-214}$-reactive CD8+ T-cells are completely excluded from insulitic lesions in the absence of local expression of IGRP$_{206-214}$; and that (ii) initiation and progression of spontaneous T1D in NOD mice does not require the accumulation of IGRP$_{206-214}$-reactive CD8+ T-cells into pancreatic islets.

Severely impaired recruitment of naïve IGRP$_{206-214}$-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*-pre-activated IGRP$_{206-214}$-reactive 8.3-CD8+ T-cells could undergo activation in the pancreatic lymph nodes (PLN) and/or home to the inflamed islets of pre-diabetic 10-12 wk-old NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts (i.e. in response to pre-existing 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 MLN and spleen) of insulitic NOD mice within a wk after adoptive transfer (Fig. 2A and B). Analysis of the islet-infiltrates of these insulitic NOD hosts 1, 2 and 3 wk after T-cell transfer revealed rapid recruitment (within 1 week) of actively proliferating 8.3-CD8+ T-cells (Figs. 2C-F). Notably, almost all the 8.3-CD8+ T-cells found within islets at this stage had undergone >2 cell divisions, and most of the cells that had only divided <3 times were found exclusively in the PLNs (Fig. 2C and Fig. S2), suggesting that recruitment of autoreactive

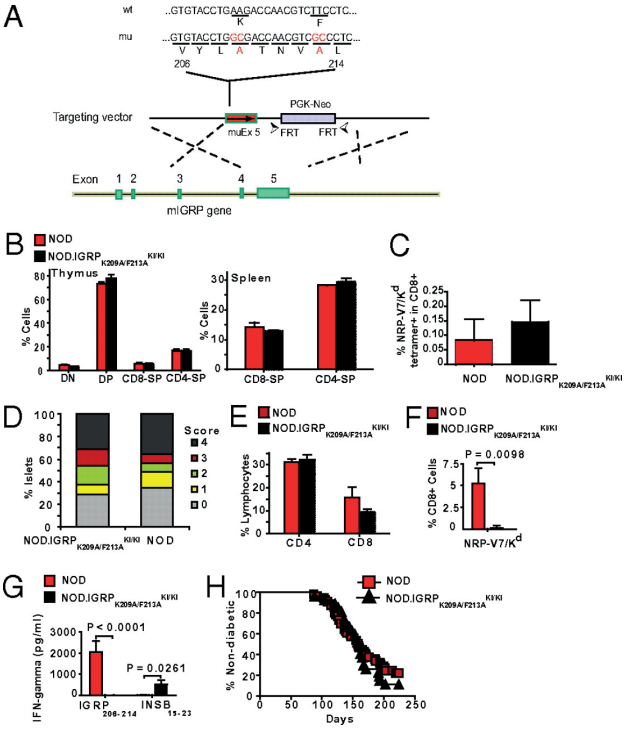


Figure 1 | NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice develop insulitis and diabetes without recruiting IGRP$_{206-214}$-reactive CD8+ T-cells into pancreatic islets.

(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.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) and CD4-SP (CD4 single positive). **(C)** Frequency of NRP-V7 reactive CD8+ T-cells in peripheral blood. PBMC from 10 wk-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 1-5 mice/experiment. **(D)** Insulitis scores. Pancreata from non-diabetic 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 IGRP$_{206-214}$-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 mM) irradiated NOD splenocytes. 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.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 ± SEM. P values in F and G were obtained with Mann-Whitney U.

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, E, and Fig. S2), and total 8.3-CD8+ T-cells (Fig. 2D, 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 pre-diabetic 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, insulitic NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts. Whereas the transfused 8.3-CD8+ T-cells readily homed to the spleen, PLNs and MLNs of insulitic NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts (Fig. 2C,D), they did not proliferate in the PLNs (Figs. 2A-C and Fig. 2E, F, and Figs. S2 and S3), confirming that this event requires crosspresentation of b cell-derived IGRP$_{206-214}$. 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, C), suggesting that some of the T-cells that are activated in the PLNs and/or islets (or the activating IGRP$_{206-214}$-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 insulitic 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% (~10^4) of all 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, non-specific inflammatory cues emanating from insulitic lesions cannot single-handedly (in the absence of local cognate pMHC) recruit naive bystander IGRP$_{206-214}$-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 IGRP$_{206-214}$-reactive CD8+ T-cell population, we tracked the recruitment of adoptively transferred naive Thy1.2+ LCMV gp33-specific CD8+ T-cells [a non-autoreactive, T1D-irrelevant T-cell population from LCMV gp33-TCR-transgenic (P14) NOD donor mice] in insulitic NOD.Thy1.1 hosts. This was done by co-transfusing 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,B). Accordingly, naive IGRP$_{206-214}$-reactive CD8+ T-cells need to engage cognate pMHC on local APCs and/or on b cells to access and/or accumulate (owing to retention and/or proliferation) in inflamed tissue.

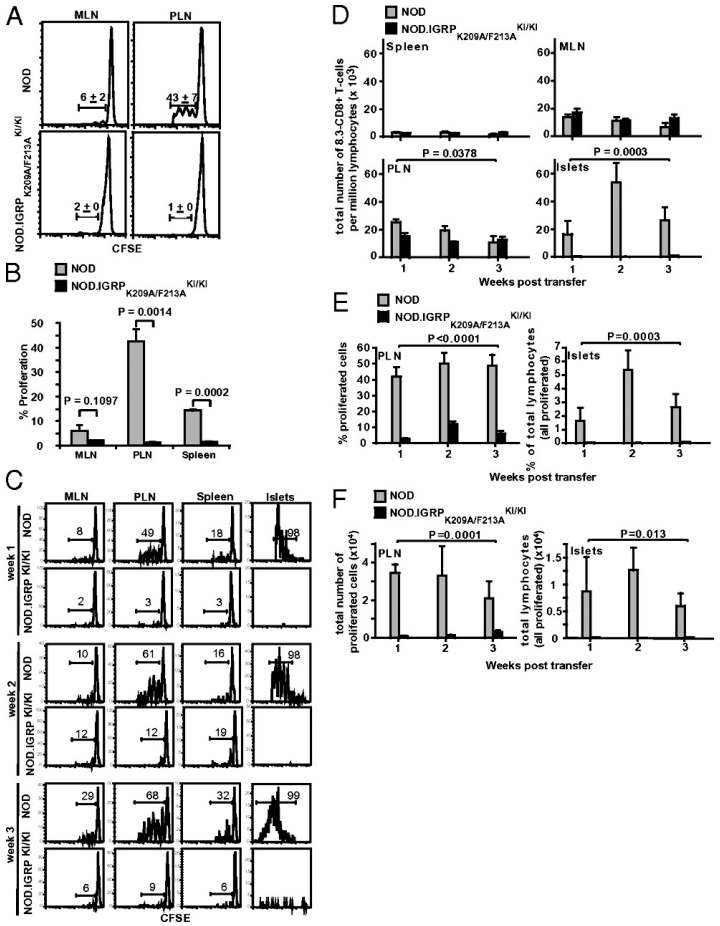


Figure 2 | Naive 8.3-CD8+ T-cells are not recruited into the inflamed pancreatic islets of NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice.

(A) Proliferation of naïve 8.3-CD8+ T-cells in the PLN. CFSE-labeled naïve 8.3-CD8+ T-cells were transfused into 10-12 wk-old NOD (n=3) or NOD.IGRP$_{K209A/F213A}^{KI/KI}$ (n=3) recipients (3 independent experiments, each using both host types). Dilution of CFSE was measured by flow cytometry 7 days post-transfer. Values correspond to the average percentage of proliferated CD8+ T-cells ± SEM. (B) Summary of data described in (A). P values were obtained with Mann-Whitney U. (C) Recruitment and proliferation of naïve 8.3-CD8+ T-cells from 8.3-NOD.*Thy1.1* donor mice in the lymphoid organs and islets of 10-12 wk-old insulitic NOD and NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts 1, 2 and 3 wk 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 3-6 experiments/time point and host type (1 mouse/time point/host in each experiment). (E) Mean ± 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) (3-6 experiments/time point and host type; 1 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 ± SEM) (3-6 experiments/time point and host type; 1 mouse/time point/host in each experiment). P values in D, E and F were obtained by two-way ANOVA.

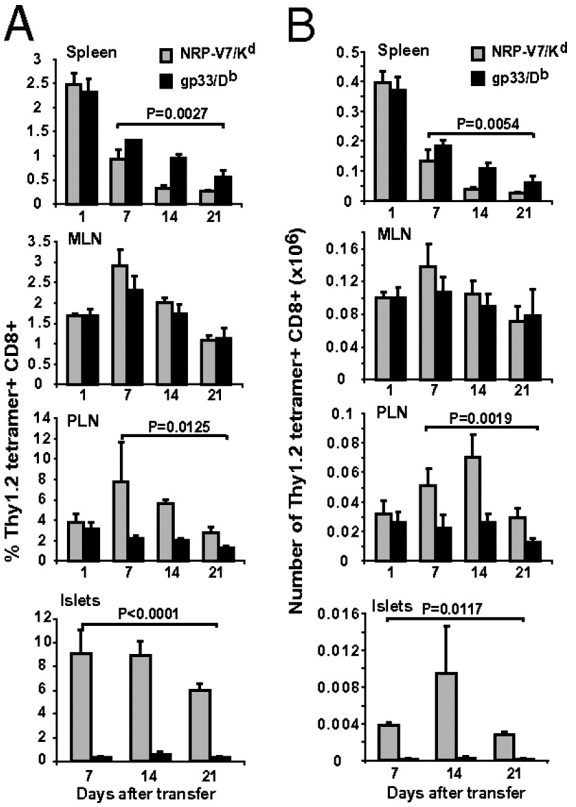


Figure 3 | Selective recruitment of cognate autoreactive T-cells into pancreatic islets of NOD mice. **(A)** Naïve 8.3-CD8+/Thy1.2+ and P14-CD8+/Thy1.2+ T-cells were mixed at 1:1 (8 x 10^6 cells each) and injected i.v. into 10-12 wk-old NOD.*Thy1.1* hosts. Hosts were sacrificed at various time points 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 3 mice/time point (n=12 mice) and 3 independent experiments (1 mouse/time point in each experiment) and are shown as mean ± SEM. **(B)** Data from (A) presented as absolute numbers of cells (mean ± SEM). P values were obtained with two-way ANOVA.

Pre-activated $IGRP_{206-214}$-specific Cytotoxic T lymphocytes also fail to home to the insulitic 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 non-inflamed islets, we transfused CFSE-labeled, *in vitro*-differentiated Thy1.1+ 8.3-CTLs (1.5 x 10^7) into non-insulitic (3 week-old) or insulitic (10-12 week-old) NOD and NOD.$IGRP_{K209A/F213A}^{KI/KI}$ hosts. Whereas CFSE+ 8.3-CTLs were rapidly recruited into non-insulitic NOD islets (Fig. 4A), leading to rapid loss of insulin-producing b cells and rampant development of diabetes in all hosts within 5 days (Fig. 4B), they were neither recruited to islets, nor caused any obvious b cell loss or diabetes in any non-insulitic NOD.$IGRP_{K209A/F213A}^{KI/KI}$ hosts for up to 6 weeks after transfer (Fig. 4A and B), suggesting that activated CD8+ T-cells cannot home to non-inflamed

tissue in the absence of local cognate pMHC. Similar results were obtained when 8.3-CTLs were transfused into insulitic hosts. 8.3-CTL were progressively recruited to, and accumulated in the PLNs and MLNs of both types of mice during the first 3 days after transfer (~2-3-fold on day 3 vs. day 1; Fig. 4C,D). In contrast, whereas CFSE+ 8.3-CTL accumulated in the islets of insulitic NOD mice (~7-9 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. 4C,D), confirming a critical role for cognate pMHC on retention and accumulation of IGRP$_{206-214}$-specific CTL in non-lymphoid 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 extra-lymphoid tissues, such as pancreatic islets in diabetes, contain a mixture of both cognate and non-cognate (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 non-specifically, such as in response to cytokine-induced chemokine receptor ligands like CXCL9, CXCL10 and CXCL11 (30, 31). Rather, our data strongly argues that, in the absence of cognate pMHC, non-specifically 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 co-expressing 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 non-inflamed islets (32). However, whether bystander naïve 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 b-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 beta cells express significantly reduced levels of pMHC class I (34), and NOD mice with a beta cell-specific disruption of beta-2 microglobulin, which cannot display pMHC class I complexes on the surface (35), recruit CD8+ T-cells to pancreatic islets.

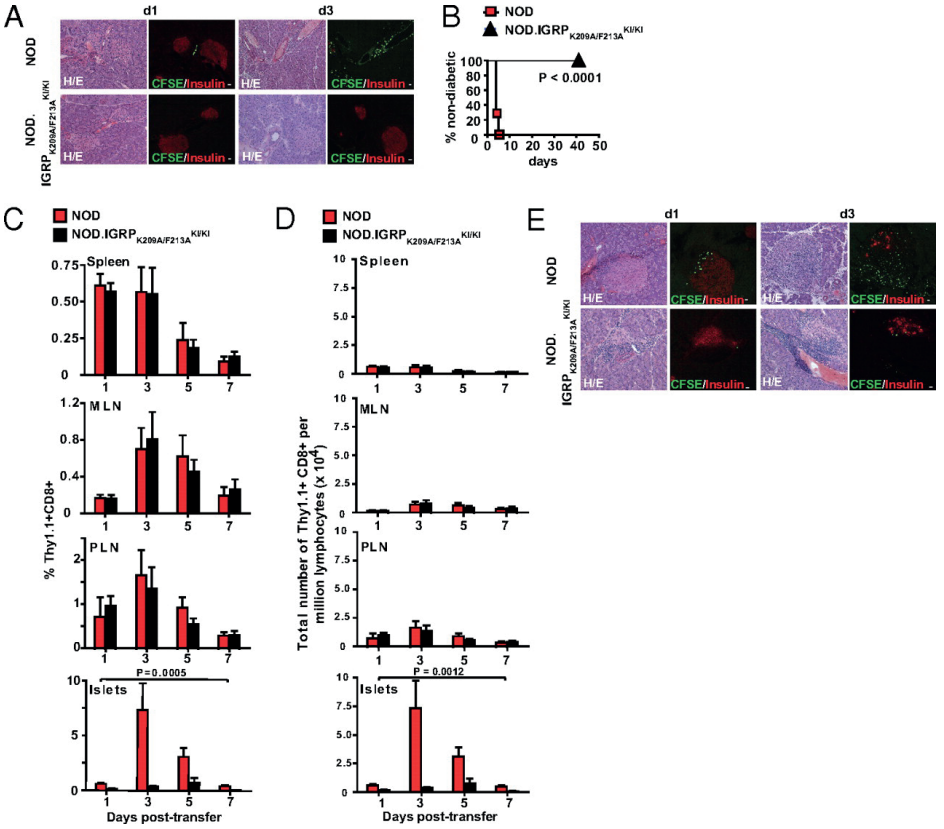


Figure 4 | The pancreatic islets of NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice also fail to recruit activated 8.3-CD8+ T-cells, even when inflamed.

(A) *In vitro*-activated, CFSE-labeled 8.3-CD8+ T-cells were injected i.v. into 3 wk-old NOD or NOD.IGRP$_{K209A/F213A}^{KI/KI}$ 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 (3 mice/time point; 10-20 islets/mouse; 3 independent experiments). Panel shows representative images. An adjacent tissue section was stained with H&E (left). White bars represent 20 mm. On NOD hosts' day 1 samples, CFSE+ T-cells were predominantly found in the periinsular 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 wk-old NOD (n=7) or NOD.IGRP$_{K209A/F213A}^{KI/KI}$ recipients (n=6) of 8.3-CTL (2 independent experiments, each including 3-4 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 10-12 wk-old NOD or NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts. Hosts were analyzed for presence of Thy1.1+ CD8+ T-cells in different organs. Data correspond to 3-4 independent experiments/time point and strain (1 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 pancreas sections from 10-12 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 (3 independent experiments; 1 mouse/time point/strain in each experiment). Images of islets are representative of severe insulitis.

MATERIALS AND METHODS

Mice. 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 (Jackson Lab, Bar Harbor, ME). To generate $IGRP_{K209A/F213A}^{KI/KI}$ mice, we transfected an *igrp* targeting construct carrying a mutated exon 5 (encoding an $IGRP_{206-214}$ epitope in which the two TCR contact residues were replaced by Ala: (VYLATNVAL; K209A/F213A) into CK35 129/Sv-derived murine 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 Avr II or Apa I-digested DNA and 5′ and 3′-specific probes differentiating wild-type (11.6 and 11.1 kb for 5′ and 3′ arms, respectively) versus 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 6 generations. Genome-wide 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, tetramers. The peptides $IGRP_{206-214}$ (VYLKTNVFL), $INSB_{15-23}$ (LYLVCGERG), NRP-V7 (KYNKANVFL), TUM (KYQAVTTTL), LCMV GP33 (KAVYNFATM) and the corresponding tetramers (PE-labeled) were prepared as described (27, 28). Briefly, the peptides were folded with recombinant human b2m 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 mg/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-CD8a (5 mg/mL) for 30 min at 4°C. For other stains, thymuses, 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, Inc.).

Specificity of islet-associated CD8+ T-cells. Islet-infiltrating cells from NOD or NOD.IGRP$_{K209A, F213A}^{KI/KI}$-mice mice were cultured for 7 days in the presence of 0.5 U/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 mM) irradiated NOD splenocytes for 48h. The IFNγ content in the supernatants was measured by ELISA (Duoset, R&D systems, Minneapolis, MN). 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 manufacture's protocols, labeled with CFSE (2.5 mM), and injected i.v. (10^7 CD8+ T-cells). In co-transfer experiments employing P14 CD8+ T-cells, each mouse received a mixture of 8 x 10^6 8.3-CD8+ and 8 x 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 mM) 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 mM CFSE and transfused i.v. (15 x 10^6) into unmanipulated 3 week or 10-12 week-old hosts. Mice were killed 1, 3, 5, 7, 14 or 21 days later and their spleens, PLN and MLN examined for presence of donor CD8+ T-cells (Tetramer+ and thy1.2+) or for dilution of CFSE in the CD8+ gate.

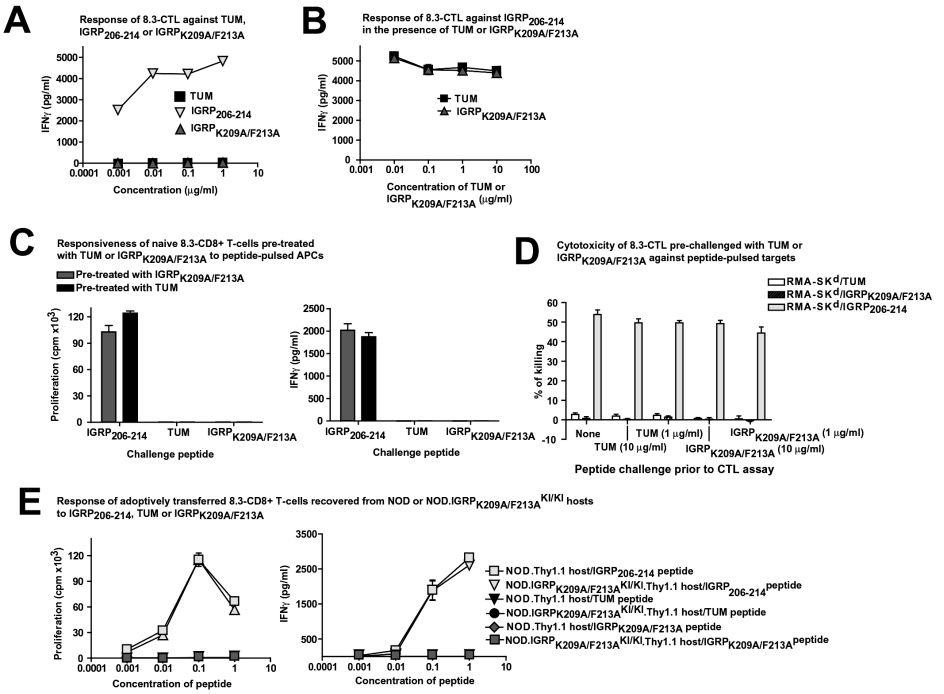
Immunopathology. Formalin-fixed, paraffin-embedded pancreas sections were stained with H&E and scored for insulitis (see below). To examine pancreatic islets for infiltration by transfused CFSE+ T-cells, pancreata were embedded in OCT medium and frozen in a dry ice/acetone bath. Cryosections (5 mm) were fixed with 3% paraformaldehyde, stained with guinea pig anti-insulin antibodies and Cy3-conjugated rabbit anti-guinea pig antibodies (Invitrogen, Carlsbad, CA). 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 2 consecutive readings greater than or equal to 3+. The average blood glucose levels in mice diagnosed as diabetic using this criteria are 21.96 $\pm$ 3.8 mM, and none of these mice have blood glucose levels below 16 mM.

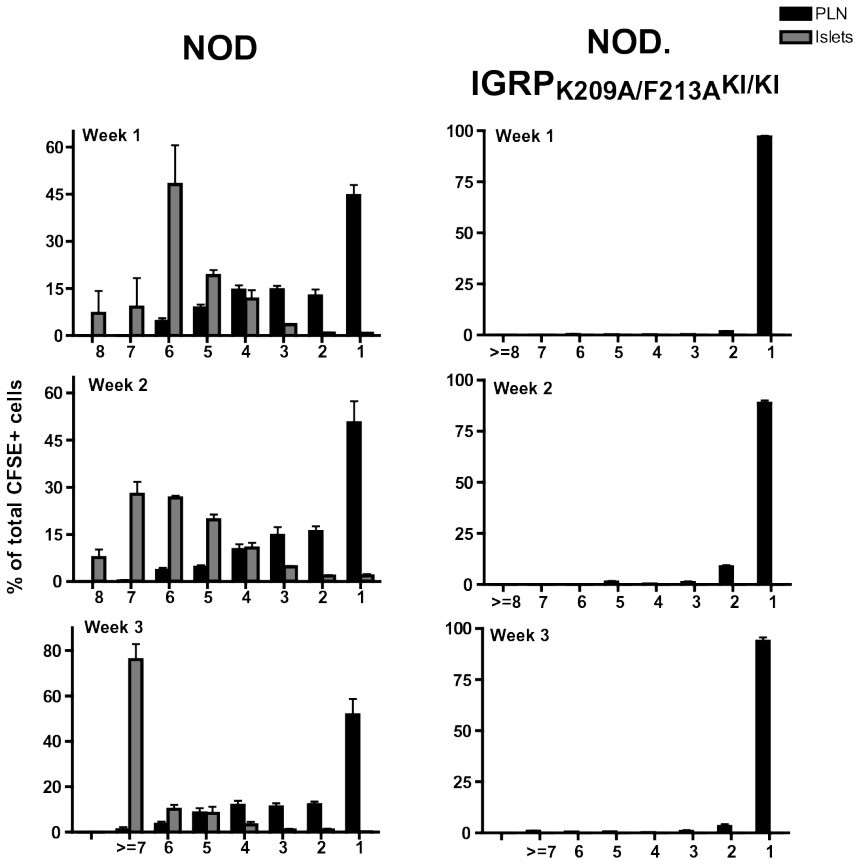
Statistical analyses. Data were compared by two-tailed Mann-Whitney U, Chi-Square, or two-way ANOVA tests. Statistical significance was assumed at $P < 0.05$.

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*. 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*$^{KI/KI}$ hosts.



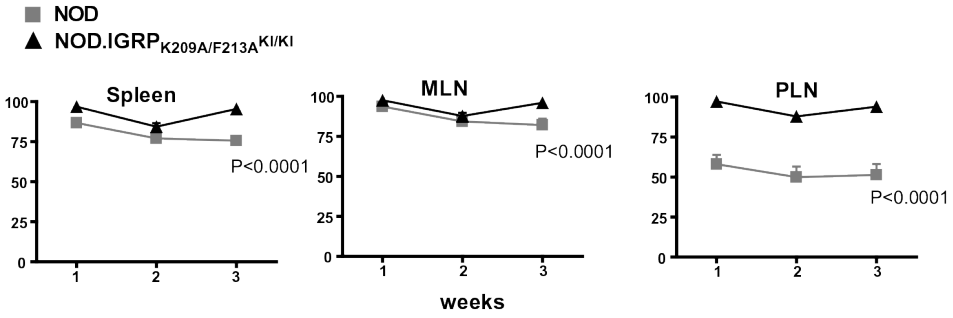
Supplementary figure 1 |

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-SKd targets pulsed with $IGRP_{206-214}$. The 8.3-CD8+ CTLs were pre- incubated 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 51Cr-release assay using RMA-SKd cells pulsed with 10 μg/mL of $IGRP_{206-214}$, TUM or $IGRPK_{209A/F213A}$ at an 8:1 effector:target ratio in triplicate wells. Percentage of killing was calculated as (51Cr in test well − spontaneous release)/(maximum release − spontaneous release) × 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.$IGRP_{K209A/F213A}^{KI/KI}$.Thy1.1 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.



Supplementary figure 2 |

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 (107) from 8.3-NOD.Thy1.1 donor mice in the lymphoid organs and islets of insulitic NOD and NOD. IGRPK209A/F213AKI/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.



Supplementary figure 3 |
Naive 8.3-CD8+ T cells fail to proliferate in the PLN of NOD.IGRPKI/KI hosts. Proliferation of adoptively transferred naive 8.3-CD8+ T cells (107) from 8.3- NOD.Thy1.1 donor mice in the lymphoid organs of insulitic NOD and NOD.IGRPK209A/F213AKI/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).

ACKNOWLEDGMENTS

We thank N. Ghyselinck for the pFlEx vector; S. Bou, M. DeCrom, M. Foote, B. Han, T. Irvine, H. Metselaar and S. Thiessen 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. Santamaria is a Scientist of the AHFMR and a JDRF Scholar. The authors have no conflicting financial interests.

AUTHOR CONTRIBUTIONS

J.W. and S.T. designed and executed experiments along with A.S., J.Y. and G.A. S.T. characterized the NOD.IGRP$_{K209A/F213A}^{KI/KI}$ strain. A.S. performed the experiments shown on Fig. 3. P.S. conceived the study, supervised the experiments, evaluated the data and wrote the manuscript along with J.W.

REFERENCES

1. von Andrian UH, Mackay CR.T-cell function and migration. N Engl J Med 2000; 343:1020-1034
2. Savinov AY, Wong FS, Stonebraker AC et al. Presentation of antigen by endothelial cells and chemoattraction are required for homing of insulin-specific CD8+ T cells. J Exp Med 2003;197:643-656
3. Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. Trends Immunol 2006;27:235-243
4. Stephens R, Randolph DA, Huang G et al. Antigen-nonspecific recruitment of Th2 cells to the lung as a mechanism for viral infection-induced allergic asthma. J Immunol 2002;169:5458-5467
5. Ostler T, Pircher H, Ehl S. 'Bystander' recruitment of systemic memory T cells delays the immune response to respiratory virus infection. Eur J Immunol 2003;33:1839-1848
6. Chen AM, Khanna N, Stohlman SA et al. Virus-specific and bystander CD8 T cells recruited during virus-induced encephalomyelitis. J Virol 2005;79:4700-4708
7. Galkina E, Thatte J, Dabak V et al. Preferential migration of effector CD8+ T cells into the interstitium of the normal lung. J Clin Invest 2005;115:3473-3483
8. Karin N, Szafer F, Mitchell D et al. Selective and nonselective stages in homing of T lymphocytes to the central nervous system during experimental allergic encephalomyelitis. J Immunol 1993;150:4116-4124
9. Picarella DE, Kratz A, Li CB et al. Transgenic tumor necrosis factor (TNF)-alpha production in pancreatic islets leads to insulitis, not diabetes. Distinct patterns of inflammation in TNF-alpha and TNF-beta transgenic mice. J Immunol 1993;150:4136-4150
10. Lee MS, von Herrath M, Reiser H et al. Sensitization to self (virus) antigen by in situ expression of murine interferon-gamma. J Clin Invest 1995;95:486-492
11. Faveeuw C, Gagnerault MC, Kraal G et al. Homing of lymphocytes into islets of Langerhans in prediabetic non-obese diabetic mice is not restricted to autoreactive T cells. Int Immunol 1995;7:1905-1913
12. Cantagrel A, Lambert N, Alam A.T cell receptor gene in synovial tissues of rheumatoid arthritis. Int Rev Immunol 1998;17:323-337
13. Peterson JD, Berg R, Piganelli JD et al. Analysis of leukocytes recruited to the pancreas by diabetogenic T cell clones. Cell Immunol 1998;189:92-98
14. Christen U, Benke D, Wolfe T et al. Cure of prediabetic mice by viral infections involves lymphocyte recruitment along an IP-10 gradient. J Clin Invest 2004;113:74-84
15. Carvalho-Pinto C, Garcia MI, Gomez L et al. Leukocyte attraction through the CCR5 receptor controls progress from insulitis to diabetes in non-obese diabetic mice. Eur J Immunol 2004;34:548-557
16. Rhode A, Pauza ME, Barral AM et al. Islet-specific expression of CXCL10 causes spontaneous islet infiltration and accelerates diabetes development. J Immunol 2005;175:3516-3524

17. Archambault AS, Sim J, Gimenez MA et al. Defining antigen-dependent stages of T cell migration from the blood to the central nervous system parenchyma. Eur J Immunol 2005;35:1076-1085

18. Chen J, Fujimoto C, Vistica BP et al. Active participation of antigen-nonspecific lymphoid cells in immune-mediated inflammation. J Immunol 2006;177:3362-3368

19. Smorodchenko A, Wuerfel J, Pohl EE et al. CNS-irrelevant T-cells enter the brain, cause blood-brain barrier disruption but no glial pathology. Eur J Neurosci 2007;26:1387-1398

20. Lieberman SM, DiLorenzo TP. A comprehensive guide to antibody and T-cell responses in type 1 diabetes. Tissue Antigens 2003;62:359-377

21. Tsai S, Shameli A, Santamaria P. CD8+ T cells in type 1 diabetes. Adv Immunol 2008;100:79-124

22. Verdaguer J, Schmidt D, Amrani et al. Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. J Exp Med 1997;186:1663-1676

23. Anderson B, Park B, Verdaguer J et al. Prevalent CD8+ T cell response against one peptide/MHC complex in autoimmune diabetes. Proc Natl Acad Sci USA 1999;96:9311-9316

24. Amrani A, Verdaguer J, Serra P et al. Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. Nature 2000;406:739-742

25. Amrani A, Serra P, Yamanouchi J et al. Expansion of the antigenic repertoire of a single T cell receptor upon T cell activation. J Immunology 2001;167:655-666

26. Verdaguer J. Yoon JW, Anderson B et al. Acceleration of spontaneous diabetes in TCRbeta-transgenic nonobese diabetic mice by beta cell-cytotoxic CD8+ T-cells expressing identical endogenous TCRalpha chains. J Immunology 1996;157:4726-4735

27. Lieberman S, Evans AM, Hn B et al. Identification of the beta-cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes. Proc Natl Acad Sci USA 2003;100:8384-8388

28. Trudeau J, Kelly-Smith C, Verchere CB et al. Autoreactive T cells in peripheral blood predict development of type 1 diabetes. J Clin Invest 2003;111:217-223

29. Wong FS, Karttunen J, Dumont C et al. Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library. Nat Med 1999;5**:**1026-1031

30. Loetscher M, Gerber B, Loetscher P et al. Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. J Exp Med 1996;184:963-969

31. Sallusto F, Lanzavecchia A, Mackay CR. Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. Immunol Today 1998;19:568-574

32. Lennon GP, Bettini M, Burton AR et al. T cell islet accumulation in type 1 diabetes is a tightly regulated, cell-autonomous event. Immunity 2009;31:643-653

33. Marelli-Berg FM, James MJ, Dangerfield et al. Cognate recognition of the endothelium induces HY-specific CD8+ T-lymphocyte transendothelial migration (diapedesis) in vivo. Blood 2004;103:3111-3116

34. Yamanouchi J, Verdaguer J, Han B et al. Cross-priming of diabetogenic T cells dissociated from CTL-induced shedding of beta cell autoantigens. J Immunol 2003;171:6900-6909

35. Hamilton-Williams EE, Palmer SE, Charlton B et al. Beta cell MHC class I is a late requirement for diabetes. Proc Natl Acad Sci USA 2003;100:6688-6693

36. Jarchum I, Takaki T, DiLorenzo TP. Efficient culture of CD8+ T cells from the islets of NOD mice and their use for the study of autoreactive specificities. J Immunol Methods 2008;339:66-73