CD4 T Cells Reactive to Hybrid Insulin Peptides Are Indicators of Disease Activity in the NOD Mouse

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Diabetes 2018;67:1836–1846 | https://doi.org/10.2337/db18-0200

We recently established that hybrid insulin peptides (HIPs), formed in islet β-cells by fusion of insulin C-peptide fragments to peptides of chromogranin A or islet amyloid polypeptide, are ligands for diabetogenic CD4 T-cell clones. The goal of this study was to investigate whether HIP-reactive T cells were indicative of ongoing autoimmunity. MHC class II tetramers were used to investigate the presence, phenotype, and function of HIP-reactive and insulin-reactive T cells in NOD mice. Insulin-reactive T cells encounter their antigen early in disease, but they express FoxP3 and therefore may contribute to immune regulation. In contrast, HIP-reactive T cells are proinflammatory and highly diabetogenic in an adoptive transfer model. Because the frequency of antigen-experienced HIP-reactive T cells increases over progression of disease, they may serve as biomarkers of autoimmune diabetes.

Islet-reactive CD4 T cells are major contributors to the pathogenesis of autoimmune type 1 diabetes (T1D). BDC-2.5, a chromogranin A (ChgA)–reactive, diabetogenic CD4 T-cell clone isolated from a diabetic NOD mouse, has been a valuable tool for studying the contribution of CD4 T cells to disease (1–3). We recently established that ChgA-reactive T cells are key to the development of T1D, as mice deficient in this protein are protected from disease (4). ChgA is expressed in various endocrine organs (adrenals, pituitary glands, and pancreas), and proteolytic cleavage of ChgA leads to the formation of several peptides, including vasostatin, pancreastatin, and WE14. The latter is a 14–amino acid peptide that is weakly antigenic for the T-cell clone BDC-2.5 and for T cells from patients with newly diagnosed diabetes (3,5). We recently described a posttranslational modification by which WE14 is covalently bound to a fragment of insulin C-peptide, resulting in formation of a hybrid peptide that is a very potent ligand for BDC-2.5 (6). Similarly, a peptide from the second propeptide region of another β-cell secretory granule protein, islet amyloid polypeptide (IAPP), forms a hybrid peptide with the same C-peptide fragment and is highly antigenic for the diabetogenic T-cell clone BDC-6.9 (6). The presence of these hybrid insulin peptides (HIPs) in β-cell tumors was confirmed by mass spectrometric analysis, and it was also demonstrated that HIP-reactive T cells could be found in the islets of human patients with T1D (6,7). Thus, HIPs are present in the periphery and provide an explanation of how proteins expressed under physiological conditions can become neoantigens in an organ-specific manner.

Although the development of HIP-reactive T-cell lines from tissue of deceased human patients with T1D has confirmed their presence in islets (6,8), the more compelling question is whether they can serve as biomarkers of the disease process. With the development of I-A^{g7}/HIP tetramers, we could take a comprehensive approach to investigating HIP-reactive T cells as biomarkers in the NOD mouse model in which it is possible to compare what is happening in the target organ to the phenotype of HIP-reactive T cells present in the blood. Islet-reactive CD4 T cells present in the islets of NOD mice have diverse specificities, and it has been well established that T cells reactive to insulin comprise part of this population (9–11). The B:9-23 epitope from insulin has been demonstrated to be essential for the initiation of autoimmune diabetes in the NOD model (12), and several B:9-23–reactive

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Received 15 February 2018 and accepted 20 June 2018.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db18-0200/-/DC1.

CD4 T-cell clones are diabetogenic (4,9). The discovery of HIPs sheds new light on the role of insulin as an autoantigen in T1D. Using MHC class II tetramers to track antigen-specific T cells, we investigated the relative contributions of insulin B:9-23 versus HIP-reactive T cells in the pathogenesis of T1D. By comparing the frequency and phenotype of each population in the target organ, the pancreatic lymph nodes (pLNs), and the blood of mice at different ages, we show that HIP-reactive T cells are indicators of the disease process in the NOD model.

RESEARCH DESIGN AND METHODS

Mice

NOD and NOD.*scid* breeding mice were acquired from The Jackson Laboratory, bred, and housed in specific pathogen-free conditions at the University of Colorado School of Medicine or National Jewish Health (Denver, CO). NOD.ChgA$^{-/-}$ mice were bred in our colony by backcrossing C57BL/6.129.ChgA$^{-/-}$ mice onto the NOD background as previously described (4). Breeding mice and experimental animals were monitored for development of disease by urine glucose testing (Diastix; Bayer) and hyperglycemia confirmed by blood glucose testing using the OneTouch Ultra glucometer (LifeScan). Mice were considered diabetic when blood glucose levels were >15 mmol/L (270 mg/dL) for two consecutive readings. All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

Culture, Assay, Isolation, and Adoptive Transfer of Murine CD4 T-Cell Clones

T-cell clones were restimulated every 2 weeks as previously described with a granule-enriched membrane fraction obtained from β-cell tumors as a source of antigen, referred to as "β-membrane," in the presence of interleukin (IL)-2 (13). We isolated the Vβ12 BDC-11.1 and Vβ6 BDC-9.H1 CD4 T-cell clones by FACS using a MoFlo XDP cell sorter (three consecutive single-cell sorts were performed to purify the 2.5HIP tetramer-positive [tet$^+$] populations) followed by cloning by limiting dilution. These T-cell lines were stimulated biweekly with antigen/MHC as described above. T-cell responses were assessed as described previously (13). Forms of antigens used included islet cell suspensions (1×10^4 cells) from either NOD or NOD.ChgA$^{-/-}$ mice, β-membrane, whole insulin (Sigma-Aldrich), or synthetic peptides: the 2.5HIP or the 6.9HIP. After 18 h, interferon-γ (IFN-γ) concentrations were determined in culture supernatants by ELISA. To investigate diabetogenicity, T-cell clones were expanded in secondary cultures with additional IL-2, and $1–1.4 \times 10^7$ cells were injected i.p. into young (<14-day-old) NOD mice.

Peptides

Peptides were obtained from CHI Scientific at a purity >95%. Peptides used in this study were: 2.5HIP (LQTLALWSRMD), 6.9HIP (LQTLALNAARDP), HEL(AMKRHGLDNYRGYSL), ins B:9-23 (SHLVEALYLVCGERG), insp8G (HLVERLYLVCGGEG), insp8E (HLVERLYLVCGEEG), or an ins B:9-23 mimotope (SHLVEVLYLVAGEEG).

Flow Cytometry, Mass Cytometry, and FACS

Antibodies used in this study are described in Supplementary Table 1. DAPI, fixable viability dye eFluor780, or 7-aminoactinomycin D was used to discriminate live cells. Gating strategies are indicated in each figure; the lymphocyte gate was based on forward light scatter (FSC)/side scatter properties, and the singlets gate was based on the FSC-area and FSC-height. Samples were run on a BD Fortessa X-20 flow cytometer, and data were analyzed using FlowJo software V10 (Tree Star). Tetramer staining was performed at 37°C for 1 h as previously described (13). For intracellular staining, cells were fixed and permeabilized using a kit from eBioscience.

Antibodies used for mass cytometry were purchased from Fluidigm or conjugated in-house (CD62L and KLRG-1). Splenocytes were first enriched for CD4 T cells using a negative selection kit from STEMCELL Technologies and then mixed 1:1 with unfractionated splenocytes. Cells were then stained with phycoerythrin (PE)- or allophycocyanin-conjugated tetramer. After a 1-h incubation at 37°C, cells were washed three times and incubated with metal-conjugated antibodies (shown in Supplementary Fig. 2). PE-labeled cells were detected using an anti-PE antibody (Fluidigm). After 30 min, cells were washed three times and resuspended in a 1.6% paraformaldehyde solution containing the Cell-ID intercalator (Fluidigm). Cells were then analyzed on a Helios Mass Cytometer.

The peptides used to make the tetramers used in this study were the $HEL_{11–25}$, 2.5HIP (C-pep:ChgA hybrid) (6), 6.9HIP (C-pep:IAPP hybrid) (6), and two insulin B:9-23 mimotope peptides (insp8G and insp8E) (10). Because the two insulin tetramers stain distinct populations of B:9-23–reactive CD4 T cells (10), in most experiments, both tetramers conjugated to the same fluorophore were used in combination to detect as many B:9-23–reactive cells as possible.

Isolation of Murine Peripheral Blood Mononuclear Cells

Peripheral blood from mice was collected (100–150 μL) by submandibular bleeding in the presence of heparin. Peripheral blood mononuclear cells (PBMCs) were isolated using Lympholyte Mammal (Cedarlane Laboratories) according to the manufacturer's recommendations.

ELISpot Analysis

The murine IFN-γ ELISpot (BD Biosciences) was used according to the manufacturer's recommendations. Briefly, 0.5×10^6 splenocytes were used per well and incubated with 10 μmol/L of indicated peptide. After 72 h, the number of IFN-γ spots was counted using an ImmunoSpot reader.

T-Cell Receptor Sequencing

Total RNA was directly extracted from T-cell lines and clones using the RNAeasy Mini Kit (Qiagen), and cDNA was prepared using the SMARTer RACE cDNA Amplification Kit (Clontech). The full length of T-cell receptor (TCR) α and β chain variable region genes was amplified by two

steps of PCR using primer sets listed below, followed by sequencing on the 454 GSJR sequencer as previously described (14). The Universal Primer A Mix (contained in the SMARTer kit) along with a primer specific for the α or β constant region (5′-GGGTGCTGTCCTGAGACCGAG-GATC-3′ for α, 5′-AGCCCATGGAACTGCACTTGGCAGCG-3′ for β) were used for the first PCR amplification. The nested PCR amplification was then performed using a forward primer (5′-CCTATCCCCTGTGTGCCTTGGCAGTCT-CAGAAGCAGTGGTATCAACGCAGAGT-3′) and a reverse primer specific for the α or β constant region (5′-CCATCT-CATCCCTGCGTGTCTCCGACTCAG-3′, five nucleotides of multiple identifier, 5′-GTACACAGCAGGTTCTGGGTTCTG-G-3′ for α, 5′-CCATCTCATCCCTGCGTGTCTCCGACTCA-G-3′, five nucleotides of multiple identifier, and 5′-CCTGG-CCAAGCACACGAGGG-3′ for β).

Statistical Analysis

Statistical analysis included Wilcoxon rank-sum test, one-way ANOVA test, and Student *t* test. Statistical significance was defined as: $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

RESULTS

HIP-Reactive T Cells Are Detected in the Islets of NOD Mice

We used I-A^{g7} tetramers containing either HIPs or insulin mimotopes to study the immune response to these antigens. I-A^{g7} tetramers were loaded with the following peptides: the 2.5HIP (a C-pep:ChgA HIP), the 6.9HIP (a C-pep:IAPP2 HIP), ins B:9-23 mimotopes (insp8G and insp8E), and, as a negative control, $HEL_{11–25}$ (a peptide from hen egg lysozyme). The percentages of 2.5HIP tet$^+$, 6.9HIP tet$^+$, and ins tet$^+$ cells were first analyzed in whole pancreas of NOD mice, and a representative example of staining with these tetramers is shown in Fig. 1*A* with gating strategy in Supplementary Fig. 1*A*. For these experiments, the pancreata of prediabetic (3- to 10-week-old) versus diabetic NOD mice were analyzed. CD4 T cells were detected in the pancreas of mice at all ages and compared with the 3- and 6-week-old mice; there was a significant increase in the number of CD4$^+$ cells in the 10-week-old group (Supplementary Fig. 1*B*). Whereas tet$^+$ cells could not be detected in the pancreas of 3-week-old NOD mice (Supplementary Fig. 1*C*), we observed a significant increase of the percentage of 2.5HIP and 6.9HIP tet$^+$ cells starting at 10 weeks of age (Fig. 1*B*). Finally, the number of 2.5HIP tet$^+$ cells present in the pancreas was higher than 6.9HIP or ins tet$^+$ cell numbers at 10 weeks (Supplementary Fig. 1*C*). There were no significant differences between the percentage of tet$^+$ cells between the 10-week-old and the diabetic group. These results indicate that most of the HIP tet$^+$ cell infiltration is occurring in mice between 6 and 10 weeks of age. Furthermore, these results show that HIP tet$^+$ cells are among the first islet-reactive CD4 T cells that infiltrate the pancreas of NOD mice and are then retained in the pancreas throughout the progression of disease. To demonstrate the specificity of the 2.5HIP or 6.9HIP tetramer in polyclonal T-cell populations, each tetramer was conjugated to two different fluorophores. As exemplified in the bottom left panels of Supplementary Fig. 1*A*, our data show that the new HIP tetramers were very specific in their binding to T cells, as nearly all tet$^+$ cells were located in the predicted diagonal.

To confirm that tet$^+$ cells were actually infiltrating the islets rather than being restricted to the exocrine pancreas, islets were isolated from nondiabetic NOD mice and examined for the presence of tet$^+$ cells. Because it is technically difficult to prepare islets from either very young mice (islets are still developing) or diabetic mice (very few islets are remaining), only 6- to 20-week-old nondiabetic mice were examined. The ins tet$^+$ and 6.9HIP tet$^+$ cells were present in the islets, averaging ∼1% of the total CD4 population (Fig. 1*C*). In contrast, the 2.5HIP tet$^+$ cells were much higher in abundance and averaged ∼4.5% of the total CD4 T cells present in the islets. Furthermore, in the nondiabetic group, the frequency of intraislet 2.5HIP tet$^+$ cells was significantly higher than in the whole pancreas ($P < 0.05$), indicating that whole pancreas analysis might underestimate the actual frequency of tet$^+$ cells present in the islets (Fig. 1*B* vs. Fig. 1*C*).

2.5HIP tet$^+$ Cells Are Highly Activated in the pLNs Early in Disease

From studies in BDC-2.5 TCR-transgenic (TCR-Tg) mice, it has been suggested that in the initiating phase of T1D, antigen-presenting cells in the islets pick up antigen from β-cells and migrate to the pLNs, where they present antigen to autoreactive T cells (15). Others have shown that NOD mice in which the pLN are removed within 3 weeks after birth do not develop diabetes (16), demonstrating a critical role of these draining LN in the activation of pathogenic T cells. However, TCR-Tg models have several limitations, including interclonal competition (17), T cells escaping from clonal deletion (18), and a bias in T-cell fate (19). More importantly, TCR-Tg mice do not allow for comparing activation of T cells of different specificities. It is therefore important to re-examine this question in the context of polyclonal CD4 T cells.

I-A^{g7}/HIP or I-A^{g7}/ins tetramers were used to examine the activation status of HIP-reactive T cells present in the pLNs of 3-, 6-, and 10-week-old NOD mice compared with T cells in brachial LNs (bLNs) as a control. We used the $CD44^{hi}CD62L^{lo}$ cell surface phenotype as a measure of antigen-induced activation as shown in Fig. 2*A*. We observed that in all age-groups, compared with the bulk CD4 population, the majority of tet$^+$ cells showed an antigen-experienced phenotype in the pLN (Fig. 2*B*), and this was particularly significant in the 2.5HIP tet$^+$ cells ($P < 0.01$ in all age-groups). Results presented in Fig. 2*B* indicate that already at 3 weeks of age, in some mice the majority of 2.5HIP tet$^+$ cells display an antigen-experienced phenotype. By 6 weeks of age, ∼50% of the 2.5HIP tet$^+$ cells present in the pLN are $CD44^{hi}CD62L^{lo}$, and the percentage of activated 2.5HIP tet$^+$ cells was even greater in the

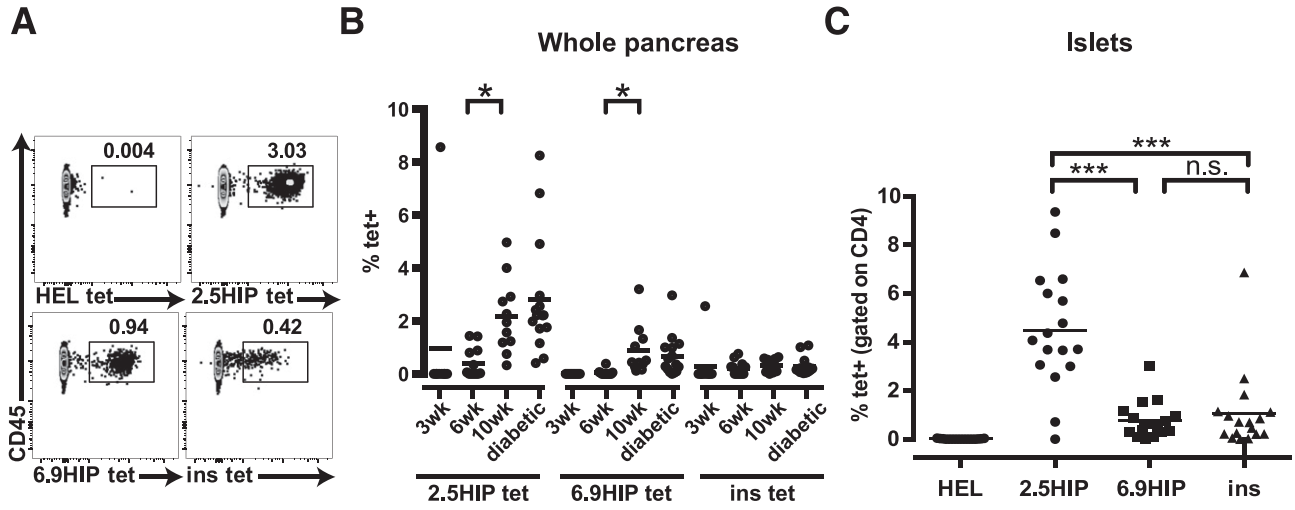

Figure 1—HIP tet$^+$ cells are present in the islets of NOD mice. *A* and *C*: Single-cell suspensions were prepared from the islets of nondiabetic NOD mice and stained with antibodies and tetramers. Tetramers contained the following peptides: HEL ($HEL_{11–25}$), 2.5HIP (LQTLALWSRMD), 6.9HIP (LQTLALNAARDP), and ins B:9-23 mimotopes (insp8G [HLVERLYLVCGGEG] and insp8E [HLVERLYLVCGEEG]). Gates were set on live, CD45$^+$, lineage-negative (Lin$^-$), CD4$^+$ cells, as shown in Supplementary Fig. 1. *A*: Representative example of tetramer staining from the islets of one NOD mouse. Data are representative of five independent experiments. *B*: Single-cell suspensions were prepared from whole pancreas of young (3-, 6-, and 10-week-old) or diabetic NOD mice and stained with antibodies and tetramers. Each symbol represents an individual mouse, and a summary of six independent experiments is provided. *C*: Tetramer analysis in the islets of 17 nondiabetic NOD mice (aged 6–20 weeks); data are a summary of four independent experiments. *$P < 0.05$; ***$P < 0.001$.

10-week-old group, averaging 75% (Fig. 2*B*). The percentages of activated 2.5HIP tet$^+$ cells in the spleen and pLN were comparable (data not shown), suggesting that once activated, these cells rapidly leave the pLN and circulate as a memory population. The 2.5HIP tet$^+$ cells remained naive in the bLN in the 6- and 10-week-old age-groups. For both 6.9HIP or ins tet$^+$ cells, the percentage of cells expressing CD44^{hi}CD62L^{lo} was similar in the pLN compared with the bLN in all age-groups. Because we observed that tet$^+$ cells with an activated phenotype could be found in the pLN of 3-week-old NOD, prior to their infiltration in the target organ, our data suggest that HIP and ins B:9-23 tet$^+$ cells are first activated in the pLN, probably before 3 weeks of age. In particular, the 2.5HIP tet$^+$ cells are among the first antigen-specific CD4 T cells to be activated in the pLN of NOD mice.

HIP-Reactive T Cells Display an Antigen-Experienced, Inflammatory Phenotype

To gain further insight into the immunophenotype of 2.5HIP tet$^+$ cells, splenocytes from diabetic NOD mice were analyzed by mass cytometry. This methodology allows simultaneous analysis of multiple parameters (Supplementary Fig. 2*A*), therefore offering high dimensional analysis of rare populations of cells, such as tet$^+$ cells. Our data shown in Supplementary Fig. 2*B* demonstrate that a sizable population of 2.5HIP tet$^+$ T cells could be found in the spleen of NOD mice and that the percentages of tet$^+$ cells were comparable to those obtained by flow cytometry (data not shown). We examined markers affected by T-cell activation (CD44, CD49d, and CD62L), surface markers of functional regulatory T cells (Tregs; CD25$^+$CD127$^-$ and KLRG-1) and programmed death-1 (PD-1), a receptor that plays an important role in regulating T-cell responses and is rapidly upregulated on activated T cells (20). Compared with the bulk CD4 population, CD44, CD49d, and PD-1 were upregulated on 2.5HIP tet$^+$ cells, whereas levels of CD62L were decreased, indicating that 2.5HIP tet$^+$ cells were antigen experienced. In Supplementary Fig. 2*C*, a Spanning-tree Progression Analysis of Density-normalized Events analysis shows the cellular heterogeneity in the spleen of NOD mice and compares functional markers between the different cell types. 2.5HIP tet$^+$ cells clustered in two distinct populations, both expressing high levels of CD44 and CD49d. Interestingly, our analysis shows that a population of CD25$^+$CD127$^-$ cells was absent in the 2.5HIP tet$^+$ cells, possibly indicating a lack of Tregs in this population.

CD4 T cells expressing the transcription factor FoxP3 (Treg) play a critical role in preventing systemic autoimmunity (21), and we and others have demonstrated that this regulation happens in an antigen-specific manner (22–24). To determine whether HIP-reactive T cells contribute to regulation of disease in the NOD mouse model, we analyzed the spleen and pancreas of NOD mice for the presence of HIP tet$^+$FoxP3$^+$ T cells (Fig. 3*A*). To investigate mice with significant amounts of islet infiltration, we analyzed nondiabetic (12- to 20-week-old) mice individually (Fig. 3*B*). In the spleen of NOD mice, the percentage of HIP tet$^+$ and ins tet$^+$ cells were markedly reduced compared with the bulk CD4$^+$ population. In contrast, in the pancreas of these mice, the percentage of ins tet$^+$ cell expressing FoxP3 was significantly higher than that of HIP tet$^+$ cells,

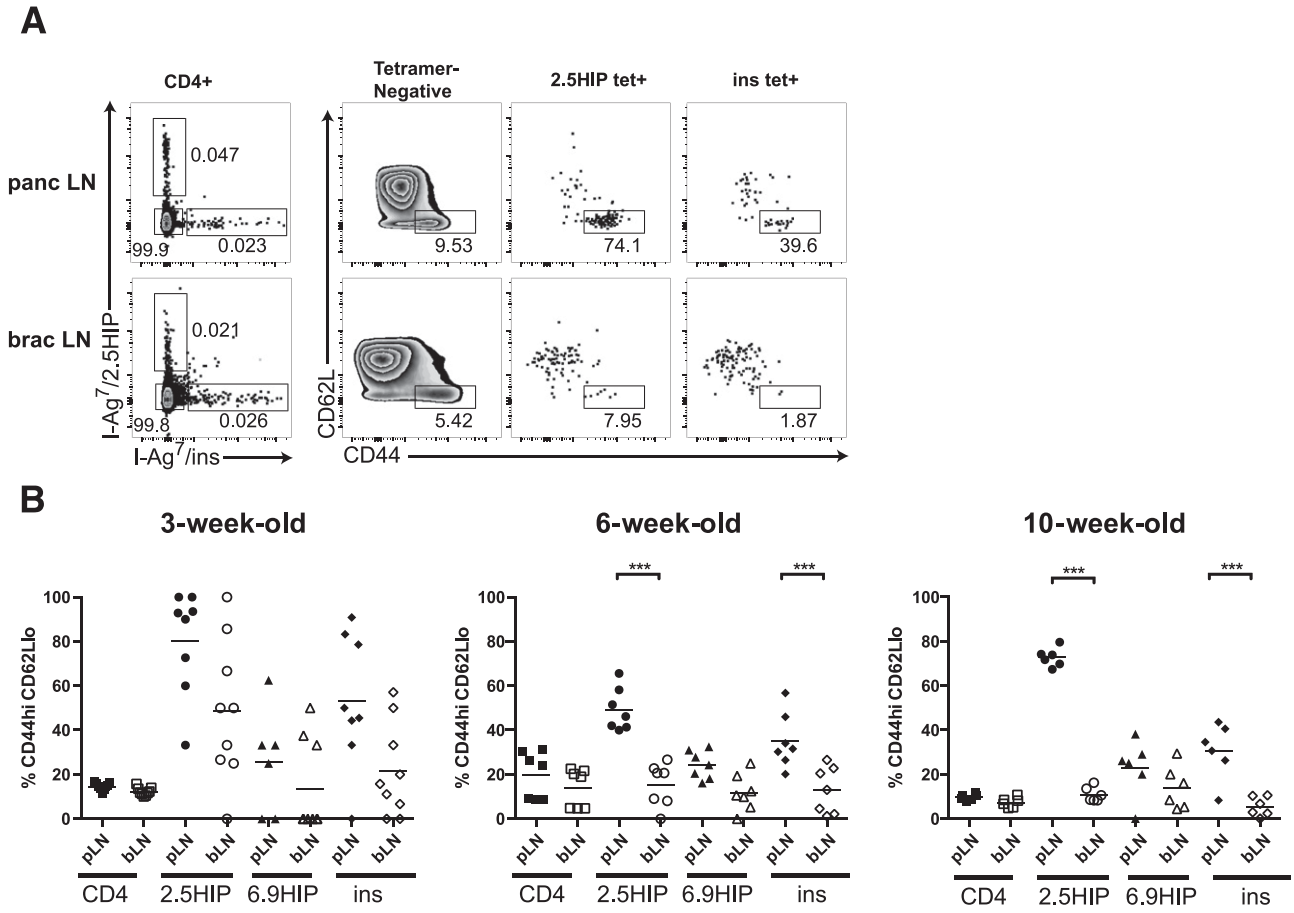

Figure 2—Phenotype of tet$^+$ cells in the pLN vs. bLN at 3, 6, and 10 weeks in NOD mice. *A*: Single-cell suspensions were prepared from pLN (panc LN) or bLN (brac LN) from a 10-week-old NOD mouse. Cells were stained with antibodies and tetramers and then analyzed by flow cytometry. Tetramers contained the following peptides: 2.5HIP (LQTLALWSRMD), 6.9HIP (LQTLALNAARDP), and ins B:9-23 mimotopes (insp8G [HLVERLYLVCGGEG] and insp8E [HLVERLYLVCGEEG]). Gates were set on live, CD45$^+$, lineage-negative (Lin$^-$), CD4$^+$ cells. The percentage of CD44^{hi}CD62L^{lo} is reported for each population (numbers within boxes). *B*: Summary of three independent experiments per age-group for 3-week-old (n = 9), 6-week-old (n = 12), and 10-week-old (n = 11) NOD mice. Each symbol represents an individual mouse. ***$P < 0.001$.

indicating a possible immunoregulatory role of ins tet$^+$ cells, but not HIP tet$^+$ cells, at the site of the autoimmune attack. To investigate whether changes in FoxP3 expression on tet$^+$ cells were influenced by antigen exposure, we compared FoxP3 expression on 2.5HIP tet$^+$ cells in NOD mice sufficient or deficient for ChgA (Fig. 3*C*). Our data show that in ChgA-sufficient mice, compared with the bulk CD4 population, there was a significant decrease in FoxP3 expression on 2.5HIP tet$^+$ cells, both in the pancreas and spleen. In contrast, in the absence of ChgA, no significant differences in FoxP3 expression were observed between CD4 and 2.5HIP tet$^+$ cells, indicating that FoxP3 expression on 2.5HIP tet$^+$ cells is antigen dependent.

All of the CD4 T-cell clones from the BDC panel exhibit a T helper type 1 (Th1) phenotype, secreting large amounts of IFN-γ and tumor necrosis factor-α in response to antigen, both in vitro and ex vivo (25). We used an IFN-γ ELISpot assay to ask whether polyclonal HIP-reactive T cells acquire an inflammatory phenotype at disease onset. Splenocytes from diabetic NOD mice were stimulated with the 2.5HIP, 6.9HIP, or an insulin mimotope peptide. Phorbol myristic acid/ionomycin was used as a positive control, and the $HEL_{11–25}$ peptide was used as a negative control. Our data indicate that IFN-γ–secreting 2.5HIP- and 6.9HIP-reactive T cells were present in the spleen of diabetic NOD mice (Fig. 3*D* and *E*). In addition, the number of spots obtained after 2.5HIP stimulation of splenocytes correlated with the number of events obtained by flow cytometry (Fig. 3*F*). Insulin-reactive cells were also detected in the spleen of diabetic NOD mice but at lower frequency, recapitulating our data obtained with tetramer analysis. Together, our data indicate that HIP-reactive T cells have a proinflammatory but not a regulatory phenotype at the site of inflammation, which might explain their role in disease pathogenesis.

T Cells Selected by HIP Tetramers Are Diabetogenic

To determine the diabetogenic potential of 6.9HIP and 2.5HIP tet$^+$ cells, we screened uncloned BDC T-cell lines (BDC-9, BDC-10, and BDC-11) isolated from diabetic NOD

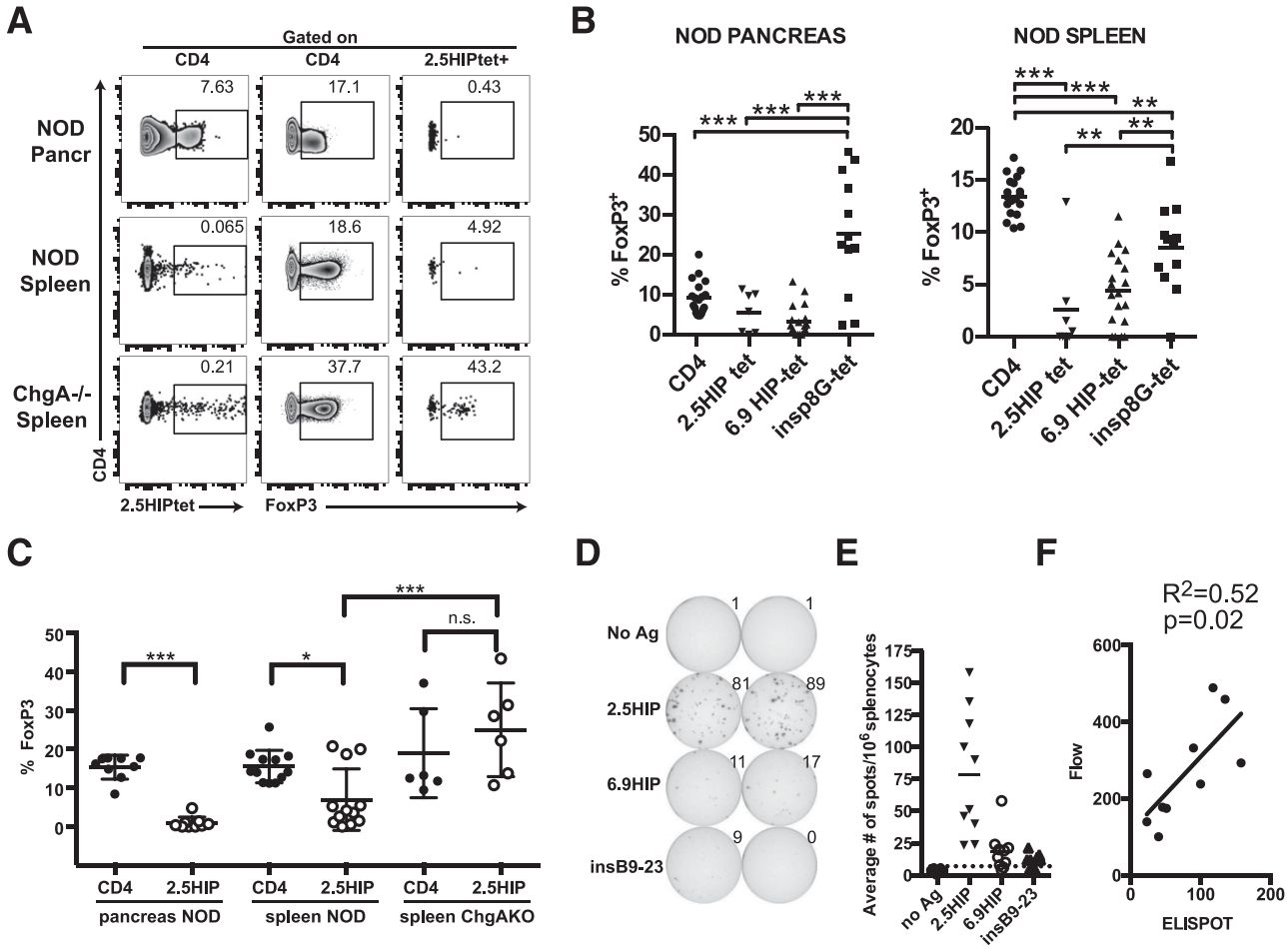

Figure 3—HIP-reactive T cells are devoid of Tregs and secrete IFN-γ. *A*: FoxP3 expression in the spleen and pancreas (Pancr) of NOD or NOD.ChgA$^{-/-}$ mice from a representative experiment. Gates were set on live, CD45$^+$, lineage-negative (Lin$^-$), CD4$^+$ cells. *B*: FoxP3 expression in the pancreas and spleen in nondiabetic (12- to 20-week-old) NOD mice. Data are a summary of at least three independent experiments. Tetramers contained the following peptides: 2.5HIP (LQTLALWSRMD), 6.9HIP (LQTLALNAARDP), or ins B:9-23 mimotopes (insp8G [HLVERLYLVCGGEG] and insp8E [HLVERLYLVCGEEG]). *C*: FoxP3 expression in ChgA-sufficient or ChgA knockout (ChgAKO) mice. Experiments were performed using diabetic wild-type NOD mice or aged-matched NOD.ChgA$^{-/-}$ mice. *D–F*: Splenocytes from diabetic NOD mice were cultured with or without antigen (Ag) in an IFN-γ ELISpot assay and the number of spots (cytokine-secreting cells) are reported. *D*: Assay plate wells from a representative experiment. Numbers to the upper right of each well indicate the number of spots counted. Duplicate wells are shown. *E*: Summary of three independent experiments. n = 10 mice. *F*: Correlation between the number of 2.5HIP tet$^+$ events obtained by flow cytometry vs. number of spots obtained by ELISpot. Data are expressed per 10^6 splenocytes. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

mice for the presence of 2.5HIP and 6.9HIP tet$^+$ cells (Fig. 4*A*). To investigate the Vβ usage of these lines, 454 deep sequencing was performed, and our results indicate that although BDC-11 was oligoclonal, containing only three different Vβ populations, BDC-9 and BDC-10 were polyclonal (Supplementary Fig. 3). Each line was tested for the presence of 2.5HIP and 6.9HIP tet+ cells by flow cytometry. We observed 6.9HIP tet$^+$ cells in the BDC-9 and BDC-10 lines, whereas all three lines contained 2.5HIP tet$^+$ cells (Fig. 4*A*). Table 1 shows the Vα and Vβ usage of 6.9HIP and 2.5HIP tet$^+$ T cells in these T-cell lines. Three consecutive FACSs were performed to isolate pure populations of 6.9HIP or 2.5HIP tet$^+$ cells, yielding two new T-cell clones termed BDC-9.H1 (Vβ6$^+$) (Fig. 4*B* and *C*) and BDC-11.1 (Vβ12$^+$) (Table 1). Like the BDC-6.9 T-cell clone, which is reactive to the 6.9HIP (a HIP containing a sequence from IAPP), the BDC-9.H1 clone did not react to IAPP-deficient islets but did respond to NOD islets (Fig. 4*D*). The insulin-reactive T-cell clone BDC-4.38 responded to islets from both strains (Fig. 4*D*). The BDC-9.H1 clone also responded to the 6.9HIP peptide at concentrations as low as 10 nmol/L (Fig. 4*E*), but not to insulin C-peptide or IAPP2 peptide, confirming that BDC-9.H1 was indeed 6.9HIP reactive. Similarly, the BDC-11.1 clone responded specifically to the 2.5HIP peptide (Fig. 4*F*) and stained with the 2.5HIP tetramer (Supplementary Fig. 4). To investigate their diabetogenicity in vivo, BDC-9.H1 or BDC-11.1 CD4 T cells were adoptively transferred into wild-type NOD mice. All recipients developed diabetes within a few weeks after transfer (Fig. 4*G*), demonstrating the diabetogenic potential of T cells selected for reactivity to HIPs.

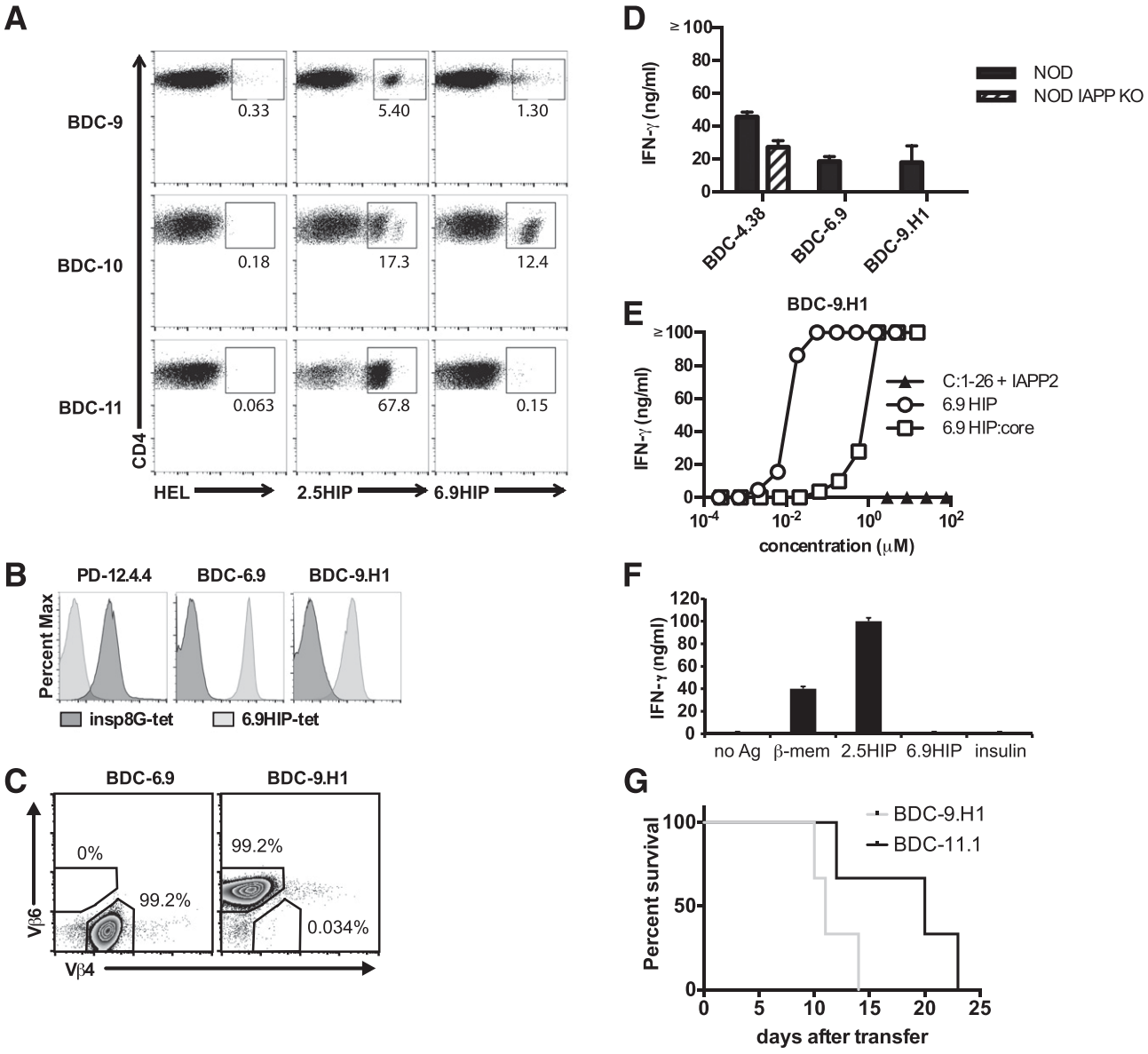

Figure 4—Antigen specificity and diabetogenicity of HIP-reactive T cells. *A*: tet$^+$ cells are present in three BDC T-cell lines. Single-cell suspensions were stained with I-A^{g7} tetramer. After 45 min, cells were counterstained with anti-CD4 and a viability dye before flow cytometry analysis. Gates were set on live CD4$^+$ cells, and data are representative of two independent experiments for each T-cell line. Our results indicate that all three lines contained 2.5HIP tet$^+$ cells, and both BDC-9 and BDC-10 contained 6.9HIP tet$^+$ cells. tet$^+$ cells were then sorted by FACS to obtain pure populations of tet$^+$ cells. *B*: The islet-reactive T-cell clones PD-12.4.4 (insulin-reactive), BDC-6.9 (6.9HIP-reactive), and BDC-9.H1 were stained with antibodies and tetramers. Gates were set on live CD4$^+$ cells, and data are representative of three independent experiments for each T-cell clone. *C*: To verify clonality, BDC-6.9 and BDC-9.H1 were stained with Vβ4 or Vβ6 antibodies. Gates were set on live CD4$^+$ cells, and data are representative of at least four independent experiments for each T-cell clone. *D*: BDC-4.38 (insulin-reactive), BDC-6.9, or BDC-9.H1 T-cell clones from the BDC panel were challenged with NOD or NOD.IAPP$^{-/-}$ islets, and IFN-γ secretion was measured by ELISA. Data are representative of three independent experiments. *E*: The T-cell clone BDC-9.H1 was challenged with peptide antigens, and IFN-γ was measured by ELISA. Peptides used were insulin C-peptide and IAPP2 (added together in the same culture well), the 6.9 HIP core peptide (LQTLALNAARDP), or the full-length 6.9HIP (EVEDPQVAQLELGGGPGAGDLQTLALNAARDPNRESLDFLLV). Data are representative of three independent experiments. *F*: The T-cell clone BDC-11.1 is 2.5HIP reactive. The T-cell clone BDC-11.1 (2×10^4) was challenged with antigen (Ag) and antigen-presenting cells (2×10^4). After 24 h, supernatants from cell cultures were tested for the presence of IFN-γ by ELISA. Results are from duplicate wells from two independent experiments. *G*: The T-cell clones BDC-9.H1 or BDC-11.1 were adoptively transferred into young (6–14 days old) NOD mice ($n = 3$ recipient mice/group from two independent experiments per T-cell clone), and mice were monitored daily for signs of hyperglycemia. Mice were considered diabetic when blood glucose levels rose above 15 mmol/L. KO, knockout.

Table 1—TCR usage of 6.9HIP- and 2.5HIP-reactive T cells from BDC lines

Cloned line	TCRβ	TCRα	Peptide ligand
BDC-2.5	TRBV2 (Vβ4)	TRAVD-6	2.5HIP
BDC-10.1	TRBV20 (Vβ15)	TRAV17	2.5HIP
BDC-9.46	TRBV13-1 (Vβ8.3)	TRAV16	2.5HIP
BDC-11.1	TRBV15 (Vβ12)	TRAV16	2.5HIP
BDC-10.3	TRBV15 (Vβ12)	TRAV13-1	2.5HIP
BDC-9.2	TRBV1 (Vβ2)	TRAV7-3	2.5HIP
BDC-6.9	TRBV2 (Vβ4)	TRAV5D4	6.9HIP
BDC-9.3	TRBV2 (Vβ4)	TRAV5D4	6.9HIP
BDC-9.H1	TRBV19 (Vβ6)	TRAV10	6.9HIP

2.5HIP tet$^+$ Cells in the Blood Acquire an Antigen-Experienced Phenotype Early in the Disease Process

Our data indicate that there is a high frequency of HIP tet$^+$ cells in the target organ of NOD mice, but in the human setting, only peripheral blood can be used to assess the presence of islet-reactive T cells. A key question is whether circulating T cells reflect the disease process in the target organ. To determine whether tet$^+$ cells detected in the blood of NOD mice could be indicative of disease activity, we investigated the frequency and phenotype of 2.5HIP, 6.9HIP, and ins tet$^+$ cells in PBMCs over time. PBMCs were analyzed weekly for 2.5HIP tet and ins tet staining from a cohort of NOD female mice, starting at 5 weeks of age; analysis of 6.9HIP tet$^+$ cells was added starting at 13 weeks. Diabetes incidence in this cohort of 14 mice was 50% at 30 weeks, and mice started to become diabetic at 14 weeks of age (Fig. 5*A*). Our results show that the frequency of 2.5HIP tet$^+$ cells increases over time in the blood of NOD mice, considerably more so than either the ins tet$^+$ or 6.9HIP tet$^+$ cells (Fig. 5*B*, center panels). We compared the phenotype of each tet$^+$ cell population to that of the bulk CD4 T-cell population present in the blood by looking at the presence of antigen-experienced CD44^{hi}CD62L^{lo} cells (as shown in Fig. 5*C*). Compared with the bulk CD4 population, the percentages of CD44^{hi}CD62L^{lo} 2.5HIP tet$^+$ cells were elevated, a difference that was already significant by 10 weeks (Fig. 5*C*, right panel). 6.9HIP tet$^+$ cells also gained an antigen-experienced phenotype, but at later time points (starting at ~15 weeks of age). The phenotype of ins tet$^+$ cells differed from that of the bulk CD4 T-cell population only at the 8-week-old time point (Fig. 5*C*, right panel). These results suggest that the phenotype of 2.5HIP and 6.9HIP tet$^+$, but not ins tet$^+$, T cells in the blood can be an indicator of the autoimmune process.

DISCUSSION

In this study, we used MHC class II tetramers to examine the role of CD4 T cells reactive to HIPs in the pathogenesis of autoimmune diabetes in the NOD mouse. Our data demonstrate that T cells reactive to the 2.5HIP, a hybrid peptide formed by the fusion of a C-peptide fragment to WE14 (a natural cleavage product of ChgA), are present in high frequency in the pancreas of both nondiabetic and diabetic NOD mice. Similarly, T cells reactive to the 6.9HIP, an HIP formed through fusion of the same C-peptide fragment to IAPP2 (a natural cleavage product of pro-IAPP), were also present in the pancreas of NOD mice. We found that the frequency of 2.5HIP tet$^+$ cells was consistently higher than the frequency of T cells bound by ins B:9-23 tetramers (Fig. 1).

When we assessed the phenotype of 2.5HIP tet$^+$ cells in the pLN, we observed that these cells acquired an antigen-experienced phenotype early in the disease process (Fig. 2), well before HIP tet$^+$ cells infiltrate the pancreas (Fig. 1). Because we find that antigen-experienced HIP-reactive T cells are present in the pLN as early as 3 weeks of age, our data suggest a pattern by which these cells are first activated in the pLN, where they become CD62L^{lo}, CD44^{hi}, CD127$^+$, and CD49d$^+$ (Supplementary Fig. 2). Upon losing CD62L expression, HIP-reactive T cells exit the pLN to enter the peripheral blood, where they can be detected at increasing frequency throughout disease progression (Fig. 5). The upregulation on HIP-reactive T cells of CD49d, an integrin responsible for the extravasation of lymphocytes into inflamed organs, would favor their entry into the islets, where they could coordinate the immune response leading to the destruction of β-cells. We have also found that a majority of 2.5HIP tet$^+$ cells express PD-1 (but not KLRG-1). The PD-1/programmed death ligand-1 (PD-L1) axis is another pathway that has been the subject of intense research in autoimmune diabetes. PD-1 is a coinhibitory receptor that has been associated with T-cell activation and exhaustion. Importantly, disruption of the PD-1/PD-L1 pathway using antibody blockade has been shown to accelerate disease in both NOD mice (26,27) and humans (28,29). Our data indicating that HIP-reactive T cells are PD-1$^+$ suggest that they could potentially be tolerized through this pathway, perhaps through overexpression of PD-L1 on islet cells (30). Finally, the expression of CD127, the receptor for IL-7, might contribute to the self-renewal and persistence of HIP-reactive T cells in the islets.

The diabetogenic potential of BDC T cells has been well established through adoptive transfer of T-cell clones (2,31) and transgenic (32) and retrogenic (33) mouse models. The pathogenic potential has been attributed to a Th1 inflammatory phenotype, and in this study, we also observed by ELISpot a high frequency of cells secreting IFN-γ in response to the 2.5HIP peptide in diabetic NOD mice (Fig. 3). In addition, using tetramers to characterize T-cell lines isolated from diabetic NOD mice, we have isolated new HIP-reactive T-cell clones and demonstrated their diabetogenic potential (Fig. 4) Very few 2.5HIP-reactive T cells express FoxP3 in the pancreas at the time of disease onset, indicating that restoring immune regulatory function in the 2.5HIP population could be an interesting avenue for immunotherapy. In contrast, insulin-reactive T cells in the pancreas appear to have varying phenotypes

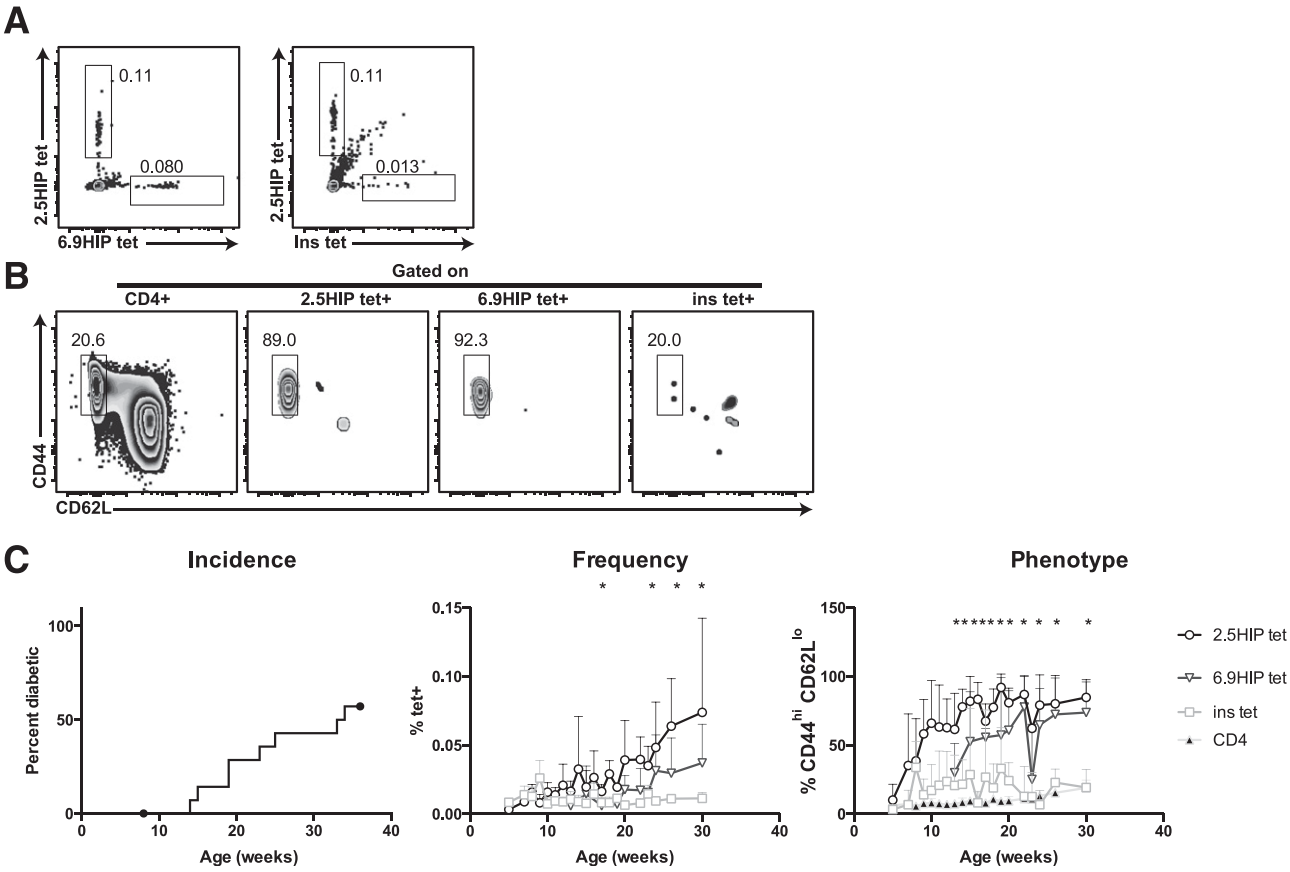

Figure 5—Frequency and phenotype of tet$^+$ cells in the blood of NOD mice over time. Peripheral blood from NOD mice was collected by submandibular bleed, and PBMCs were isolated on a Ficoll gradient. PBMCs were then stained with antibodies and tetramers. Tetramers contained the following peptides: 2.5HIP (LQTLALWSRMD), 6.9HIP (LQTLALNAARDP), and ins B:9-23 mimotopes (insp8G [HLVERLYLVCGEEG] and insp8E [HLVERLYLVCGEEG]). *A*: A representative example of the frequency of tet$^+$ cells in the PBMCs from a mouse in the cohort. *B*: A representative example of CD44 vs. CD62L expressed on mouse PBMCs. Gates were set on live, CD45$^+$, CD4$^+$, lineage-negative (Lin$^-$) cells or on tet$^+$ cells (as indicated). *C*: Disease incidence in the cohort of 14 mice is shown in the left panel. The percentage of tet$^+$ cells is shown in the center panel, and the percentage of CD44^{hi}CD62L^{lo} cells is represented in the right panel. Asterisks indicate significant differences compared with insulin tet$^+$ cells ($P < 0.05$). *$P < 0.05$.

and functions, including pathogenic Th1 cells (4,9,34,35), anergic T cells (11), and FoxP3$^+$ T cells (36,37). Our data indicate that insulin tet$^+$ cells expressed FoxP3 at a percentage that was higher than that of the bulk CD4 population, particularly in the pancreas, suggesting that this population can also play a regulatory role in disease. These observations could also explain why in our study, very few insulin-reactive T cells isolated from the spleen produce IFN-γ (Fig. 3*E*). Collectively, our data demonstrate that HIP-reactive T cells play a role in the pathogenesis of NOD diabetes that differs from that of insulin-reactive T cells.

There are compelling reasons to investigate T cells that are reactive to the newly identified HIPs as possible biomarkers. Efforts have been ongoing for over two decades to establish islet antigen-reactive T cells as biomarkers of disease, but it has been difficult to demonstrate clear and convincing differences between patients with T1D and control subjects in their T-cell responses to various candidate autoantigens. We propose that HIP-reactive T cells could serve as indicators of disease progression and that targeting HIP-reactive T cells during the pathogenesis of disease might be key to restoring immune tolerance. Because 2.5HIP tet$^+$ cells are one of the predominant populations in the pancreas, it is possible that targeting one antigen might be enough in antigen-specific therapies. For example, a recent report has shown that nanoparticles coated with MHC class II molecule loaded with a BDC-2.5 mimotope peptide induced antigen-specific tolerance through the induction of a TR1-like population (38). Other variations on approaches used to induce antigen-specific tolerance could also be effective in this setting (27,39,40). The studies to date, however, have all used BDC-2.5 peptide mimotopes; using HIPs as the tolerizing peptides could target a broader repertoire of islet-reactive T cells and therefore would possibly be more effective in the setting of a polyclonal population, either in NOD mice or even more so in humans. Finally, better understanding of HIP reactivity in the NOD mouse and the discovery of new HIPs could be essential to our understanding of the disease

and our ability to more accurately predict disease onset and intervene in an antigen-specific fashion.

Acknowledgments. The authors thank the National Institutes of Health Tetramer Core Facility for providing all of the MHC tetramers mentioned in this study.

Funding. This study was supported by American Diabetes Association (ADA) grant 1-14-BS-089 (to K.H.), ADA Junior Faculty Award 1-15-JF-04 (to R.L.B.), ADA Pathway to Stop Diabetes 1-15-ACE-14 (to T.D.), JDRF grant 2-2012-197 (to R.S.F.), and National Institutes of Health research grants R21-AI-133059 (R.L.B.), R01-DK-081166 (to K.H.), R01-DK-111733 (to R.S.F.), and R01-DK-099317 (to M.N.).

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

Author Contributions. R.L.B. performed flow cytometry experiments, ELISpot analysis on mouse splenocytes, and analyzed data. R.L.B. and K.H. conceived and provided oversight of the project and wrote the manuscript. B.L.J. and T.A.W. performed flow cytometry, disease transfer, and CD4 T-cell cloning experiments. B.L.J., T.A.W., T.D., and R.S.F. reviewed the manuscript and provided comments. R.S.L. collected blood from NOD mice for the longitudinal study, isolated islets, and performed flow cytometry analysis. G.B. provided assistance in cell culture of the BDC T-cell clones. B.B. performed adoptive transfer of disease in NOD mice and monitored mice for hyperglycemia. M.N. performed TCR analysis of mouse T-cell lines and clones. K.H. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the 76th Scientific Sessions of the American Diabetes Association, New Orleans, LA, 10–14 June 2016.

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