Title: A Humanized Mouse Model of Autoimmune Insulitis

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**Abstract:**

Many mechanisms of and treatments for Type 1 diabetes studied in the NOD mouse model have failed to replicate in human disease. Thus, the field of diabetes research remains hindered by lack of an *in vivo* system in which to study the development and onset of autoimmune diabetes. To this end, we have characterized a system using human CD4+ T cells pulsed with autoantigen-derived peptides. Six weeks after injection of as few as 0.5x10^6 antigen-pulsed cells into the NOD-Scid IL2rg/- mouse expressing the human HLA-DR4 transgene, infiltration of mouse islets by human T cells was seen. While islet infiltration occurred with both healthy and diabetic donor antigen-pulsed CD4+ T cells, diabetic donor injections yielded significantly greater levels of insulitis. Additionally, significantly reduced insulin staining was observed in mice injected with CD4+ T cell lines from diabetic donors. Increased levels of demethylated β cell-derived DNA in the bloodstream accompanied this loss of insulin staining. Together, these data show that injection of small numbers of autoantigen-reactive CD4+ T cells can cause a targeted, destructive infiltration of pancreatic β cells. This model may be valuable for understanding mechanisms of induction of human diabetes.
Introduction

The development of Type 1 diabetes involves a combination of genetic and environmental factors governing susceptibility to and/or protection from disease (1). NOD mice, the most widely studied model of human Type 1 diabetes, share a number of disease characteristics including autoantigens, the chronicity of the autoimmunity, and MHC homology, but significant differences between the two still remain (e.g., the time of progression from insulitis to clinical diabetes and the sex bias of disease incidence) (2). Because of these differences and others, many mechanisms and treatments that have been verified in NOD mice have failed to translate into successful treatments in humans (3,4). It therefore is imperative to develop model systems in which human cells that are involved in diabetes can be directly studied.

The antigens that are involved in Type 1 diabetes have largely been identified through autoantibodies that are found in individuals at risk for and with the disease. They include preproinsulin (PPI), glutamic acid decarboxylase 65 (GAD65), and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), as well as other antigens recognized by polyclonal antibodies (islet cell antibodies, ICA) (5). T cells that are directed against these antigens are believed to cause β cell destruction, but there is little direct evidence that this is the case. The technical problems in studying the functions of autoreactive T cells include difficulties in growing and maintaining autoantigen-reactive lines and the lack of a suitable model system in which they can be studied.

Previous studies have analyzed histopathology (6–8) and T cell tetramer staining (9) of pancreata from cadaveric diabetic donors. In these studies, CD8+ T cells that are reactive with
IGRP were detected by immunohistochemical staining. However, staining of pre-diabetic insulitic lesions in humans is still conspicuously missing from the literature. Better visualization and understanding of these earliest events is of great significance, as it is unknown how the cellular composition of these lesions may have changed up to the point of clinical Type 1 diabetes diagnosis, let alone over a lifetime of disease in an individual. Knowledge of these very early events could allow for design of therapeutics aimed at prevention, as well as treatment of Type 1 diabetes.

Herein, we tested whether CD4+ T cells, derived from HLA-matched diabetic and healthy donors and expanded on diabetes antigens, could cause insulitis and β cell destruction in NOD mice devoid of endogenous T cells, B cells and NK cells (NOD-\textit{Scid} \textit{Il2rγ/-} mice, referred to here as NSG mice) and also transgenic for human HLA-DR4 (10,11) (referred to as NSG.DR4). Parallel injections of PBMC from diabetic or healthy control individuals were also performed, allowing for direct comparisons of both the extent of insulitis and non-specific organ involvement of the two systems.

We show that injections of antigen-pulsed expanded CD4+ T cells from patients with Type 1 diabetes resulted in varying degrees of islet infiltration, from peri-insulitis to severe insulitis. In these mice, there was a significant loss of insulin and increased levels of demethylated \textit{Ins1} DNA and Caspase-3 staining compared to control mice, reflecting β cell death. Interestingly, we isolated increased numbers of mouse CD45+ cells from the pancreata of mice injected with diabetic donor CD4+ T cells, suggesting that in this model cells from diabetic patients are able to establish an inflammatory environment in which murine leukocytes collaborate. These studies are the first to show β cell destruction mediated by human cells in a
hybrid humanized mouse system. This model will be useful for studies of early insulitis and β cell destruction mediated by human immune cells.

**Research Design and Methods**

**HLA Haplotype Determination**

PBMC were collected from patients with Type 1 diabetes and non-diabetic donors via leukopheresis or whole blood collection. Lymphocytes were isolated via Ficoll gradient. DNA was isolated from each prospective donor (Qiagen DNeasy Blood & Tissue Kit) and MHC haplotype was determined using the DRDQ 2 Test SSP UniTray Kit (Invitrogen). Only donors determined to be DRB1*0401 were used for further analysis. HLA-DR typing and disease status of donors is shown in Table 1. Written informed consent was obtained from all donors. The use of human cells was approved by the Yale Institutional Review Board.

**MACS-based cell sorting**

To isolate CD4+ T cells, PBMC were incubated with a depleting “antibody cocktail”, and processed according to manufacturer’s instructions (CD4+ T Cell Isolation Kit II – human, Miltenyi Biotech). The effluent population was considered CD4+ T cell-enriched and the population remaining on the column to be CD4+ T cell-depleted; this second population was used as donor-autologous APCs. Purity of the isolated CD4+ T cell population was routinely >93% by FACS.

**Peptides and T cell culture**

Peptides used for T cell priming, previously described (12,13), were produced by the Keck Biotechnology Research Laboratory, Yale University: IGRP<sub>23-35</sub> (YTFLNFMSNVGD), IGRP<sub>247-259</sub> (DWHIDTPFAGL), PPI<sub>76-90, 88S</sub> (SLQPLALEGSLQSRG).
For generation of antigen-pulsed lines, previously described protocols were amended (12–14). APCs generated by MACS-based sorting were adhered to plastic for 2 hours. Adherent cells were collected and irradiated (5000 Rad) after being loaded with 10µg/ml peptide, in AIM V medium (Invitrogen). 20U IL-2 was replenished every four days until immortalization.

Cultures were restimulated every two weeks with irradiated, peptide-pulsed donor-matched APC at ratios of 10:1 (CD4:APC). After four stimulations, CD4+ T cells were infected with a human telomerase (hTERT)-expressing murine leukemia virus (MuLV)-based retroviral vector (15,16), three days post-stimulation in order to generate continuous cell lines. Cells were incubated in T cell medium with virus plus 8µg/ml polybrene (hexadimethrin bromide, Sigma) for 6 hours.

**Transfer of T cells**

NSG.DR4 mice were obtained from the laboratory of Li Wen, Yale University. Six-to-eight-week old NSG.DR4 mice were injected with 1.0-3.0 x 10^6 HLA-DR4 donor PBMC or 0.5-5.0 x 10^6 antigen-pulsed CD4+ T cells. For a given donor and line, there were no differences in tissue reconstitution or insulitis scores observed in conjunction with variations in the numbers of cells injected. The use of animals was approved by the Yale University Institutional Animal Care and Use Committee. Mice were sacrificed six weeks post-injection. Single cell preparations were made from the blood, spleen, and pancreas. The percent reconstitution (Fig. 1D) with human cells was calculated based on the results of staining with human anti-CD45 (APC) and mouse anti-CD45 (FITC) (BD Biosciences) as:

\[
% = \left(\frac{\text{% human CD45-positive cells}}{\text{% human CD45-positive cells} + \text{% mouse CD45-positive cells}}\right) \times 100.
\]

Characterization of mouse cells in the pancreas was done using anti-mouse CD11b (PE, BD Biosciences) and anti-mouse CD11c (PeCy7, eBioscience).
**Histology – preparation and analysis**

Pancreatic tissues were stained for human CD45RO and insulin. For CD45RO, antigen retrieval was followed by incubation with a 1:50 dilution of mouse anti-human CD45RO (Invitrogen) for 60 minutes, and a biotinylated goat anti-mouse IgG (KPL) for 30 minutes. For insulin staining, a 1:50 dilution of guinea pig anti-insulin (Invitrogen) was incubated for 60 minutes, followed by peroxidase rabbit anti-guinea pig (Invitrogen, 1:200 in PBS).

**Insulin staining quantification**

Insulin staining of pancreas sections was performed as described above for all mice which showed positive insulitis scores. Photographs of all islets from one section were then taken for each such mouse. ImageJ software (http://rsbweb.nih.gov/ij/) was used to measure the total area of each islet. The total area of each islet staining positive for insulin was then determined using the “Analyze Particles” function and taking into account pixel size 500-Infinity and circularity 0.00-1.00. The percent positive insulin staining was then determined for each islet by calculating:

\[
\% = \left( \frac{\text{area staining insulin-positive}}{\text{total islet area}} \right) \times 100.
\]

**Analyzing and scoring tissue**

For each mouse, every islet on one slide was scored according to amount of CD45RO+ cell invasion, using guidelines in Supplementary Table 1. For scoring the small intestine, only very well oriented villi were considered. Slides were scored as described in Supplementary Table 2. Scoring of graft-versus-host disease (GVHD) in the liver was based upon the number of portal tracts affected/involved, using guidelines in Supplementary Table 3. Scoring of the acinar tissue was performed to determine the nature of any pancreatic infiltration, using guidelines in
Supplementary Table 4. Balding and skin conditions at the time of harvest were scored using guidelines in Supplementary Table 5.

**Tetramer staining and flow cytometry**

IGRP$_{247-259}$ and PPI$_{76-90}$, 88S tetramers were produced by the Tetramer Core Laboratory, Benaroya Research Institute at Virginia Mason, using methods previously described (17).

Fourteen days after generation, 5x10$^6$ antigen-pulsed cells were stained with tetramer (1μl tetramer/10$^5$ cells) in AIM-V medium plus 2% human serum for 2.5 hours, at a concentration of 1000 cells/μl. Cells were then washed and stained with anti-human CD25-FITC and anti-human CD4-APC (BD Biosciences) for an additional 45 minutes. Cells were analyzed/sorted on the BD FACS Vantage SE using the following guidelines: CD4+ cells were first gated. Cells were then examined using CD25 and tetramer staining. Cells staining tetramer-positive and CD25mid were then collected as the final target population.

**Analyzing beta cell death**

Relative levels of serum-derived demethylated *Ins1* DNA were measured by RT-PCR, using methods previously described (18).

Caspase-3 staining was performed by Yale Pathology Tissue Services, using rabbit anti-Caspase-3 (abCam) and Biotin-sp-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch). For each islet evaluated, the total number of cells as well as the number of cells staining Caspase-3-positive were counted, and these values used to calculated the percentage of Caspase-3-positive cells in each islet.
**Statistical analysis**

Graph Pad Prism software was used for statistical analysis. Statistical significance was determined using the unpaired t-test or ANOVA analysis as noted, except in the case of the linear regression analysis shown in Figure 5B.
Results

Reconstitution of NSG.DR4 mice with PBMC or antigen-pulsed CD4+ T cell lines from healthy and diabetic donors

We stimulated antigen-reactive T cells from PBMC from healthy controls or Type 1 diabetic HLA-DR4+ donors using diabetes antigens preproinsulin (PPI_{76-90,\text{88S}}), IGRP_{23} and IGRP_{247}. Tetramer staining was used to identify the percentages of antigen-specific cells in pulsed lines after two weeks (Figures 1A,B). Cells were immortalized (15,16) and ³H-based proliferation assays were used to verify continued antigen-specific T cell expansion in the lines post-immortalization (Supplementary Figure 1).

NSG.DR4 mice were injected with 1.0-3.0x10⁶ HLA-DR4 donor PBMC or 0.5-5.0x10⁶ HLA-DR4 donor antigen-pulsed CD4+ cells and were sacrificed 6 weeks post-injection. Single cell preparations were made from the blood, spleen and pancreas of each mouse, and cells were stained for expression of both human and mouse CD45 to determine the extent of reconstitution of a given tissue.

For both healthy and diabetic donors the average level of human CD45 cell reconstitution of blood (Figure 1C, top left, $P < 0.001$) and spleen (Figure 1C, top right, $P < 0.001$) was greater in mice injected with PBMC than in those injected with antigen-pulsed CD4+ T cell lines.

We observed differences in the infiltration of cells into the pancreas when the NSG.DR4 mice received CD4+ T cell lines expanded with islet antigens. There was significantly greater pancreatic infiltration of human CD45+ cells in recipients of the CD4+ lines from diabetic donors than with those from healthy donors ($P < 0.05$, Figure 1C, bottom).
Comparison of insulitis in mice reconstituted with PBMC or CD4+ T cells from healthy controls or patients with Type 1 diabetes

We next sought to determine if the human CD45+ cells detected in the pancreas were homing to the islets; human CD45RO staining of pancreatic sections was performed. Examples of human lymphocyte infiltration of mouse islets are shown in Figure 2A. The accumulated islet scores of all diabetic and healthy control donor CD4+ T cell-injected mice are shown in Figure 2B. Injection of diabetic donor T cells yielded significantly higher insulitis scores ($P < 0.05$). Further, severe insulitis was only observed with diabetic donor CD4+ T cell injections. The differences in the cellular infiltrates were not due to general differences in the viability of the CD4+ T cells prior to transfer, as the lines responded to antigen stimulation and expanded with similar kinetics. In addition, the expansion of the CD4+ T cells also did not reflect a difference in MHC gene dosing since all of the diabetic donors and healthy control donors were heterozygous for DR4 (Table 1).

Lymphocytic infiltration of the extra-islet pancreas and other organs

To determine whether the infiltrates we had seen with the diabetes antigen-specific CD4+ cell lines were specific for islets, we determined whether there were infiltrates in non-islet tissues (small intestine, liver, pancreatic acinar tissue and skin). Tissues were scored for each mouse with any islet scores $>0$ (Figure 3). There was evidence of minor GVHD-like response in the small intestine of mice injected with PBMC; this was not the case in mice injected with CD4+ T cell lines (Figure 3A). Liver infiltration and pancreatic acinar tissue infiltration were considerable in mice injected with PBMC, but not in mice that received CD4+ T cell lines (Figure 3B,C). The majority of mice ($>70\%$) injected with PBMC showed some level of balding
at the time of sacrifice (Figure 3D) but this was not seen in the recipients of the CD4+ T cell lines.

**Variations in peptide response in Type 1 diabetic donors**

We next compared the islet infiltration with CD4+ T cell lines grown on different diabetes associated peptides and found that the islet infiltration varied by cell donor and by islet antigen (Figure 4). With injections from patient T1D001, for example, PPI88S-pulsed cells show the greatest infiltration of mouse islets. CD4+ T cells from donor T1D584 also showed a dominant response to PPI88S, although all three peptide-pulsed lines caused insulitis in mice. T1D029 showed a dominant response to IGRP23, with only minor insulitis associated with the PPI88S line and no infiltration with the IGRP247 line injections. These findings also indicate that variation in donor responses are islet- or insulin-peptide-driven and not merely a by-product of non-specific *in vitro* stimulation, as donor lines were prepared and stimulated in parallel and injected in equal numbers and yet showed different infiltration capabilities *in vivo*.

**Insulin staining is reduced in islets exhibiting insulitis**

Next, we wished to determine if the significant pancreatic infiltration affected insulin production in the islets. ImageJ (19) was used to analyze all mice with islet scores >0, and the percent of the total islet area that was positive for insulin staining was determined for each islet.

On average, the insulin-positive area from islets in untreated control mice was 62%, consistent with previously published observations (20). There was no statistically significant decrease in the insulin-positive area in mice that received PBMC from healthy control subjects but there was a small reduction in insulin-positive area in recipients of PBMC from Type 1 diabetes patients (*P*<0.01, Figure 5A). Recipients of antigen-pulsed diabetic CD4+ T cell lines showed less insulin staining than did mice in any other category examined (mean % insulin-
positive = 47.6%), and significantly less ($P <0.0001$) compared to CD4+ T cell lines from healthy donors, which did not cause a significantly reduced expression of insulin.

Linear regression analysis was performed to determine if there was a relationship between the average % insulin positivity and the average islet score of mice injected with CD4+ T cell lines from diabetic donors (Figure 5B). This analysis showed that average insulin staining was negatively correlated with insulitis scores ($r^2 = 0.176, P =0.015$). However, none of the recipient mice developed hyperglycemia.

**Analysis of the mechanism of islet destruction in the recipients of antigen-reactive CD4+ T cells**

Since the mice did not develop overt diabetes, it was not clear whether the infiltrating cells were actually killing β cells or alternatively whether the reduced insulin staining was due to functional impairment of the β cells, resulting in decreased insulin staining (21). To determine if the transfer of the CD4+ T cells from patients with Type 1 diabetes caused β cell death, we used a quantitative PCR-based assay to detect circulating β cell-derived demethylated Ins1 DNA released from dying β cells. We have previously shown that this assay can detect β cell death in streptozotocin (STZ)-induced diabetes in mice, and in NOD mice prior to hyperglycemia (18). Serum samples from twelve mice injected with CD4+ T cell lines from Type 1 diabetic donors and resulting in marked insulin loss were compared to serum samples from mice injected with HD CD4+ T cell lines with insulitis scores >0 (none of the HD line-injected mice showed marked insulin loss). Control samples were taken from age-matched mice injected with PBS only, as well as STZ-treated mice. For CD4+ T cell- and STZ-injected mice, the % increase in Δ ($C_{\text{meth}} - C_{\text{demeth}}$) was compared to the same for control NSG mice treated with PBS. There were significantly greater levels of demethylated Ins1 DNA in the serum of recipients of antigen-
expanded CD4+ T cell lines from patients with Type 1 diabetes compared to HD CD4+ T cell line-injected control mice ($P<0.05$, Figure 5C). Caspase-3 staining of pancreatic sections corroborated these results. Mice injected with CD4+ T cell lines from diabetic donors had the highest percentage of Caspase-3-positive cells on a per-islet basis (Figure 5D).

To better elucidate the mechanism of β cell killing observed in mice injected with T1D CD4+ T cell lines, we next isolated PPI$_{88S}$ antigen-specific clones from healthy and Type 1 diabetic donor CD4+ T cell lines, using a modified version of previously described methods (14). Fourteen clones from healthy donors and four from patients with Type 1 diabetes were isolated and verified as antigen-specific using $^3$H-based proliferation assays (*data not shown*). We compared the cytokine production by the clones in response to PMA/Ionomycin stimulation and found that the average levels of GM-CSF, IFNγ and TNFα production were comparable in cells from patients and HD, but the cells from HD produced higher levels of IL-4, IL-5 and IL-13 (Supplementary Figure 2).

It has been previously shown that antigen-specific CD4+ T cells with a Th2-like cytokine phenotype are less capable of recruiting cells to the pancreas than are cells with a Th1-like phenotype, and are overall unable to promote islet destruction in the NOD mouse (22–25). Consequently, we hypothesized that a difference in cytokine production on a cellular level might lead to a less inflammatory environment in mice injected with HD cells. A hallmark of any inflammatory lesion is the recruitment of antigen-non-specific cells. Therefore we looked for another indicator of inflammation, the presence of mouse CD45+ cells, in mice injected with T1D vs. HD CD4+ T cell lines. We evaluated overall pancreatic cell counts and mouse CD45 staining (by FACS) of pancreas at harvest. We found that mice injected with T1D CD4+ T cells
show a marked increase in mouse CD45 cell homing to the pancreas, in comparison with mice injected with HD CD4+ T cells (Figure 6A, $P=0.016$).

FACS analysis was performed on cells recovered from the pooled pancreata of mice injected with T1D001 PPI$_{88S}$ lines. Approximately 90% of mouse CD45+ cells analyzed were CD11b+, and approximately 1/3 of these cells were also CD11c+. These CD11b+/CD11c+ cells correspond to a mouse islet antigen-presenting dendritic cell (DC) population previously described by Calderon and Unanue (26) (Figure 6B). The remaining CD11b+CD11c- population likely contains pancreas-infiltrating macrophages, which have previously been identified in diabetic islet infiltrates (27).

Histological staining of mice injected with diabetic donor CD4+ T cells revealed large numbers of F4/80 positive-staining cells in the islets (Figure 6C, left). Similar staining was also performed in HD-injected and PBS-injected NSG.DR4 mice; in these cases F4/80+ cells were present only in very small numbers, or not at all (Figure 6C, center and right).
**Discussion**

Here, we characterized a system using autoantigen-pulsed human CD4+ T cells, whose homing was readily observed in a mouse expressing the human HLA-DR4 transgene. We observed infiltration of mouse islets by human cells; severe insulitis was only observed in mice injected with CD4+ T cell lines from diabetic donors (Figure 2). The infiltration was specific for the islets (Figure 3) and was associated with reduced insulin staining (Figure 5A). Insulin staining in mice injected with HD CD4+ T cells was not reduced in this system. Because only mice with positive insulitis scores were included in this analysis it can be concluded that although healthy donor T cells can infiltrate the pancreas, they do not disrupt insulin production in this model. Although other groups have observed islet infiltration using injection of either human PBMC alone or human PBMC followed by a human CD8+ T cell clone, in the NSG HLA-A2+ mouse (28,29), to our knowledge, this is the first demonstration of CD4+ T cell-associated β cell death in a humanized mouse model.

Some insulitis was observed with transfer of diabetic donor PBMC, although insulin staining was significantly more decreased (and Caspase-3 staining significantly more increased) with diabetic donor CD4+ T cell line transfer than with PBMC transfer. However, due to high levels of infiltration of the exocrine pancreas associated with PBMC transfer (as well as advanced balding and increased levels of disease in the liver and intestine) it seems the actions of PBMC are non-specific in nature in this model. A more appropriate label for the infiltration observed in the pancreata of PBMC-injected mice would thus be “GVHD,” as opposed to “insulitis”.

It was possible that reduced insulin staining in diabetic donor CD4+ T cell line-injected mice was the consequence of inflammatory infiltrates and not the result of β cell killing, since
dysfunctional β cells that can recover with immune therapy have been identified previously by our group (21). However, along with reduced insulin staining and increased Caspase-3 staining, we found increased levels of circulating demethylated β cell-derived DNA in recipients of CD4+ T cell lines from patients with Type 1 diabetes, indicating that the cellular infiltrates contributed to β cell killing (Figure 5C). Islet destruction and insulin loss may occur before the six-week termination point of our experiments. Because the window for demethylated DNA detection in the process of β cell death may be limited, its possible that the levels of β cell death may be even greater than estimated by the analyses discussed herein.

In spite of the reduced insulin-positive area and evidence of β cell killing, the mice did not develop overt hyperglycemia, suggesting that additional cells (such as CD8+ T cells or others) may be required for complete β cell destruction. Van Belle et al. recently showed in the human insulin promoter-lymphocytic choriomeningitis (Ins-LCMV) mouse model of Type 1 diabetes that autoreactive CD8+ T cells depend on CD4+ T cell-mediated recruitment to the pancreatic islets (30). Indeed, the importance of CD4+ T cells and MHC class II expression in Type 1 diabetes pathogenesis is well documented. Several groups have determined that allelic variations in MHC Class II genes result in odds ratios for diabetes susceptibility much greater than any other known gene or chromosomal region (31–33). Specifically, HLA-DR4 expression (especially in conjunction with co-expression of HLA-DR3/HLA-DQ8) results in greatly increased diabetes susceptibility (34). Further, Arif and colleagues demonstrated that CD4+ T cells appear in a greater percentage of islets and in significantly greater numbers in individuals recently diagnosed with Type 1 diabetes, as opposed to long-term diabetics, highlighting their importance early in disease (35). While CD4+ T cells do not appear capable of initiating
fulminant diabetes in our model, our findings do reinforce the potential of diabetes antigen-reactive CD4+ T cells to establish a critical pathogenic environment in the pancreatic islets.

To this end, we observed by FACS significantly increased levels of mouse CD45+ cells in the pancreata of mice injected with T1D CD4+ T cells, relative to mice injected with HD CD4+ T cells (Figure 6A). In a healthy intact pancreas, a baseline level of mouse sentinel leukocyte homing is present in the islets (36). In this model, it appears that the combination of large numbers of antigen-specific infiltrating human CD45RO cells (Figure 2B) and a pro-inflammatory cytokine environment (Supplementary Figure 2) leads to increased mouse leukocyte homing to and accumulation in the islet, which in turn could lead to antigen release by dying beta cells and to further recruitment of murine cells capable of antigen presentation and beta cell killing. This explanation would also account for the lack of mouse CD45+ cell recruitment in mice injected with HD cells, as HD cells do not accumulate significantly in the islets and clones derived from these cells display a more Th2-like cytokine profile relative to T1D clones.

We also observed a dominant antigenic response leading to insulitis that differed between subjects (Figure 4). This may reflect an increased proportion of antigen-specific cells or growth advantage of cells with particular antigen specificity. It has been argued that insulin autoimmunity is a base requirement for initiation of Type 1 diabetes (37–39). Here we observed that a mutant preproinsulin (PPI88S) peptide was often the dominant epitope tested, in terms of the levels of insulitis observed. In one diabetic donor (T1D029) however, the IGRP23 epitope clearly dictated the dominant response. In light of these observations, it could be theorized that the roles of different autoimmune targets are different between individuals or even wax and wane over the course of disease in a given individual, so that autoimmune responses (or a lack thereof)
targeting different antigens may direct the course of disease at various time points. Further, this observation serves as a proof of concept for our model. If the insulitis observed was due simply to non-specific activation \textit{in vitro} before injection, one would expect to find comparable levels of infiltration with all three lines from the same donor; this was clearly not the case.

The results of experiments outlined above show that injection of autoantigen-pulsed CD4+ T cells leads to targeted infiltration of the pancreas, insulin loss, and β cell killing. This model system may be useful for testing the ability of immune modulatory agents to prevent β cell destruction. Such interventions could include co-injection of donor-matched regulatory T cells (40), cytokine therapy (30,41,42) or chemokine/chemokine receptor blockade (43,44). Furthermore, by defining the dominant antigens that are recognized by the pathologic T cells, a personalized approach to immune therapy may be developed.

**Author Contributions**

A.V.M. designed and performed experiments and wrote the manuscript. S.M. and J.L. performed experiments. J.G. consulted on scoring pathology and reviewed/editied the manuscript. L.W., N.R., and K.H. contributed to discussion and reviewed/editied the manuscript. A.B. designed experiments and wrote the manuscript.

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A.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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<td>T1D584</td>
<td>F</td>
<td>T1D</td>
<td>10</td>
<td>14</td>
<td>DRB1<em>0401/DRB1</em>1301</td>
</tr>
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*Donor has also been diagnosed with autoimmune thyroiditis

**Table 1. Summary of HLA-DR4 donors.** Disease status, sex, age at Type 1 diabetes diagnosis (if applicable), age at donation, and HLA-DR haplotype is shown for each donor used in these studies.
Fig. 1. Injection of antigen-specific CD4+ T cell lines into NSG.DR4 mice. (A) CD4+ T cells from two healthy and two diabetic donors were stained for tetramer (as described in Research Design and Methods) at two time points: 24 hours after isolation (when they had not yet been APC/peptide stimulated) and two weeks after APC+peptide stimulation. Percentages of CD4+ T cells staining tetramer-positive are shown. After 24 hours, tetramer staining fell in the range of 0.06-0.20%. Tetramer staining after peptide pulsing for two weeks ranged between 0.53 and 3.80%, with antigen-specific fold-inductions in the range of 4x to 43x. (B) “Mismatched” tetramer staining of antigen-expanded lines was also performed, to determine that the increased tetramer staining at two weeks was due to a true expansion of antigen-specific cells, and not non-specific staining. Mismatched tetramer staining was found to be at or below background levels; an example of such staining (from donor T1D029) is shown. (C) Six-to-eight-week old NSG.DR4 mice were injected with 1.0-3.0 x 10^6 HLA-DR4 donor PBMC (diabetic (T1D) or healthy control, n=41 and 15 mice, respectively) or 0.5-5.0 x 10^6 antigen-pulsed CD4+ T cells (diabetic (T1D) or healthy control, n=87 and 21 mice, respectively) in 150µl PBS, or PBS alone, via a retro orbital intravenous route. Injection numbers were determined based upon cell availability, and no significant differences in reconstitution were observed over either range. Mice were sacrificed 6 weeks post-injection. Single cell preparations were made from the blood (top left) and spleen (top right) and pancreas (bottom) of each mouse, and were stained for expression of both human and mouse CD45, as described in Research Design and Methods. The percentage of live cells staining positive for human CD45 is graphed along the y-axis. Open circles= T1D CD4 line injections; open squares= HD CD4 line injections; filled circles= T1D PBMC injections; filled squares= HD PBMC injections. In this figure, PBMC injections from all donors and CD4+ T cell line injections from donors HD003, HD004, T1D001, T1D029 and T1D584 are analyzed. Each data point represents one animal and significance was determined using non-parametic ANOVA analysis; overall ANOVA – P <0.0001. Here, * - P <0.05, *** - P <0.001.
Fig. 2. Lymphocytic infiltration of the pancreatic islets at termination. (A) Islet scoring was based on human CD45RO staining of paraffin slides, as described in Research Design and Methods. Shown are examples of pancreata from mice injected with T1D CD4⁺ T cell lines, highlighting different levels of insulitis. Here, human CD45RO⁺ lymphocytes are staining dark red. (B) Cumulative scoring of islets is shown for injections of T1D CD4⁺ T cell lines (n=3 donors, 87 mice, 277 islets) healthy control CD4⁺ T cell lines (n=2 donors, 21 mice, 99 islets), T1D PBMC (n=6 donors, 41 mice, 131 islets) and healthy control PBMC (n=3 donors, 15 mice, 64 islets). Each data point represents one islet. In this figure, PBMC injections from all donors and CD4+ T cell line injections from donors HD003, HD004, T1D001, T1D029 and T1D584 are analyzed. Statistical significance was determined using non-parametric ANOVA analysis; overall ANOVA – P <0.0001. Here, * - P <0.05, *** - P <0.001.
Fig. 3. Lymphocytic infiltration outside the pancreatic islets. For evaluation of non-specific tissue infiltration by lymphocytes, a 6cm section of small intestine was removed just below the duodenum and a 0.5x1.0cm section was removed from the tip of the median lobe of liver at the termination of experiments. Shown here is lymphocytic infiltration of the small intestine (A), liver (B) and pancreatic acinar tissue (C), and skin health (D) at termination, in mice injected with PBMC (n=41) or CD4⁺ (n=39) T cell lines. Only those mice with islet scores >0 were included in analysis. Healthy control and diabetic donors were grouped together for analysis, as no differences were observed between the grouped cohorts. For liver, small intestine and pancreatic acinar tissue, scoring was based on H&E staining of paraffin slides, as described in Research Design and Methods. Open circles= CD4 line injections; filled circles= PBMC injections. In this figure, PBMC injections from all donors and CD4⁺ T cell line injections from donors HD003, HD004, T1D001, T1D029 and T1D584 are analyzed. Significance was determined using non-parametric ANOVA analysis.
Fig. 4. Lymphocytic infiltration of the islets at termination, by donor. Examples of lymphocytic infiltration of the pancreatic islets at termination, in mice injected with PBMC (n=56) or CD4+ T cell lines (n=108). Each data point represents one individual islet. A breakdown of the islet scores resulting from CD4+ line injections for each donor is shown. Filled circles= IGRP23-pulsed CD4 T cell lines; filled squares= IGRP247-pulsed CD4 T cell lines; filled upward-facing triangle= PPI88S-pulsed CD4 T cell lines; filled downward-facing triangles= IGRP23/IGRP247 combination-pulsed CD4 T cell lines. Statistical significance was determined using non-parametric ANOVA analysis. For T1D001, overall ANOVA – P=0.0142; for T1D029, overall ANOVA – P=0.002; for T1D584, overall ANOVA – P=0.0019; for HD004, overall ANOVA – P=0.0048. Here, * - P <0.05, ** - P <0.01.
**Fig. 5.** Insulin loss and β cell death in mice injected with CD4+ T cell lines from Type 1 diabetic donors. Shown in panel (A) is the cumulative analysis of insulin staining in mice with islet scores >0. The percent of each islet staining positive for insulin (see Research Designs and Methods) is graphed along the y-axis; each data point represents one islet. The % insulin-positive mean, number of mice analyzed and number of islets analyzed for each cohort are given below. Diabetic (T1D) CD4+ Lines: n=32 mice, 206 islets (mean % insulin-positive = 47.6%), healthy CD4+ Lines: n=6 mice, 44 islets (mean % insulin-positive = 57.3%), diabetic (T1D) PBMC: n=27 mice, 147 islets (mean % insulin-positive= 51.7%), healthy PBMC: n=14 mice, 50 islets (mean % insulin-positive = 56.8%), PBS only (“non-injected”) control: n= 3 mice, 23 islets (mean % insulin-positive = 62.0%), STZ-treated: n=3 mice, 4 islets (mean % insulin-positive = 3.0%). Control, STZ-treated mice were injected via an intraperitoneal route for five consecutive days with 40mg/kg body weight STZ. In this figure, PBMC injections from all donors and CD4+ T cell line injections from donors HD004, T1D001, T1D029 and T1D584 are analyzed. Significance was determined using parametric ANOVA analysis; overall ANOVA – P <0.0001. Here, * - P <0.05, *** - P <0.001, **** - P <0.0001, n/s – results did not reach statistical significance. (B) In mice injected with CD4+ T cell lines from diabetic donors, average insulin staining is significantly reduced in mice with higher average islet scores (p=0.0150), with a slope of -4.044 +/- 1.570. Each data point represents one mouse (n=32 mice). In this figure, CD4+ T cell line injections from donors T1D001, T1D029 and T1D584 are analyzed. (C) There is an increase in beta cell death in recipients of diabetic donor antigen-expanded cell lines. The relative level of demethylated Ins1 DNA (i.e. Δ= \( \Delta Ct^{\text{meth}}-Ct^{\text{demeth}} \)) was measured by RT-PCR from 150μl serum isolated from mice 6 weeks after receiving cell lines from healthy control subjects or patients with Type 1 diabetes, using methods previously described (18). Control STZ-treated mice were injected via an intraperitoneal route with 200mg/kg body weight STZ and harvested 24 hours later. The % increase in Δ was compared to the same for control NSG mice treated with PBS. The % increase in Δ for mice treated with STZ ranged from 38-118%. Filled circles= HD CD4+ T cell line injections; filled squares= T1D CD4+ T cell line injections. In this figure, CD4+ T cell line injections from donors HD004, T1D001, T1D029 and T1D584 are analyzed. Significance was determined using the unpaired t-test. Here, * - P<0.05. (D) There is an increase in the percentage of islet cells staining Caspase-3-positive in mice injected with CD4+ T cell lines from diabetic donors. Mice with islet scores >0 were included in analysis. For each such mouse, the total number of cells as well as the number of Caspase-3-staining cells was counted for each islet. The % of each islet staining Caspase-3-positive is shown along the y-axis on the graph. Each data point represents one islet. For diabetic (T1D) CD4 Lines: n=32 mice, 111 islets; healthy CD4 Lines: n=6 mice, 53 islets; diabetic (T1D) PBMC: n=27 mice, 137 islets; healthy PBMC: n=14 mice, 29 islets; PBS only (“non-injected”) control: n= 3 mice, 11 islets. In this figure, PBMC injections from all donors and CD4+ T cell line injections from donors HD004, T1D001, T1D029 and T1D584 are analyzed. Significance was determined using parametric ANOVA analysis; overall ANOVA – P <0.0001. Here, * - P <0.05, *** - P <0.001, **** - P <0.0001, n/s – results did not reach statistical significance.
Mu CD45+ Cell Homing to Pancreas

# of cells (in millions)

T1D CD4 Lines  HD CD4 Lines

*p=0.0160

Mouse CD45+ population

Mouse CD45- population

B

C
Fig. 6. Mouse CD45 cells are recruited to the pancreas by CD4+ T cells from Type 1 diabetic donors. (A) Recruitment of mouse leukocytes to the pancreas was determined using cell counts in half a pancreas at the time of harvest, in conjunction with mouse CD45 staining by FACS. The number of mouse CD45 cells (in millions) is shown along the y-axis. Recruitment of mouse leukocytes was significantly higher in mice injected with T1D CD4 lines (n=87 mice) than in mice injected with HD CD4 lines (n=21 mice), (P=0.0160). Each point represents one mouse. In this figure, CD4+ T cell line injections from donors HD003, HD004, T1D001, T1D029 and T1D584 are analyzed. (B) Single cell suspensions were isolated and pooled from mice injected with T1D001 PPI88S lines. Cells were stained for expression of mouse CD45 (left panel), CD11b and CD11c (middle and right panels) to better characterize the mouse cells recruited to the pancreas in this model. Of the cells staining mouse CD45-positive, 34% also stained CD11b/CD11c double-positive, identifying them as dendritic cells (middle panel). The majority of the remaining CD45-positive cells stained CD11b single-positive (middle panel). No CD11b or CD11c staining was detected in the population staining CD45-negative (right panel). (C) Left: F4/80 staining shows large quantities of pancreas-infiltrating F4/80-positive macrophages and/or dendritic cells in a mouse injected with a CD4+ T cell line from donor T1D001. Center: an NSG.DR4 mouse injected with cells from HD004 shows little or no F4/80 staining, similar to an NSG.DR4 mouse injected with PBS only (right).