Local Autoantigen Expression as Essential Gatekeeper of Memory T-Cell Recruitment to Islet Grafts in Diabetic Hosts

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It is generally believed that inflammatory cues can attract noncognate, "bystander" T-cell specificities to sites of inflammation. We have shown that recruitment of naive and in vitro activated autoreactive $CD8^+$ T cells into endogenous islets requires local autoantigen expression. Here, we demonstrate that absence of an autoantigen in syngeneic extrapancreatic islet grafts in diabetic hosts renders the grafts "invisible" to cognate memory (and naive) T cells. We monitored the recruitment of islet-specific glucose-6-phosphatase catalytic subunit-related protein $(IGRP)_{206–214}$-reactive $CD8^+$ T cells into $IGRP_{206–214}$-competent and $IGRP_{206–214}$-deficient islet grafts in diabetic wild-type or $IGRP_{206–214}^{-/-}$ nonobese diabetic hosts (harboring either naive and memory T cells or only naive $IGRP_{206–214}$-specific T-cells, respectively). All four host–donor combinations had development of recurrent diabetes within 2 weeks. Wild-type hosts recruited $IGRP_{206–214}$-specific T cells into $IGRP_{206–214}^{+/+}$ but not $IGRP_{206–214}^{-/-}$ grafts. In $IGRP_{206–214}^{-/-}$ hosts, there was no recruitment of $IGRP_{206–214}$-specific T cells, regardless of donor type. Graft-derived $IGRP_{206–214}$ activated naive $IGRP_{206–214}$-specific T cells, but graft destruction invariably predated their recruitment. These results indicate that recurrent diabetes is exclusively driven by autoreactive T cells primed during the primary autoimmune response, and demonstrate that local antigen expression is a sine qua non requirement for accumulation of memory T cells into islet grafts. These findings underscore the importance of tackling autoreactive T-cell memory after β-cell replacement therapy. ***Diabetes*** **62:905–911, 2013**

Nonobese diabetic (NOD) mice have development of a form of type 1 diabetes that results from destruction of β cells by $CD4^+$ and $CD8^+$ T cells recognizing many autoantigenic peptides (1). A significant fraction of islet-associated $CD8^+$ cells recognize the mimotope NRP-V7 in the context of the major histocompatibility complex (MHC) molecule K^d (2). These cells are a significant component of the earliest NOD islet $CD8^+$ infiltrates (2,3), are diabetogenic (4,5), and target residues 206–214 of islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (6). The peripheral $IGRP_{206–214}$-reactive $CD8^+$ T-cell pool is sizeable (7) and, on recruitment into islets, undergoes a local avidity maturation process that contributes to disease progression (8).

Studies in infection and autoimmune disease models have suggested that recruitment of T cells into sites of extralymphoid inflammation does not require local expression of cognate peptide–MHC (pMHC) (9–11). However, we recently have shown that cues emanating from pancreatic islets undergoing spontaneous autoimmune inflammation in NOD mice cannot recruit naive or newly activated bystander T-cell specificities. This was established by monitoring the recruitment of naive or in vitro activated $IGRP_{206–214}$-specific $CD8^+$ T cells in gene-targeted NOD mice expressing a T-cell "invisible" $IGRP_{206–214}$ sequence. These mice had development of diabetes with normal incidence, but their insulitic lesions could not recruit either cell type. These results indicated that recruitment of naive T cells or effector cytotoxic T lymphocytes to a site of autoimmune inflammation results from an active process that is strictly dependent on local display of cognate pMHC (12).

Here, we asked whether this revised paradigm also applies to recruitment of memory (autoantigen-experienced) autoreactive T cells and/or recruitment of naive and memory T cells to syngeneic islet grafts. We reasoned that the "nonphysiological" lymphatic and vascular anatomy of islets grafts transplanted under the kidney capsule (13–15), coupled with a high rate of graft cell death (16), should allow recruitment of "graft-irrelevant" (i.e., nonautoreactive) memory T cells to the site in response to local inflammatory cues, including those caused by grafting. We demonstrate that recruitment of $CD8^+$ T cells to islet grafts during disease recurrence exclusively involves autoantigen-specific T cells from the memory pool, excluding a role for bystander T-cell specificities or graft antigen-activated autoreactive T cells.

RESEARCH DESIGN AND METHODS

Mice. $NOD.IGRP_{K209A/F213A}^{KI/KI}$ mice, encoding an immunologically silent $IGRP_{206–214}$ epitope, have been described (12). These studies were approved by the local Animal Care Committee.

Diabetes. Diabetes was monitored twice per week by measuring urine glucose levels and was confirmed by tail vein blood glucose measurements. All recipient mice had at least two successive blood glucose measurements >22.2 mmol/L and underwent transplantation within 1–2 weeks of diabetes onset.

Peptides and tetramers. The peptides $IGRP_{206–214}$, NRP-V7, and TUM, and the corresponding tetramers (phycoerythrin -labeled), were prepared as described (17).

Flow cytometry. Cell suspensions were stained with pMHC tetramers and FITC-conjugated or peridinin chlorophyll protein (PerCP)-conjugated anti-CD8α and anti-CD4 mAbs (BD Pharmingen) for 60 min at 4°C, fixed in 1% paraformaldehyde/PBS, and analyzed by fluorescence-activated cell sorting.

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Islet isolation. Pancreatic islets were isolated by hand-picking after collagenase P digestion of the pancreas and cultured overnight at 37°C in 5% CO_2.
Islet transplantation and graft harvest. Five hundred islets were transplanted under the left kidney capsule. Successful engraftment was defined as restoration of glycemic control for >48 h. Graft failure was defined as nonfasting blood glucose >15 mmol/L.
Specificity of islet-associated CD8$^+$ T cells. The grafts of recurrent diabetic hosts were cut into ~2-mm^3 fragments and cultured for 1 week in 0.5 units/mL rIL-2. T cells were analyzed by fluorescence-activated cell sorter as described. Measurements of interferon-γ secretion by graft-associated T cells (2×10^4/well) in response to peptide-pulsed irradiated NOD splenocytes (10^5/well) were determined by enzyme-linked immunosorbent assay (R&D Systems) and normalized to values obtained with TUM.
Adoptive transfer. Purified splenic CD8$^+$ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (2.5 μmol/L) and injected intravenously (5×10^6) 24 h after transplantation. Mice were killed 7 days later, and the grafted and nongrafted kidney-draining lymph nodes, spleens, pancreatic lymph nodes, and mesenteric lymph nodes were examined for dilution of CFSE in the CFSE$^+$CD8$^+$ gate.
Statistical analyses. Data were compared by Mann-Whitney U or χ^2 log-rank tests. Statistical significance was assumed at $P < 0.05$.
Online supplementary materials. The online supplementary materials provide supporting data on the recruitment of IGRP$_{206–214}$-reactive or InsB$_{15–23}$–reactive CD8$^+$ T cells to islet grafts, as well as representative fluorescence-activated cell sorter profiles.

RESULTS

NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice (referred to as IGRP$_{206–214}^{-/-}$ or "epitope-deficient" mice) have development of diabetes with the same incidence and kinetics as wild-type NOD ("epitope-competent") mice but cannot trigger the activation or recruitment of naive or in vitro activated IGRP$_{206–214}$-reactive CD8$^+$ T cells (12). Here, we investigated if the naive IGRP$_{206–214}$-reactive CD8$^+$ T cells of epitope-deficient hosts and/or their memory counterparts arising in epitope-expressing hosts are recruited into epitope-competent or epitope-deficient islet grafts (from NOD.*scid* and NOD.*rag2*$^{-/-}$.IGRP$_{K209A/F213A}^{KI/KI}$ donors, respectively).

We first tracked the recruitment of IGRP$_{206–214}$-reactive CD8$^+$ T cells from diabetic IGRP$_{206–214}^+$ hosts (harboring both naive and memory IGRP$_{206–214}$-reactive CD8$^+$ T cells) into IGRP$_{206–214}^+$ or IGRP$_{206–214}^{-/-}$ grafts. The presence of IGRP$_{206–214}$-reactive CD8$^+$ T cells recruited into the graft was analyzed by flow cytometry using pMHC tetramers. We also measured the amount of interferon-γ that graft-infiltrating T cells secreted in response to peptide-pulsed irradiated splenocytes as an additional read-out of T-cell recruitment.

Diabetic IGRP$_{206–214}^+$ hosts receiving IGRP$_{206–214}^{-/-}$ islets had development of recurrence of disease, but did so a few days later than those receiving IGRP$_{206–214}^+$ islets (12.7 ± 3.5 vs. 5.9 ± 0.7 days; Figs. 1*A* top, *B*, and *C* left). This indicated that recruitment of naive and/or preactivated IGRP$_{206–214}$-reactive CD8$^+$ T cells contributes to, but is dispensable for, graft destruction in diabetic IGRP$_{206–214}^+$ hosts. Importantly, however, whereas IGRP$_{206–214}$-reactive T cells accounted for a significant fraction of IGRP$_{206–214}^+$ graft-associated CD8$^+$ T cells (18.2% ± 4.7%), they were undetectable in IGRP$_{206–214}^{-/-}$ grafts (Fig. 2*A–C* and Supplementary Fig. 1*A*). Furthermore, the lymph nodes draining the grafted (left) kidney in mice receiving IGRP-$_{206–214}$–expressing islet grafts harbored more IGRP$_{206–214}$-reactive CD8$^+$ T cells than those draining the contralateral (nongrafted) kidney, and this was not seen in diabetic hosts grafted with IGRP$_{206–214}^{-/-}$ islets (Fig. 3*A*). In addition, the pancreatic lymph nodes and the spleen, and to a lesser extent the mesenteric lymph nodes, of mice grafted with IGRP$_{206–214}^+$ islets contained more IGRP$_{206–214}$-reactive CD8$^+$ T cells than those from mice grafted with IGRP$_{206–214}^{-/-}$ islets (Figs. 3*B* and *C*). This suggests that graft-derived IGRP$_{206–214}$ induces the activation and retention of host naive and/or preactivated IGRP$_{206–214}$-reactive CD8$^+$ T cells in graft-proximal lymphoid organs.

We next investigated whether the IGRP$_{206–214}$-reactive CD8$^+$ T cells recruited to the epitope-expressing grafts include naive T-cells primed by graft-derived IGRP$_{206–214}$. We followed the fate of IGRP$_{206–214}^+$ and IGRP$_{206–214}^{-/-}$ islet grafts and IGRP$_{206–214}$-reactive CD8$^+$ T cells in diabetic IGRP$_{206–214}^{-/-}$ hosts, which are unable to generate antigen-experienced IGRP$_{206–214}$-reactive CD8$^+$ T cells from an otherwise normal pool of naive T-cell precursors. IGRP$_{206–214}^{-/-}$ hosts rejected IGRP$_{206–214}^+$ and IGRP$_{206–214}^{-/-}$ islets with kinetics similar to those seen in NOD mice (Figs. 1*A–C*). Yet, IGRP$_{206–214}$-reactive CD8$^+$ T cells were barely detectable in IGRP$_{206–214}^+$ and IGRP$_{206–214}^{-/-}$ grafts implanted into IGRP$_{206–214}^{-/-}$ hosts (Figs. 2*A* and *B*), indicating that the grafts do not recruit newly primed IGRP$_{206–214}$-reactive CD8$^+$ T cells, at least within the first 2 weeks after transplantation. Interestingly, IGRP$_{206–214}^+$ grafts in IGRP$_{206–214}^{-/-}$ hosts recruited slightly more InsB$_{15–23}$-reactive CD8$^+$ T cells than in IGRP$_{206–214}^+$ hosts (Supplementary Figs. 1*B* and *C*). Although these differences were not statistically significant, they suggest that in these mice the IGRP$_{206–214}$-reactive CD8$^+$ T-cell niche is occupied by other memory T-cell specificities. In addition, the islet graft-associated CD8$^+$ T cells express markers of memory (i.e., are CD44^{high}, CD62L$^-$, and CD127$^+$; data not shown).

In agreement with these data, the proximal lymphoid organs (graft-draining lymph nodes, pancreatic lymph nodes, and spleen) and blood of IGRP$_{206–214}^{-/-}$ hosts transplanted with antigen-expressing islets contained fewer IGRP$_{206–214}$-reactive CD8$^+$ T cells than their IGRP$_{206–214}^+$ host counterparts, suggesting that graft-derived antigen does not induce a detectable peripheral expansion of naive autoreactive T cells (Fig. 3*D*). In addition, because the percentages of IGRP$_{206–214}$-reactive CD8$^+$ T cells in the graft-draining lymph nodes, pancreatic lymph nodes, and spleen of IGRP$_{206–214}^+$ hosts grafted with IGRP$_{206–214}$-deficient islets were also low (Fig. 3*D*, right panels), we conclude that the peripheral expansion of IGRP$_{206–214}$-reactive CD8$^+$ T cells seen in IGRP$_{206–214}^+$ islet-grafted IGRP$_{206–214}^+$ hosts (Figs. 3*A–C*) largely, if not exclusively, involves antigen-experienced T cells. Interestingly, NOD hosts grafted with IGRP$_{206–214}^{-/-}$ islets accumulated IGRP$_{206–214}$-reactive CD8$^+$ T cells in the bloodstream (Fig. 3*D*), suggesting that, in the absence of antigen in the graft and graft-draining lymphoid organs, preactivated IGRP$_{206–214}$-reactive CD8$^+$ T cells are "trapped" in the bloodstream.

Finally, we asked whether absence of graft-antigen-primed naive IGRP$_{206–214}$-reactive CD8$^+$ T cells in the epitope-expressing grafts of IGRP$_{206–214}^{-/-}$ hosts was caused by inability of graft-derived IGRP$_{206–214}$ to activate cognate naive CD8$^+$ T cells, or to protracted recruitment and/or accumulation of these T cells into the graft. This was performed by tracking the proliferation of naive splenic CFSE-labeled IGRP$_{206–214}$-reactive CD8$^+$ T cells from 8.3 T-cell receptor transgenic mice in NOD hosts grafted with IGRP$_{206–214}^+$ or IGRP$_{206–214}^{-/-}$ islets the previous day. Various host lymphoid organs were examined for dilution of CFSE in the CFSE$^+$CD8$^+$ gate 7 days after T-cell transfer. Naive 8.3-CD8$^+$ T cells proliferated vigorously in the lymph nodes draining IGRP$_{206–214}^+$ (but not IGRP$_{206–214}^{-/-}$) grafts and, to a lesser extent, in the pancreatic lymph node and spleen, where some of the proliferation appears to be induced by host-derived (residual) IGRP$_{206–214}$ (Fig. 4*A* and *B*). There were very few donor 8.3-CD8$^+$ T cells in IGRP$_{206–214}^+$

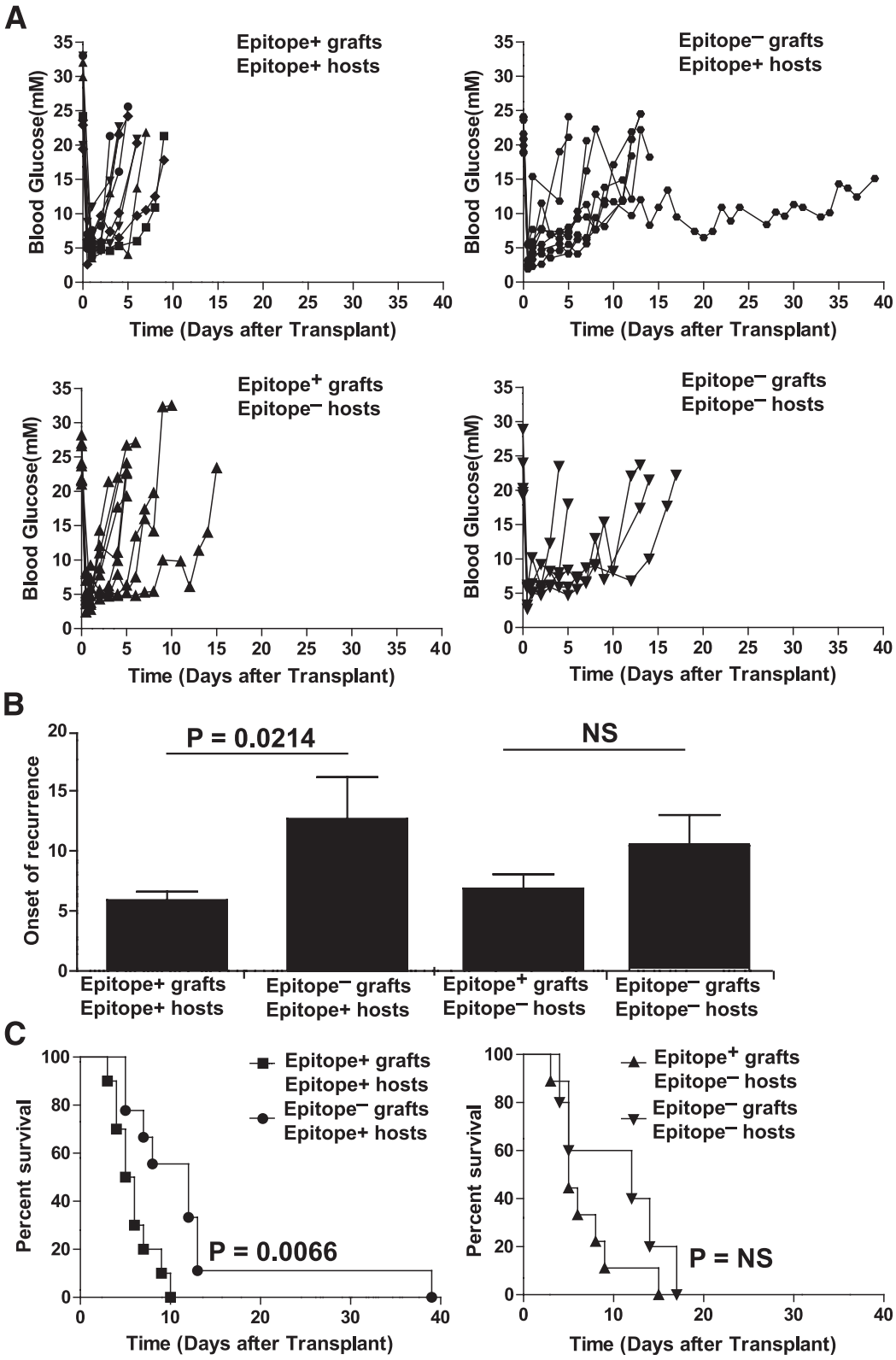

FIG. 1. Survival of islet grafts from $IGRP_{206–214}$-competent or $IGRP_{206–214}$-deficient donors in spontaneously diabetic $IGRP_{206–214}$-competent or $IGRP_{206–214}$-deficient NOD hosts. *A*: Individual blood glucose curves of diabetic NOD hosts receiving NOD.*scid* (n = 10) or NOD.*rag2*$^{KI/KI}$.$IGRP_{K209A/F213A}{}^{KI/KI}$ islets (n = 9), and diabetic NOD.$IGRP_{K209A/F213A}{}^{KI/KI}$ hosts receiving NOD.*scid* (n = 9) or NOD.*rag2*$^{-/-}$.$IGRP_{K209A/F213A}{}^{KI/Ki}$ islets (n = 5). *B*: Average onset of disease recurrence after transplantation (in days) in the four different donor/host combinations. *P* values were obtained by Mann-Whitney *U* test. *C*: Survival curves of grafts in diabetic NOD (*left*) or NOD.$IGRP_{K209A/F213A}{}^{KI/KI}$ hosts (*right*). *P* values were calculated via log-rank test. For (*B*) and (*C*), differences between epitope-positive and epitope-negative grafts in epitope-positive hosts remained statistically significant upon exclusion of the epitope-negative graft that survived to 40 days (P = 0.0395 in *B*; and P = 0.0353 in *C*).

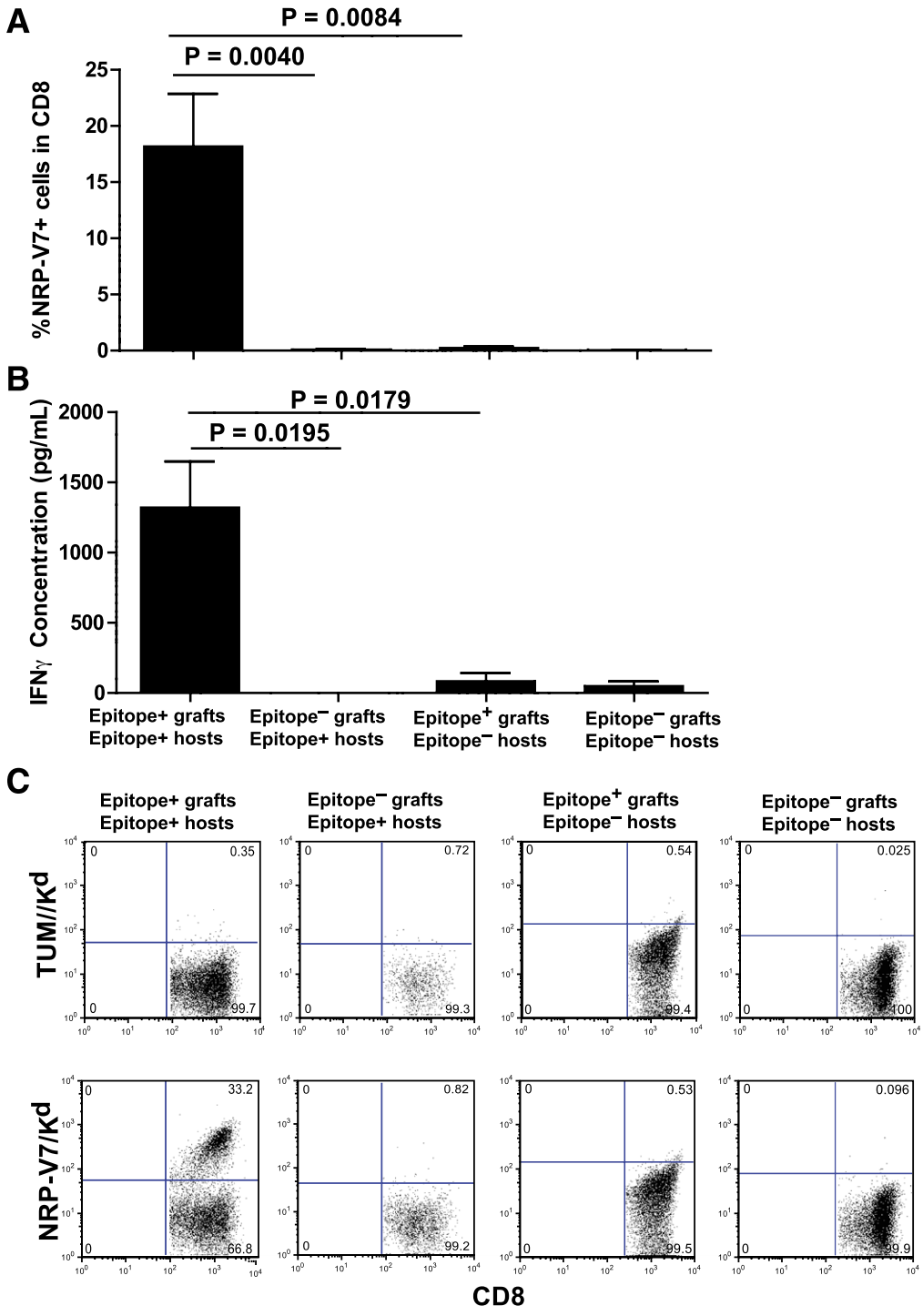

FIG. 2. Recruitment of IGRP$_{206–214}$-reactive CD8$^+$ T cells from diabetic IGRP$_{206–214}$-competent or IGRP$_{206–214}$-deficient NOD hosts into islet grafts from IGRP$_{206–214}$-competent or IGRP$_{206–214}$-deficient donors. *A*: Percentages of NRP-V7/K^d tetramer-positive in islet-graft–associated CD8$^+$ T cells. Data (average ± SEM) correspond, from left to right, to eight, four, four, and three grafts per group, respectively. *B*: Interferon-γ (IFN-γ) secretion by islet graft-associated CD8$^+$ T cells in response to NRP-V7 peptide-pulsed NOD dendritic cells. Data correspond, from left to right, to five, four, four, and three grafts per group, respectively. *C*: Representative fluorescence-activated cell sorting staining profiles of CD8$^+$ T cells isolated from islet grafts in the four different donor/host combinations. TUM/K^d was used as a negative control tetramer. *P* values in (*A*) and (*B*) were obtained with Mann-Whitney *U* test. Grafts were harvested immediately after the last blood glucose measurement in Fig. 1. (A high-quality color representation of this figure is available in the online issue.)

or IGRP$_{206–214}{}^{-/-}$ grafts (0.06% ± 0.03% vs. 0.06% ± 0.016% of CD8$^+$ cells, respectively) (Fig. 4*C*). These observations indicate that destruction of IGRP$_{206–214}{}^{+}$ and IGRP$_{206–214}{}^{-/-}$ grafts in IGRP$_{206–214}{}^{+}$ hosts (Fig. 1*A*) predates recruitment of newly activated T cells.

DISCUSSION

The data presented herein challenge a current paradigm stating that nonantigen-specific inflammatory cues can attract and retain noncognate, bystander T-cell specificities to sites of inflammation, including syngeneic islet

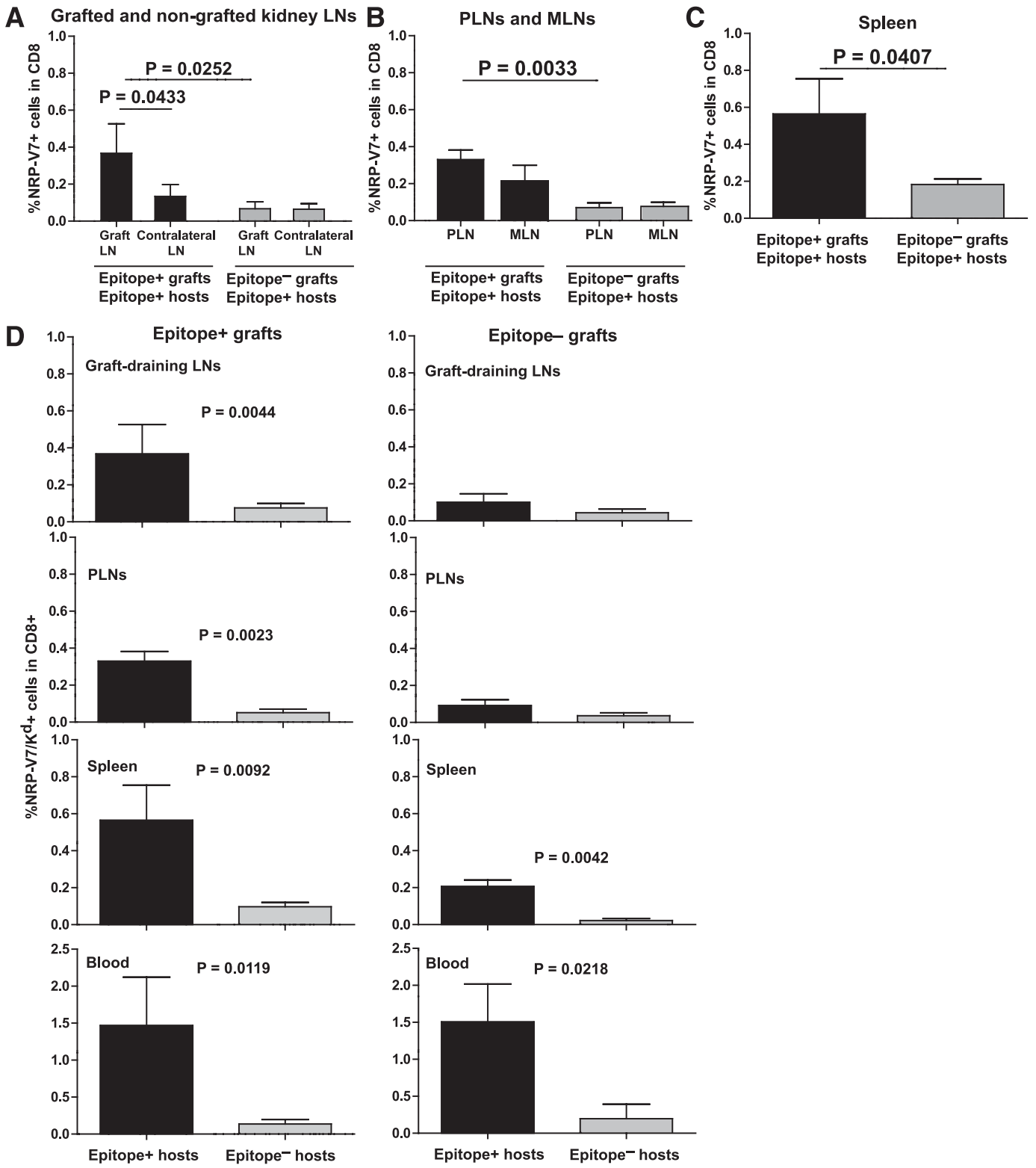

FIG. 3. Frequencies of $IGRP_{206–214}$-reactive $CD8^+$ T cells in lymphoid organs and blood of diabetic $IGRP_{206–214}$-competent or $IGRP_{206–214}$-deficient hosts grafted with $IGRP_{206–214}$-competent or $IGRP_{206–214}$-deficient islets. Percentages of NRP-V7/K^d tetramer-positive cells in $CD8^+$ T cells from lymph nodes (LNs) draining the grafted compared with contralateral kidneys (*A*), the pancreatic lymph nodes (PLNs) and the mesenteric lymph nodes (MLNs) (*B*), and the spleen (*C*). Data (average ± SEM) correspond to six and eight mice, respectively. *D*: Frequencies of $IGRP_{206–214}$-reactive $CD8^+$ T cells in lymphoid organs and blood of $IGRP_{206–214}$-competent compared with $IGRP_{206–214}$-deficient hosts grafted with $IGRP_{206–214}$-competent (*left*) compared with $IGRP_{206–214}$-deficient islets (*right*). Data (average ± SEM) correspond to six, six, eight, and five mice per group, respectively. Background staining with the negative control tetramer TUM/K^d was subtracted. *P* values were obtained with Mann-Whitney *U* test.

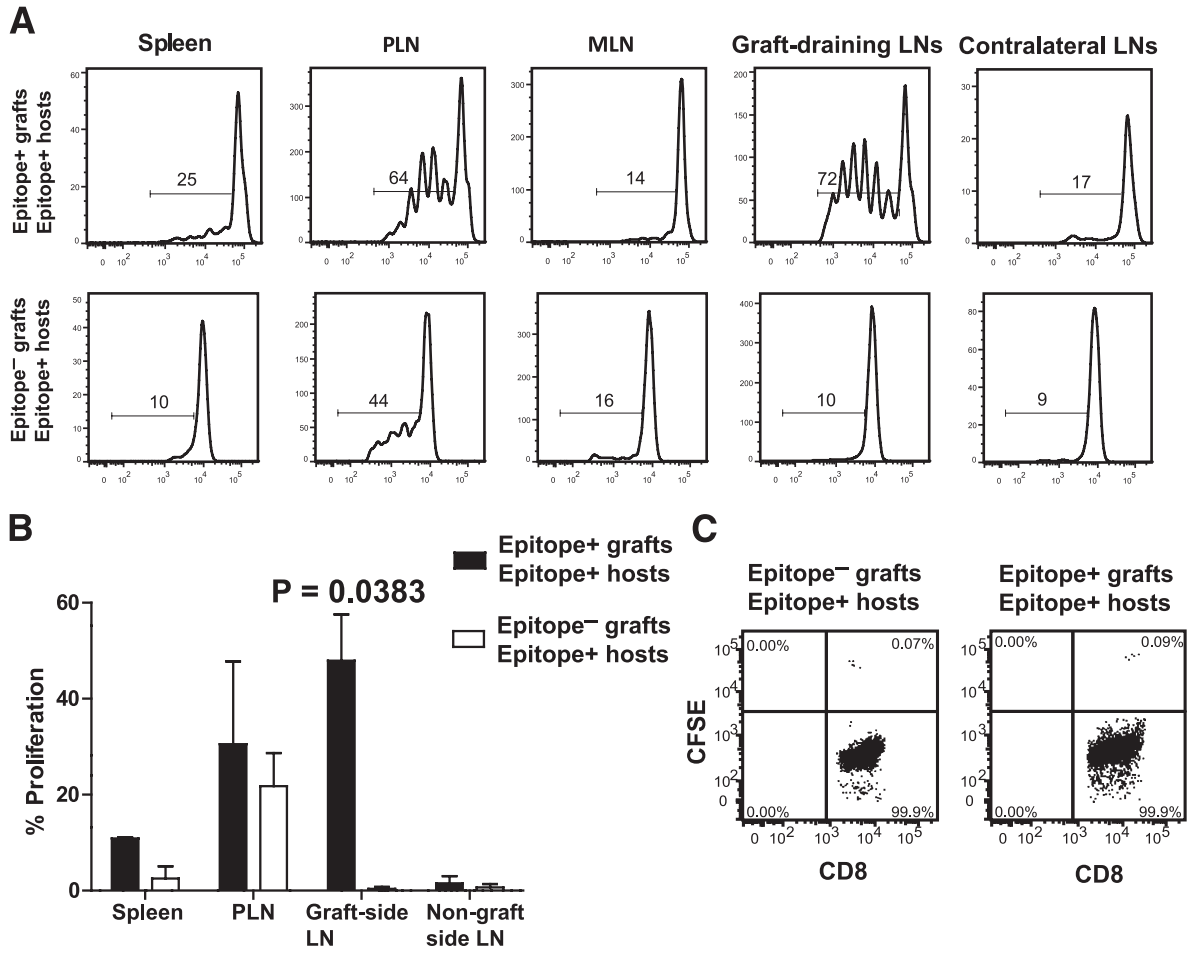

FIG. 4. Proliferation of naive CFSE-labeled 8.3-CD8$^+$ T cells in lymphoid organs of IGRP$_{206–214}$-competent hosts grafted with IGRP$_{206–214}$-competent compared with IGRP$_{206–214}$-deficient islets. *A*: Representative CFSE dilution profiles. *B*: Average ± SEM of the percentage of proliferated cells. *C*: Representative flow profiles of graft-associated CD8$^+$ T cells of these mice. Data in (*A–C*) correspond to three mice per host/donor combination. *P* values were obtained with Mann-Whitney *U* test. LN, lymph node; MLN, mesenteric lymph node; PLN, pancreatic lymph node.

transplants in diabetic mice. We demonstrate that absence of the cognate autoantigen in a syngeneic extrapancreatic islet graft in a diabetic host renders the graft invisible to cognate memory (and naive) T cells. Local antigen expression, in addition to MHC class I expression (18), is thus a sine qua non requirement for accumulation of autoreactive CD8$^+$ T cells into islet grafts.

The absolute need for local autoantigen expression is highlighted by two important considerations. First, IGRP$_{206–214}$/K^d (NRP-V7)-reactive CD8$^+$ T cells are among the most prevalent in NOD islet infiltration (8). Second, the vascular beds irrigating islet grafts, including the subcapsular kidney space, have a porous, fenestrated architecture (13–15) that could conceivably render them permeable to bystander T cells. Therefore, it is remarkable that autoantigen-experienced (i.e., memory) IGRP$_{206–214}$-reactive T cells, despite their prevalence in the periphery, do not accumulate into IGRP$_{206–214}$-deficient grafts. Recruitment of memory CD8$^+$ T cells to islet grafts thus follows the same rules that we have described for the recruitment of naive and in vitro activated CD8$^+$ T cells into endogenous islets (i.e., requiring a cognate pMHC interaction in situ). A recent report demonstrates a striking similarity in human insulitis: all the CD8$^+$ T cells found in the inflamed islets of type 1 diabetic patients bound self-pMHC complexes (19).

Our findings further imply that individual autoantigenic specificities, even when prevalent, play relatively minor roles in the anamnestic autoimmune response contributing to graft destruction in autoimmune disease-affected hosts. Our results also clearly indicate that destruction of syngeneic islet grafts in diabetic NOD mice is largely, if not exclusively, effected by autoantigen-experienced T cells primed during the primary autoimmune response. Although graft antigen-loaded antigen-presenting cells residing in the graft-draining lymph nodes can readily induce the activation of naive autoreactive CD8$^+$ T cells, graft destruction precedes recruitment of these T cells into the graft. The high physical and functional pMHC-binding avidities of antigen-experienced T cells coupled with their ability to mount rapid recall responses to limiting amounts of antigen (20) likely afford them a competitive advantage, particularly during the first 2 weeks after transplantation. Differences in the bio-distribution of memory versus naive T cells may be another contributing factor. These considerations, however, do not exclude the likely involvement of graft antigen-primed naive autoreactive T cells in chronic loss of graft function, such as, for example, in the context of partially matched islet allografts.

Recurrent autoimmunity in allogeneic islet cell transplantation has become a topic of growing interest. For example, the pretransplant peripheral frequencies of

autoreactive T cells in diabetic recipients are predictive of islet allograft fate, and posttransplant increases are associated with loss of graft function (21–24), suggesting that recurrent autoimmunity may contribute to allograft destruction. Although clinical islet transplantation is a more complex situation, our model has allowed us to dissect the specific roles of bystander immunity versus anamnestic and naive autoimmunity to islet graft rejection. Our observations emphasize the importance of developing therapies capable of preventing priming of naive alloreactive T cells causing allograft rejection, recruitment of memory autoreactive T cells causing anamnestic autoimmunity in the immediate posttransplant period, and the priming of naive autoreactive T cells causing chronic loss of graft function, giving special attention to the pretransplant autoreactivity status of the diabetic host.

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No potential conflicts of interest relevant to this article were reported.

G.M.A., X.C.-C., S.T., and J.R.W. researched data and contributed to the editing of the manuscript. G.M.A., X.C.-C., S.T., J.R.W., and B.O.R. contributed to the discussion. B.-Y.X. and J.W. assisted with experiments. J.W. and B.O.R. reviewed and edited the manuscript. P.S. designed and supervised the study. P.S. is the guarantor of this work and, as such, had full access to all the data and takes responsibility for the integrity of the data and the accuracy of data analysis.

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