A hybrid insulin epitope maintains high 2D affinity for diabetogenic T cells in the periphery

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Abstract. Beta-cell antigen recognition by autoreactive T cells is essential in type I diabetes (T1D) pathogenesis. Recently, insulin hybrid peptides (HIPs) were identified as strong agonists for CD4 diabetogenic T cells. Here, using BDC2.5 transgenic and NOD mice, we investigated T cell recognition of the HIP2.5 epitope, which is a fusion of insulin C-peptide and Chromogranin A (ChgA) fragments, and compared it with the WE14 and $ChgA_{29\text{-}42}$ epitopes. We measured *in situ* 2D affinity on individual live T cells from thymus, spleen, pancreatic lymph nodes, and islets prior to and post diabetes. Although preselection BDC2.5 thymocytes possess higher affinity than splenic BDC2.5 T cells for all three epitopes, peripheral splenic T cells maintained high affinity only to the HIP2.5 epitope. In polyclonal NOD mice, a high frequency (~40%) of HIP2.5-specific islet T cells were identified at both prediabetic and diabetic stages comprised by two-distinct high and low affinity populations that differed in affinity by 100-fold. This high frequency of high and low affinity HIP2.5 T cells in the islets potentially represents a major risk factor in diabetes pathogenesis.

Introduction

Type 1 diabetes (T1D) is a complex T cell-mediated autoimmune disease. Consistent with the requirement of CD4 T cells for T1D development, HLA-DR4 and DQ8 (human) and I-A^{g7} (murine) are key genetic risk factors [1, 2]. Despite great progress in T1D research, we still do not understand how CD4 T cells break tolerance to β-cell autoantigens and cause disease. Numerous MHC II-restricted epitopes have been identified from various islet proteins such as insulin and Chromogranin A (ChgA) among others [3, 4]. Many corresponding diabetogenic CD4 TCRs have been cloned, including the well-characterized BDC2.5 TCR that has been made into a transgenic (Tg) mouse model [5]. Insulin and ChgA have surfaced as two prime autoantigen targets in NOD mice since a point mutation in the insulin B chain (replacing a tyrosine at position 16 to an alanine, Y16A) [6] or deficiency of ChgA ($ChgA^{-/-}$) [7] protects NOD mice from diabetes. Although $ChgA^{-/-}$ confers complete protection, the dominant epitope of T1D has remained controversial. Islet cells from WT NOD mice are potent stimulators of the BDC2.5 clone but completely lose stimulation on the $ChgA^{-/-}$ background [7]. Two antigenic ChgA peptides have been identified, namely WE14 ($ChgA_{358–371}$) and $ChgA_{29-42}$, but both are only minimally stimulatory to BDC2.5 Tg cells. More recently, a dominant strong agonist for the BDC2.5 TCR was discovered as a hybrid insulin peptide (HIP) fusion between an insulin C peptide fragment and the MHC anchor residues from the WE14 ($ChgA_{358–362}$) peptide [8]. A series of HIPs were identified to be agonistic peptides for previously cloned CD4 TCRs from diabetic NOD mice. Importantly, HIPs also exist in human [8]. Therefore, the HIPs potentially represent an important new class of post-translationally modified (PTM) β-cell autoantigens that offer an exciting explanation for the protection from T1D found in ChgA deficient mice.

A fundamental premise of T1D research is that β-cell autoantigen recognition by diabetogenic T cells initiates and perpetuates the disease. A clear understanding of autoantigen recognition may hold the key to the ultimate goal of specifically depleting or restoring tolerance of diabetogenic T cells and at the same time sparing the functioning T cell repertoire. However, there is limited quantitative information on the binding kinetics (e.g., affinity) of the TCR-pMHC interaction as opposed to the downstream less direct measures of functional readouts such as expression of activation markers and cytokine secretion. Tetramer staining is a popular assay for enumeration of β-cell autoantigen-specific T cell populations but it provides a measure of avidity and interacts with only the highest affinity TCRs. MHC class II tetramer binding therefore underestimates the number of antigen-specific T cells by as much as 10-fold [9, 10]. The TCR/CD3 protein complex is embedded in the two-dimensional (2D) cell membrane as is their pMHC ligand. Recently, we and others have applied novel 2D techniques to measure TCR-pMHC binding kinetics on live T cells and demonstrated a dramatic role of the cellular environment on T cell antigen recognition [11-15]; more importantly, such in situ 2D TCR kinetics, notably 2D affinity, correlates with T cell functional responses and T cell fate [13-17].

Here, we tracked 2D affinity of the insulin/ChgA HIP2.5 epitope recognized by the BDC2.5 transgenic T cells and polyclonal NOD CD4 T cells during thymocyte development and peripheral activation upon disease onset, and compared it to two previously identified ChgA epitopes, i.e., WE14 and $ChgA_{29-42}$. Our results showed that the HIP2.5 peptide possesses much higher affinity than WE14 and $ChgA_{29-42}$. This was especially true in the periphery as the HIP2.5 epitope maintained high affinity in the spleen and islets. Our sensitive 2D assay also revealed a much higher percentage (~40%) of HIP2.5-specific CD4 T cells than did HIP2.5 tetramer

staining in NOD islets both prior to and post diabetes, demonstrating that there are many more HIP2.5-reactive T cells than previously realized [8].

Methods

Mice

NOD and BDC2.5 TCR transgenic (Tg) mice were bred in the Emory University and University of Utah animal facilities and used between 5 and 20 weeks of age, with 9 weeks of age being the average age used. BDC2.5 retrogenic (Rg) mice were generated in the animal facility at St. Jude Children's Research Hospital as previously described using retroviral producers encoding these TCRs [18]. For BDC2.5 Rg mice, reconstitution was verified by flow cytometric analysis 4-5 weeks post bone marrow transplant and healthy mice that exhibited engraftment and expression of the desired TCR were used for experiments. Mouse diabetes incidence was monitored weekly by testing for the presence of glucose in the urine by Diastix (Bayer, Elkhart, IN). Mice tested positive by Diastix were further tested with a Breeze2 glucometer (Bayer, Elkhart, IN) for elevated blood glucose levels and were considered diabetic if their blood glucose was >400 mg/dl. All experiments were carried out in accordance with IACUC protocols at Emory University, University of Utah, St. Jude Children's Research Hospital, and Baylor College of Medicine.

Reagents and antibodies

Cell culture media R10 was RPMI-1640 supplemented with 10% heat inactivated FBS, 2 mM L-Glutamine, 2 X 10^{-5} M 2-ME, 10 mM HEPES buffer, and 50 µg/ml Gentamicin sulfate. All media components were from Mediatech except FBS (Gibco) and 2-ME (Sigma Aldrich). Experimental additive solution 45 (EAS45) was prepared as described, sterile filtered, and frozen in aliquots at -20°C until needed [19]. Antibodies and clones used: RT1B PE (Clone Ox-6,

detects I-A^{g7}), CD4 APC (RM4-5), Vα2 FITC (B20.1), Vβ4 PE (KT4), TCRβ PE (H57-597), CD25 APC (clone PC61), CD69 BV650 (clone H1.2F3), and PD1 PE-cy7 (clone 29F.1A12) were from BD Pharmingen. All peptides (HIP2.5 – LQTLALWSRMD, Mimotope 1 (Mim1) – AHHPIWARMDA, $ChgA_{29\text{-}42}$ – DTKVMKCVLEVISD, and WE14 – WSRMDQLAKELTAE) were synthesized on a Prelude peptide synthesizer (Gyro Protein Technologies) using FMOC chemistry and resuspended in molecular grade water to 3 mM and sterile filtered through a 0.22 μm syringe filter.

T cell proliferation

Single cells suspensions of BDC2.5 Tg splenocytes were labeled with CellTrace™ Violet (ThermoFisher Scientific) according to manufacturer's instruction. Labeled BDC2.5 T cells were plated into 96-well flat-bottom plate at $6x10^5$ cells/well with a series of indicated peptide concentrations in complete RPMI media and incubated at 37°C with 5% CO_2. After 24 and 72 hours of incubation, T cells were analyzed by flow cytometry.

T Cell isolation

A CD53-based negative selection method was used to isolate double positive preselection thymocytes from thymus [20]. CD4 positive selection MACS kit from Miltenyi Biotec was used to isolate CD4 T cells from spleen and pancreatic lymph nodes. When indicated, CD4 enriched T cells were sorted based on Vα2 staining. For pancreatic T cells, pancreata of female NOD mice were perfused by injecting 3mL collagenase IV (Worthington, Lakewood, NJ) through the bile duct, harvested, and placed in 2mL collagenase IV. Pancreata were incubated at 37°C for 30 min, after which they were washed once with 10mL 5% FBS/HBSS and resuspended in 10mL 5% FBS/HBSS. Islets were handpicked and incubated at 37°C for 15 min in 1mL cell dissociation buffer (Invitrogen, Carlsbad, CA) and further dissociated by vortexing. Cells were then washed

in 10mL 5% FBS/HBSS and directly analyzed by flow cytometry or sorted based on CD4+CD5+ for 2D affinity measurements. Tetramer analysis was performed by gating on single cells within the lymphocyte gate, followed by gating on B220-CD4+CD3+ T cells. Double staining with HIP2.5 PE and APC tetramers was used to improve staining specificity. CLIP tetramer was used as a negative control.

2D affinity measurement

The micropipette adhesion frequency assay has been described previously [21]. Peptide:I-A^{g7} monomers (NIH tetramer core facility) were coupled to biotinylated human red blood cells (RBCs) via biotin-streptavidin chemistry, which serves as a surrogate antigen presenting cell. In a typical test cycle, a coated RBC aspirated onto a glass micropipette is mechanically controlled (by a piezo actuator) to approach, contact, and retract from a CD4 T cell held by an opposing static pipette. This test cycle is repeated 50 times for each RBC-T cell pair. An adhesion event is visually identified by stretching of the RBC membrane upon its retraction. Adhesion frequency (P_a) was then calculated as number of adhesion events divided by total cycle number. The frequency of antigen-specific cells was determined for the entire population by dividing number of cells with P_a greater than the cutoff for nonspecific binding ($P_a > 0.1$) by total number of cells tested. The effective 2D affinity A_cK_a was calculated using the following equation:

$$A_c K_a = \frac{-\ln(1 - P_a(\infty))}{m_{TCR} m_{pMHC}}$$

where $P_a(\infty)$ is the adhesion frequency at an equilibrium contact time (≥ 2 seconds), m_{TCR} and m_{pMHC} are the densities of TCR and pMHC respectively. TCR surface density was calculated with a PE Fluorescence Quantitation Kit from BD Pharmigen with TCR stained by either Vβ4 PE for BDC2.5 T cells or TCRβ for polyclonal CD4 T cells, and pMHC (I-A^{g7}) by RT1B PE.

Statistical analysis

Prism 6 (GraphPad Software) was used to determine significance using unpaired two-tailed t tests. Sample means were deemed significantly different if $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Results

Splenic Tg BDC2.5 T cells recognize the ChgA-related autoantigens with a broad range of affinity that correlates with their functional responses. The micropipette adhesion frequency assay utilizes a pMHC-decorated RBC to directly visualize pMHC-TCR binding on individual live T cells via deformation of the flexible RBC [14]. By varying the concentration of pMHC molecules coated on the RBC, one can ensure that adhesion events between the RBC and the T cell are infrequent and mediated by a small number of pMHC:TCR bonds, allowing derivation of 2D affinity [22]. Densities of TCR and pMHC are defined components used to calculate TCR affinity for pMHC. Using this assay, we quantified 2D affinity for WE14, $ChgA_{29-42}$ and HIP2.5 epitopes for splenic BDC2.5 T cells (**Fig. 1**). WE14 was the first discovered ChgA epitope for the BDC2.5 TCR. It however lacks two major anchor residues at positions 1 and 4 for the sole MHC II I-A^{g7} allele in NOD mice and is only minimally antigenic [23]. $ChgA_{29-42}$, with a full binding motif of I-A^{g7}, was a second ChgA epitope reported to activate BDC2.5 T cells at high concentrations [24]. We also included analysis of a high potency mimotope (Mim1) [25] as a positive control (5.6×10^{-4} μm^4) and an insulin epitope as a negative control, which confirmed antigen specificity of the BDC2.5 system with no binding (**Fig. 1B**). Among the four antigenic peptides, the HIP2.5 possesses the highest 2D affinity (1.8×10^{-3} μm^4), almost 100x greater than WE14 and 3x greater than the mimotope. Both HIP2.5 and Mim1 epitopes were of sufficiently high affinities (>10^{-4} μm^4), which we have defined as the approximate cut-off for detection by tetramers for the BDC2.5 TCR. WE14 has the lowest 2D affinity (3.0×10^{-6} μm^4) with $ChgA_{29-42}$

being marginally higher (**Fig. 1A**). These average affinities would be below what is needed for the respective peptide:MHC tetramers to detect BDC2.5 T cells [9, 10, 26]. In addition to affinity, our adhesion frequency assay also provides information on the population of responding cells in terms of the number of responding cells and the range of response, as each symbol represents a separate T cell. All of the T cells (100%) possessed affinity for the HIP2.5 and Mim1 epitopes as compared to only ~50% of tested T cells bound to either WE14 or $ChgA_{29\text{-}42}$ (**Fig. 1C**).

To examine the impact of 2D affinity on T cell responses, we measured functional outcomes of Tg BDC2.5 splenic T cells toward the four peptides, focusing on early activation markers and proliferation**.** Using cell trace violet (CTV) to track T cell proliferation, we found that even the lowest HIP2.5 peptide concentration achieved complete cell division (>6 divisions) although with increased peptide doses, more cells divided (**Fig 2**). Therefore, the strength of HIP2.5 stimulation appears to only determine rapidity and simultaneity but not a binary decision of proliferation. In addition, proliferation does not equate to upregulation of activation markers such as CD25, CD69, and PD1; rather, the strength of stimulation plays a dominant role in their upregulation. The same proliferation characteristics were observed for the Mim1 whereas neither $ChgA_{29\text{-}42}$ nor WE14 induced proliferation or increased surface marker expression (**Fig. 3**). These data demonstrated that 2D affinity for $ChgA_{29\text{-}42}$ and WE14 was too low to fully activate BDC2.5 Tg cells in our in vitro experiments.

BDC2.5 thymocytes display increased affinity for ligand. One major function of thymocyte selection is to purge high affinity T cell clones for self-antigens to avoid autoimmunity. To test whether such selection pressure may alter autoantigen recognition by BDC2.5 T cells for $ChgA_{29\text{-}42}$ and WE14, we compared 2D affinity for CD4+ CD8+ double positive (DP)

preselection BDC2.5 thymocytes to splenic T cells. The affinity of Tg preselection BDC2.5 thymocytes (3.5×10^{-4} μm^4) for $ChgA_{29-42}$ is more than 20 times higher than that of splenic T cells (**Fig. 4A**). This higher affinity is at the same level as a model foreign reactive CD4 SMARTA clone interacting with an LCMV epitope [26]. Surprisingly, preselection BDC2.5 thymocytes have even higher 2D affinity for the WE14 epitope (5.6×10^{-4} μm^4), ~200 fold higher than that of splenic cells (**Fig. 4B**). This would suggest that selective pressure decreases BDC2.5 TCR affinity for ChgA during development. To further support the notion that thymic selection alters how BDC2.5 TCR recognizes antigen, we tracked the frequency of endogenous Vα TCR chains. Because of limited availability of antibodies targeting TCR Vα chains, we tracked Vα2 expressing T cells [27]. Indeed, the percentage of cells expressing Vα2 increased forty-fold from the Tg preselection thymocytes to Tg splenic T cells (DP – 0.36% vs. SP – 14%, **Fig. 4C&D**). However, Tg splenic BDC2.5 T cells have the same affinity for WE14 or $ChgA_{29-42}$ whether from the FACS sorted Vα2+ or Vα2- counterparts (**Fig. 4A&B**), indicating that other TCRαs might contribute. To eliminate endogenous TCRα chain contributions we analyzed the affinity for both $ChgA_{29-42}$ and WE14 using retrogenic (Rg) splenic BDC2.5 T cells on a $Rag^{-/-}$ background. In this case, the affinity of splenic Rg T cells was similar to that of preselection Tg BDC2.5 T cells (**Fig. 4A&B**).

The decrease in BDC2.5 affinity for antigen(s) was also observed for the HIP2.5 and Mim1 epitopes but the decrease in affinity from DP to splenic T cells was less. The 2D affinity of preselection BDC2.5 thymocytes for HIP2.5 was ~5-fold higher than splenic T cells (1.1×10^{-2} μm^4 vs. 1.8×10^{-3} μm^4, respectively) (**Fig. 5A**). 2D affinity for the Mim1 epitope followed the same pattern as the HIP2.5 epitope (**Fig. 5B-D**). Compared to $ChgA_{29-42}$ and WE14, TCR affinity

for the Mim1 and HIP2.5 epitopes was maintained at a higher level post thymic development. Unlike ChgA, neither of these epitopes is likely expressed in the thymus.

HIP2.5 reacts with a high percentage of polyclonal NOD CD4 T cells with high 2D affinity from pre- to post-diabetes. We next used the micropipette adhesion assay to probe the islets of NOD mice for HIP2.5 reactive polyclonal T cells. We found that 40% of the islet-derived T cells possessed affinity for the HIP2.5 epitope at prediabetic time point (**Fig. 6A,** total HIP2.5-specific data). The high frequency of islet CD4 HIP2.5-reactive T cells is ~20 times higher than tetramer staining (**Fig. 6C, D, and E**). Although the frequency of HIP2.5-specific CD4 T cells trended higher in post-diabetic islets, the difference was not statistically significant. In contrast, splenic CD4 T cells did not show HIP2.5 specificity (**Fig. 6B**). This is consistent with the expected frequency of antigen reactive T cells in the periphery as opposed to the site of autoimmune disease. Of note, the HIP2.5 reactive polyclonal islet CD4 T cell populations clearly exhibited a bimodal distribution of either high or low 2D affinity separated by three orders of magnitude for both pre- and post-diabetic conditions (**Fig. 6F,** open symbols). The high affinity polyclonal TCRs for the HIP2.5 epitope was higher at both pre- (1.1×10^{-2} μm^4) and post-diabetes (0.9×10^{-2} μm^4) as compared to the BDC2.5 T cells (1.8×10^{-3} μm^4). The bimodal distribution was surprising as previous analysis of polyclonal populations has identified a normal distribution [28], which can be seen for the $ChgA_{29-42}$ and insulin mimotope p8G [29] reactive T cells (**Fig. 6F)**. About 20% of the pancreatic lymph node derived T cells interacted with $ChgA_{29-42}$ epitope were of lower affinity (**Fig. 6D,** closed symbols). Similarly, CD4 T cells specific for the insulin p8G epitope were predominantly low affinity (96%) at the prediabetic stage although high affinity binders were enriched (15%) after mice became diabetic. Overall, ~50% of islet CD4 T cells were p8G-specific with the micropipette assay. Collectively, our data showed that there are

substantial populations of high affinity and low affinity CD4 T cells for the HIP2.5 epitope before disease onset and they are maintained during overt diabetes.

Discussion

In T1D, CD4 T cell recognition of β-cell autoantigen is a key event in disease pathology. Among identified β-cell-derived CD4 epitopes, HIP2.5 is a potential prime target of murine CD4 diabetogenic T cells that triggers and perpetuates the disease. Recent identification of HIPs in the islets of healthy hosts (e.g., BALB/c mice and control donors) [30] suggests that HIP epitopes alone are not sufficient to cause diabetes. As one of the highest genetic risk factors in T1D is MHC II, presentation of the HIP peptides may be required for activation or regulation of HIP-reactive T cells. In this study, we have characterized how transgenic and polyclonal mouse CD4 autoimmune T cells recognize the HIP2.5 epitope. Using the high sensitivity of the micropipette adhesion frequency assay, we quantified affinity and frequency of HIP2.5-specific murine diabetogenic T cells. We found that the HIP2.5 epitope (presented by IA^{g7}) binds to transgenic BDC2.5 T cells with high affinity and behaves as a strong agonist. Furthermore, in NOD islets, a high frequency of high affinity HIP2.5-specific T cells (20%) exist in the prediabetic phase and are maintained during diabetes; in comparison, high affinity p8G-specific T cells were few in the prediabetic phase but enriched after diabetes (15%). Such high frequency of high affinity autoreactive CD4 T cells for single epitopes is surprising and is ~10 times higher than that reported by tetramer staining (**Fig. 6** and [8, 29]). In addition, there is a high frequency of low affinity autoreactive CD4 T cells in the islets. Together, we found that ~ 40%, 50%, and 20% of CD4 T cells in NOD islets are specific for the HIP2.5, p8G, and $ChgA_{29-42}$ epitopes respectively. Considering other insulin hybrid peptides [8, 31] and many previously identified self-peptides from both insulin and chromogranin A [4, 32], we expect most if not all islet T cells to interact

with local antigens. These high percentages of reactive T cells would also suggest increased levels of crossreactivity between T cells.

The affinity profile of polyclonal CD4 T cells in the islet for the HIP2.5 epitope was different from BDC2.5 T cells, showing a clear bimodal distribution with high affinity binders representing nearly 50% of the total HIP2.5-specific population. The high frequency of strong HIP2.5 binders implies an absence of negative selection in the thymus, a common problem for autoantigens derived from posttranslational modifications [33]. We have recently also reported high affinity TCRs for hybrid HLA-DQ8 molecules [34]. This bimodal response to HIP2.5 from polyclonal T cells is also very different from other systems we have analyzed. Using a well-characterized dominant epitope from myelin oligodendrocyte glycoprotein (MOG), we previously demonstrated that the dynamic range in affinity of polyclonal CNS-infiltrating CD4 T cells covered a broad continuum that spans at least three logs [9], which can be described by a single normal distribution with lower mean (~10^{-5} μm^4). As expected from thymic negative selection, the peripheral CD4 T cells are predominantly of low affinity (similar as that of splenic BDC2.5 interacting with the WE14 and $ChgA_{29-42}$ epitopes). The importance and cause of the bimodal distribution to HIP2.5 remains to be determined. Of interest, we have postulated based on our analysis of myelin-specific T cells that high affinity together with low affinity T cells are key components of autoimmune disease [9, 35]. This model would be compatible with the suggestion of high affinity clones to initiate disease and lower affinity TCRs to perpetuate an anti-self response.

In Bettini et al., we reported the 2D affinity for a panel of murine CD4 clones specific for the insulin B chain epitope $InsB_{9-23}$ and found that 2D affinity correlated with ex vivo T cell activation responses [36]. The dynamic range of 2D affinity for the 7 TCR panel was limited to 1

log with all TCRs having relatively high affinity (ranging from 10^{-4} to$10^{-3}\mu m^4$). Of interest, there was no obvious correlation among these high 2D affinity TCRs with diabetes onset or incidence in retrogenic mice. Of interest, this study did not include any low affinity T cells for the insulin antigens, which likely differs from the polyclonal setting (Fig 6).

WE14 and $ChgA_{29-42}$ peptides are recognized by the BDC2.5 TCR in peripheral T cells with affinity that is two orders of magnitude lower than the HIP2.5 epitope; however, in the thymus, preselection BDC2.5 thymocytes bind to $ChgA_{29-42}$:I-A^{g7} or WE14:I-A^{g7} with much higher affinity. Thus, it seems that affinity for self $ChgA_{29-42}$ and WE14 epitopes was downregulated during thymic development, possibly as a means to limit self-reactivity in the periphery. In contrast, the affinity of the HIP2.5 epitope remains high for diabetogenic CD4 T cells in the periphery; in fact, it is still five times higher than that of a typical foreign reactive SMARTA T cells for an LCMV epitope [26]. Therefore, our data support a hypothesis of ChgA but not HIP2.5 expression in the thymus [37, 38] allowing for high affinity for the HIP2.5 epitope in the periphery to eventually trigger disease.

It is thought that the frequency and kinetics of diabetogenic T cells specific for β-cell autoantigens determine disease outcome. The first study documenting a link between TCR antigen recognition and diabetes progression utilized a mimetope (called NRP-A7) of the CD8 NY8.3 TCR (specific for an islet-specific glucose-6-phosphatase epitope presented by K^d) to quantify tetramer staining of polyclonal islet T cells and track their frequency [39]. As diabetes progressed, tetramer avidity and percentage of tetramer positive cells progressively increased within expanded islet T cells in vitro. However, results from later studies are mixed. Some reports on NOD mice and patient blood samples are supportive, showing higher frequency of autoantigen-specific T cells correlated with worsened disease [40] or diabetes progression [41-

45]. Even in these cases, there are usually significant overlaps between compared groups. Yet other studies did not reveal significant difference of frequency for autoimmune T cells between healthy controls and diabetic patients [46, 47]. A recent study making use of tetramers for analysis showed an increase in the frequency of HIP2.5-reactive T cells as diabetes progressed [31], which was not observed in the current study. The discrepancy might be due to the different tracking methods used. Our 2D assay has been proven to be much more sensitive than tetramer staining and has consistently been able to identify more antigen-specific T cells [9, 28, 48]. It is possible that tetramer staining may have missed a subpopulation of HIP2.5-reactive T cells, leading to an underestimation of HIP2.5-specific T cell frequency. Therefore, considering all available data, whether tetramer derived frequency of autoantigen-specific T cells can serve as a reliable biomarker for disease progression is still an unresolved issue in T1D.

In summary, we have determined the affinity and frequency of CD4 T cells specific to HIP2.5 and related chromogranin A autoantigens. For BDC2.5 T cells, $ChgA_{29-42}$ and WE14 affinity is greatly downregulated in the periphery during thymic development, yet peripheral BDC2.5 T cells maintain high affinity for the HIP2.5 epitope. The high affinity and optimal functional responses (Fig. 1-3) highlight HIP2.5 as a factor in BDC2.5 T1D. Importantly, we also observed a higher frequency of high affinity polyclonal T cells reactive to HIP2.5 in NOD islets than have previously been reported. It would be an interesting future avenue of research to investigate the underlying regulatory mechanism of affinity downregulation for the $ChgA_{29-42}$ and WE14 epitopes and apply such understanding to modify autoimmune CD4 T cell recognition of PTMs such as HIP2.5 as a novel intervention method in treating diabetes.

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Guarantors. Dr. Brian D. Evavold 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.

Data and Resource Availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Abbreviations used in this article: ChgA, Chromogranin A; T1D, Type 1 Diabetes; pMHC, peptide MHC; 2D, two-dimensional; Tg, transgenic; Rg, retrogenic; DP, double positive ($CD4^{+}CD8^{+}$).

Figure 1. BDC2.5 transgenic (Tg) splenic T cells recognize autoantigens with a broad range of affinity. Purified BDC2.5 splenic CD4 splenic T cells were interrogated for interaction with WE14, $ChgA_{29\text{-}42}$, HIP2.5, and Mim1 presented by $I\text{-}A^{g7}$, from which 2D affinity and antigen-specific frequency were derived. (**A**) 2D affinities of indicated antigens. Each point represents an individual cell. (**B**) Adhesion frequency for all individual cells tested. A portion of the cells did not bind to red blood cells coated with $I\text{-}A^{g7}$ presented WE14 and $ChgA_{29\text{-}42}$, demonstrating that not all BDC2.5 T cells are specific for the two weak antigens. The insulin epitope control shows the specificity of the 2D assay. Each point in Panels A&B represents an individual cell. For each epitope, data were pooled from multiple experiments with each experiment corresponding to one mouse (WE14, experiment number n=6; $ChgA_{29\text{-}42}$, n=14; HIP2.5, n=2; Mim1, n=3). (**C**) Corresponding frequency of antigen specific BDC2.5 CD4 T cells for experiments in Panels A&B. Each point represents an individual experiment. In all the panels, lines and error bars represent mean and standard error of the mean (SEM).

Figure 2. The HIP2.5 peptide is agonistic for the BDC2.5 Tg splenic T cells. BDC2.5 splenocytes were labeled with cell trace violet (CTV), followed by ex vivo activation by pulsing with the HIP2.5 peptide at indicated concentrations. Data from a representative mouse on day 3 post HIP2.5 stimulation are shown for proliferation (**A-F**) and surface marker expression of CD25 (**G-L**), CD69 (**M-R**), and PD1 (**S-X**).

Figure 3. BDC2.5 Tg splenic T cells mount strong responses to the HIP2.5 epitope but no observable responses for the WE14 and $ChgA_{29\text{-}42}$ epitopes. (**A-E**) Dose-response curves of BDC2.5 splenic T cells to WE14, $ChgA_{29\text{-}42}$, HIP2.5, and Mim1 at day 1 post stimulation by

corresponding peptides. The HIP2.5 peptide elicited similarly strong responses to the mimotope in terms of TCR downregulation (A), upregulation of CD25 (B), CD69 (C), and PD1 (D), and proliferation (E). (**F-H**) Comparison of T cell responses between day 1 and day3 post stimulation. Data shown are for 100 μM peptide concentration (no antigen as a control). Lines and error bars represent mean and SEM from three experiments (with 3, 3, and 2 mice per experiment). For CD25, CD69, and PD1, there was no statistical difference between any pair of no antigen, WE14, and $ChgA_{29\text{-}42}$ conditions or between Mim1 and HIP2.5 conditions; in contrast, there is significant difference between any one of no antigen, WE14, and $ChgA_{29\text{-}42}$ conditions and either Mim1 or HIP condition.

Figure 4. Splenic BDC2.5 Tg T cells recognize the $ChgA_{29\text{-}42}$ and the WE14 epitopes with lower 2D affinity than preselection thymocytes. (**A&B**) 2D affinity of double positive (DP, CD4+CD8+) preselection, and splenic single positive (SP, CD4+CD8-), splenic SP Vα2+ and Vα2- BDC2.5 transgenic (Tg) T cells, splenic SP BDC2.5 retrogenic (Rg) T cells for the $ChgA_{29\text{-}42}$ (A) and WE14 (B) epitopes. Each point in panels (A&B) represents one cell. For each condition, data were pooled from multiple experiments with each experiment corresponding to one mouse (WE14, Tg DP, experiment number n=6; WE14, Tg SP, n=6; WE14, Rg SP, n=2; $ChgA_{29\text{-}42}$, Tg DP, n=6; $ChgA_{29\text{-}42}$, Tg SP, n=14; $ChgA_{29\text{-}42}$, Rg SP, n=3). (**C**) Representative flow plots of Vα2 (plotted with Vβ4) expression in the preselection and splenic BDC2.5 Tg T cells from Panel D. (**D**) Vα2 expression is much higher on splenic Tg BDC2.5 T cells than preselection thymocytes. Each point in Panel D represents one mouse. In panels (A, B and D), lines and error bars represent mean and SEM.

Figure 5. BDC2.5 transgenic T cells recognize the HIP2.5 and a mimotope (Mim1) epitopes with high 2D affinity. (**A&C**) 2D affinity of preselection DP, splenic SP, and ex vivo activated BDC2.5 Tg T cells for the HIP2.5 and the Mim1 epitopes respectively. The 2D affinity of the BDC2.5 Tg SP splenic T cells are much lower than that of preselection DP T cells whereas 2D affinity partially recovered after ex vivo activation. (**B&D**) Adhesion frequency for all individual cells tested. In all panels, each point represents an individual cell. Lines and error bars represent mean and SEM. For each condition, data were pooled from multiple experiments with each experiment corresponding to one mouse (HIP2.5, preselection thymocyte, n=2; HIP2.5, splenic, n=2; HIP2.5, activated, n=2; Mim1, preselection thymocyte, n=6; Mim1, splenic, n=3; Mim1, activated, n=3).

Figure 6. Bimodal distribution of HIP2.5 affinity for polyclonal NOD T cells. (**A**) The frequency of HIP2.5-specific cells in the islets of prediabetic and diabetic NOD mice, where the line is the mean value and each dot represents data from one experiment (one mouse). In addition to the frequency of total HIP2.5-specific T cells, antigen-specificity was also reported for high and low 2D affinity T cells for the HIP2.5 defined in panel F. (**B**) NOD splenic CD4 T cells do not bind to the HIP2.5 epitope. Enriched splenic CD4 T cells from three prediabetic NOD mice were tested for HIP2.5 reactivity, but none was a binder. (**C&D**) HIP2.5 tetramer staining of prediabetic NOD islet CD4 T cells. Panel (C) shows representative flow plots (CLIP as a control). Both PE- and APC-conjugated tetramers were used to improve staining specificity. Each point in Panel (D) represents results from one mouse. (**E**) Micropipette adhesion assay detects more antigen-specific CD4 islet T cells than tetramer staining. Filled bar shows percentage of HIP2.5-specific CD4 islet T cells from prediabetic mice detected by micropipette

(from panel F) and open bar by tetramer staining (from panel D). (**F**) Effective 2D affinities for HIP2.5 (open symbols, data pooled from 3 mice pre- and 3 mice post-diabetes) and p8G (half-open symbols, data pooled from 2 mice pre- and 2 mice post-diabetes) were calculated for the islet polyclonal CD4 T cells, where lines represent the mean, error bars SEM, and each dot an individual cell. Also shown are $ChgA_{29\text{-}42}$ affinities for polyclonal CD4 T cells in the pancreatic lymph node (closed symbols).

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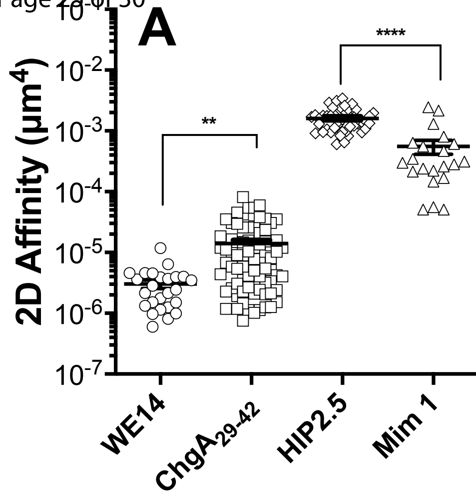
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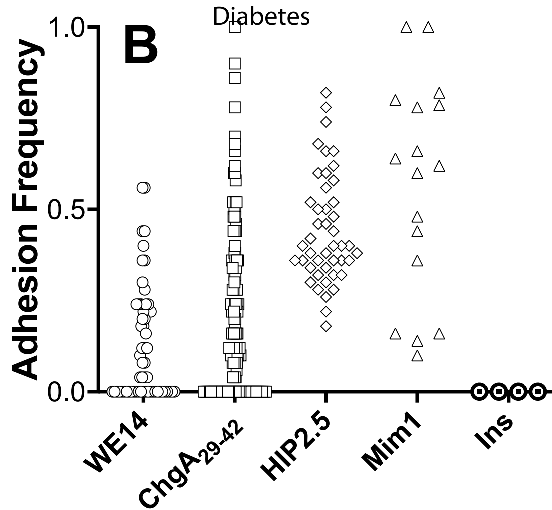
2D Affinity (μm4)
10-1
10-2
10-3
10-4
10-5
10-6
10-7
**
****
WE14
ChgA29-42
HIP2.5
Mim 1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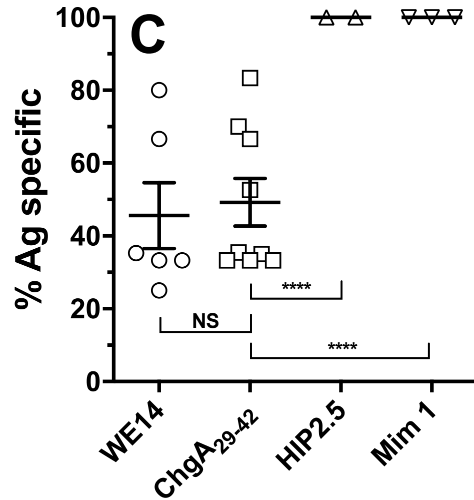
WE14
ChgA29-42
HIP2.5
Mim1
Ins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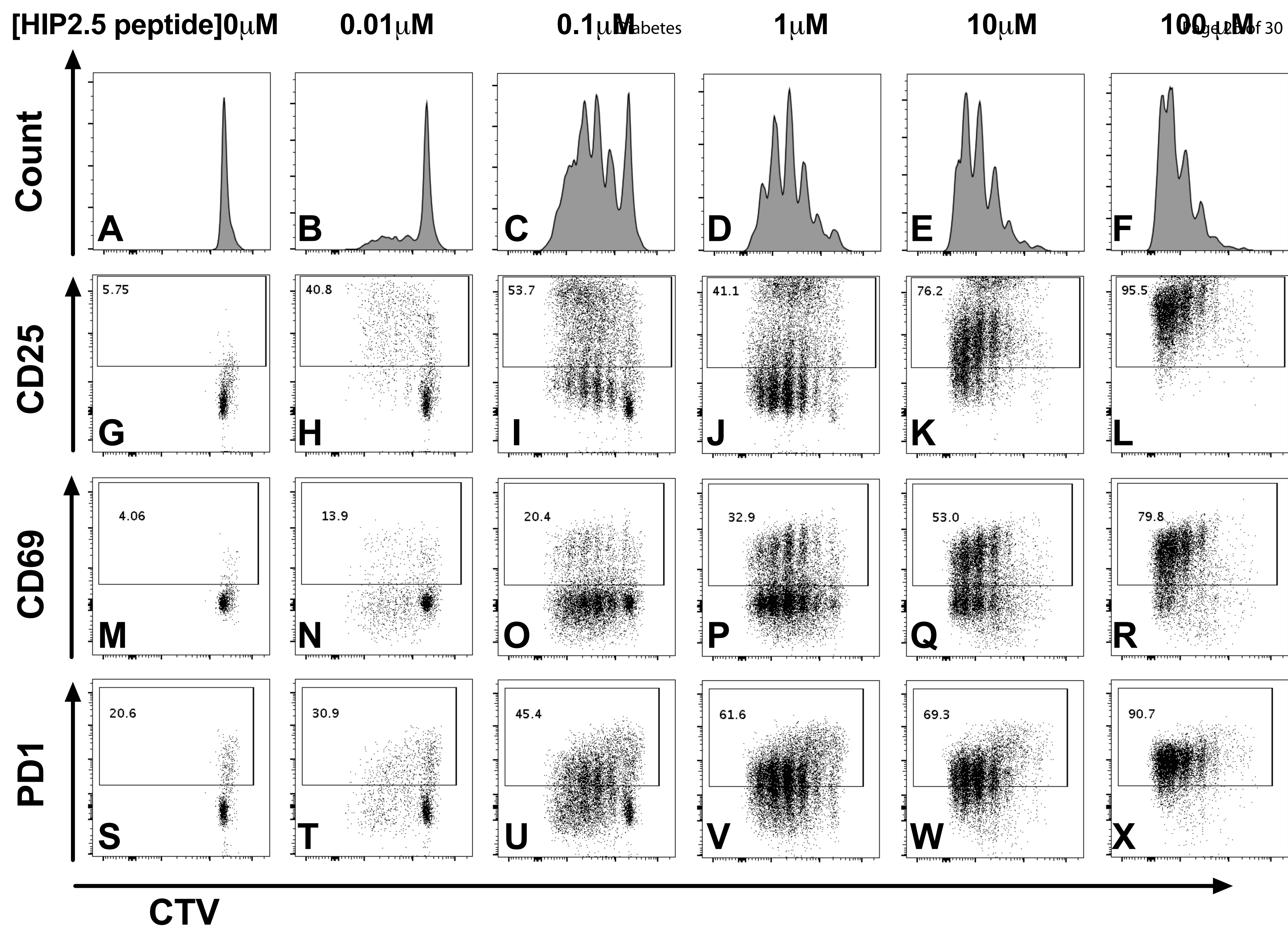
% Ag specific
100
80
60
40
20
0
****
NS
****
WE14
ChgA29-42
HIP2.5
Mim 1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0.01μM
0.1μM
1μM
10μM
100μM
Count
CD25
CD69
PD1
CTV
A
B
C
D
E
F
5.75
40.8
53.7
41.1
76.2
95.5
G
H
I
J
K
L
4.06
13.9
20.4
32.9
53.0
79.8
M
N
O
P
Q
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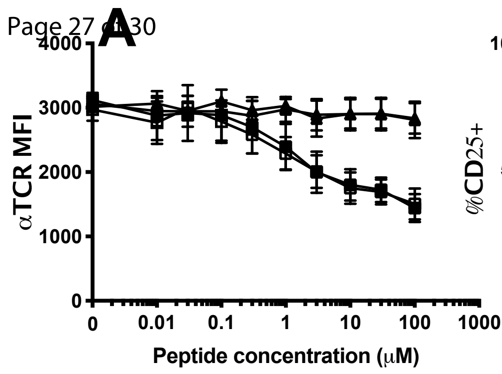
90.7
S
T
U
V
W
X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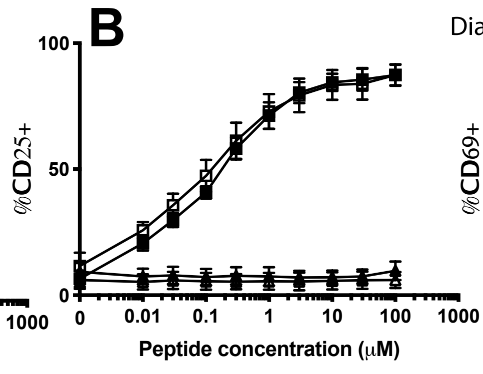
αTCR MFI
Peptide concentration (μM)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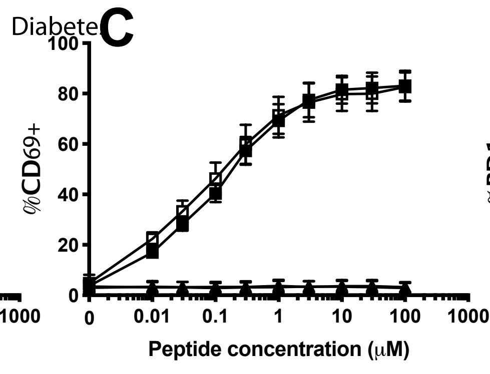
Peptide concentration (μM)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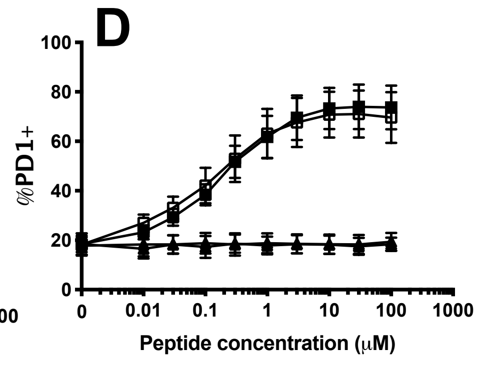
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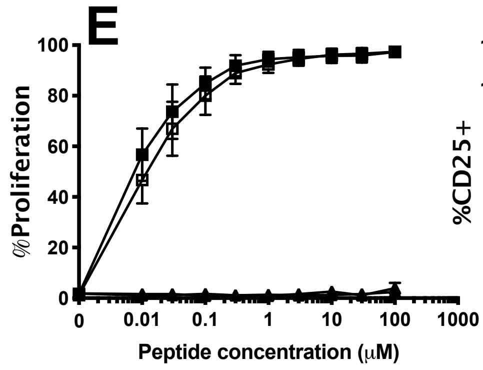
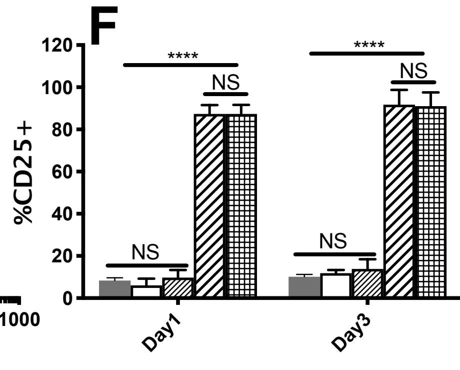
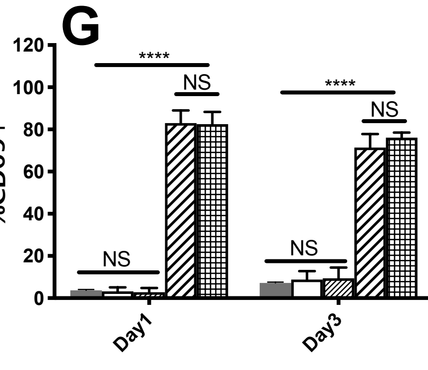
Peptide concentration (μM)


E
%Proliferation
Peptide concentration (μM)


F
%CD25+
****
NS
Day1
Day3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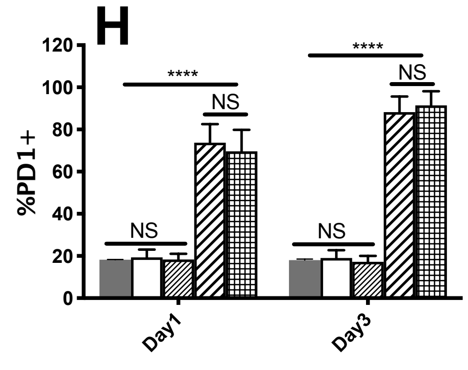
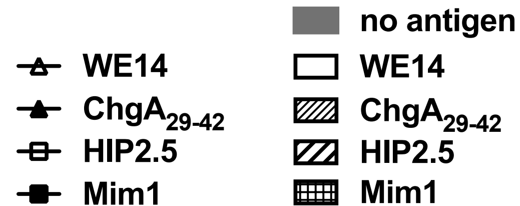
%CD69+
****
NS
Day1
Day3


H
%PD1+
****
NS
Day1
Day3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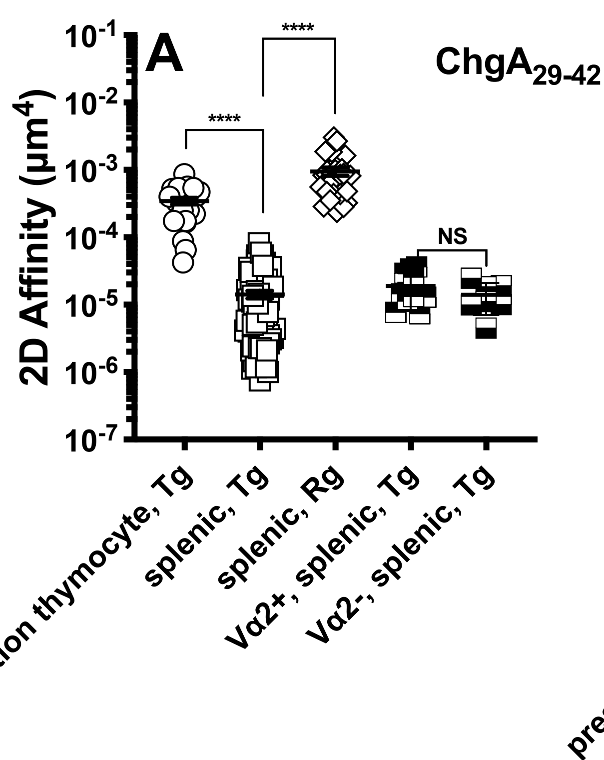
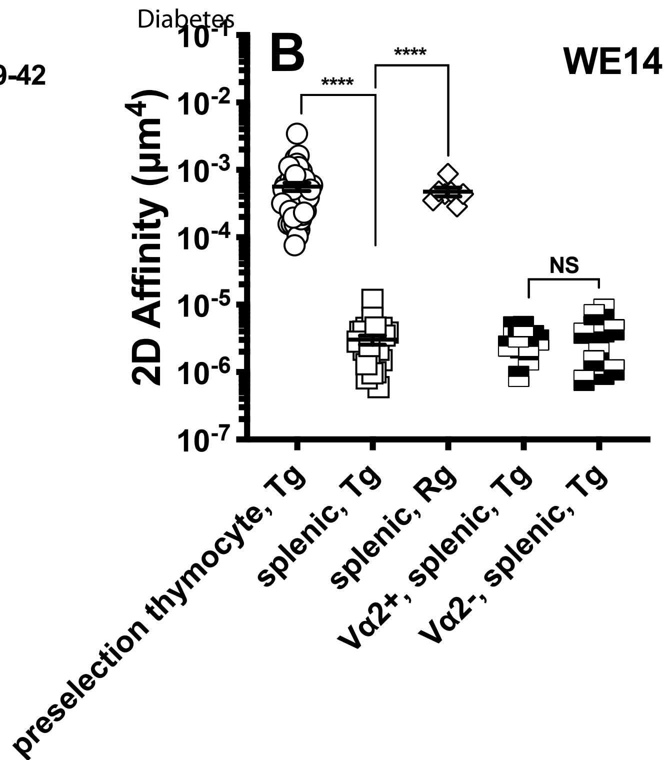
HIP2.5
Mim1
no antigen
WE14
ChgA29-42
HIP2.5
Mim1


A
ChgA29-42
2D Affinity (μm4)
10-1
10-2
10-3
10-4
10-5
10-6
10-7
****
****
NS
preselection thymocyte, Tg
splenic, Tg
splenic, Rg
Vα2+, splenic, Tg
Vα2-, splenic, Tg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WE14
2D Affinity (μm4)
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10-3
10-4
10-5
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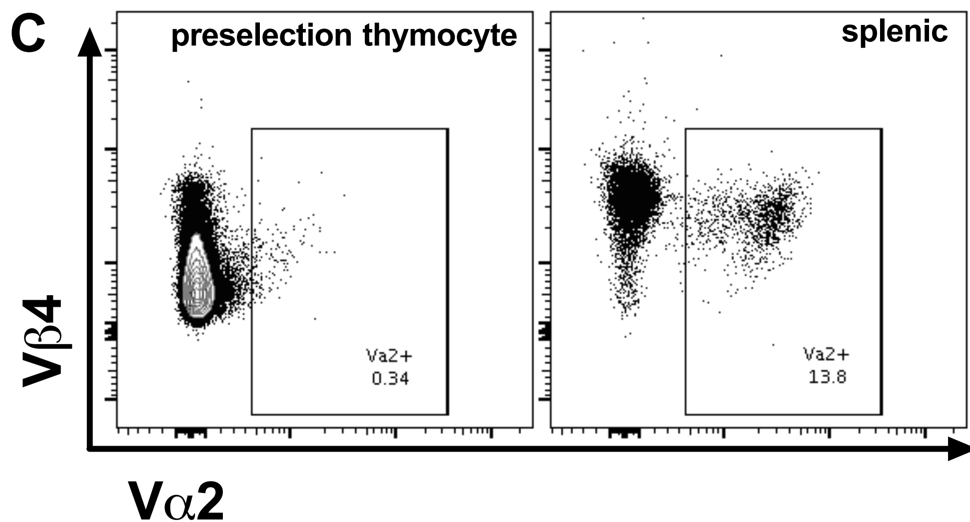
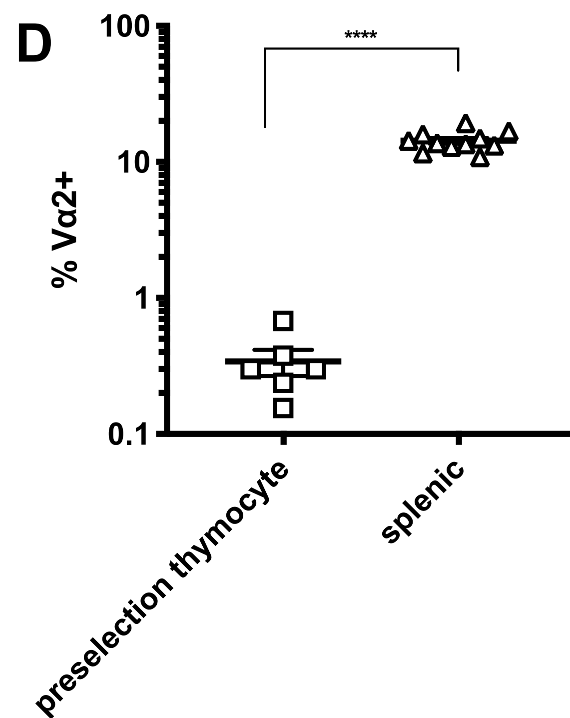
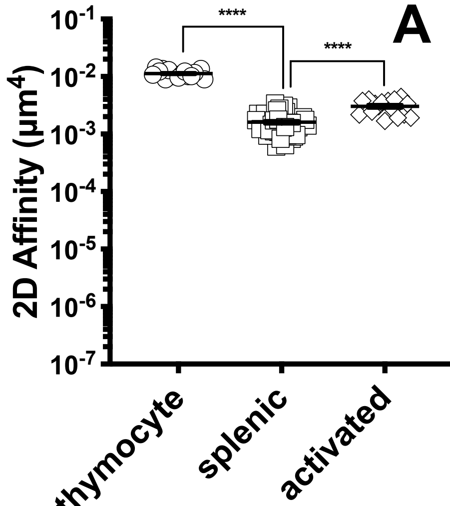
****
****
NS
preselection thymocyte, Tg
splenic, Tg
splenic, Rg
Vα2+, splenic, Tg
Vα2-, splenic, Tg


C
preselection thymocyte
splenic
Va2+
0.34
Va2+
13.8
Vβ4
Vα2


D
% Vα2+
100
10
1
0.1
****
preselection thymocyte
splenic


BDC2.5 vs HIP2.5
A
****
****
2D Affinity (μm4)
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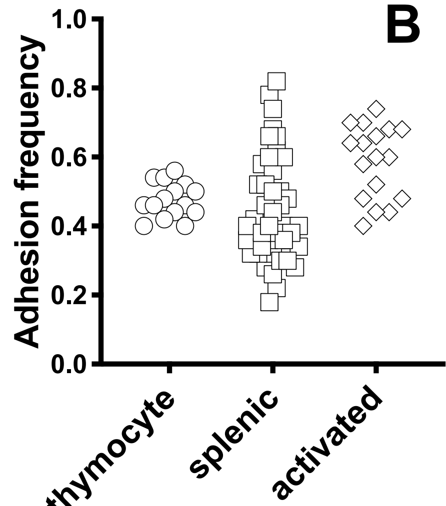
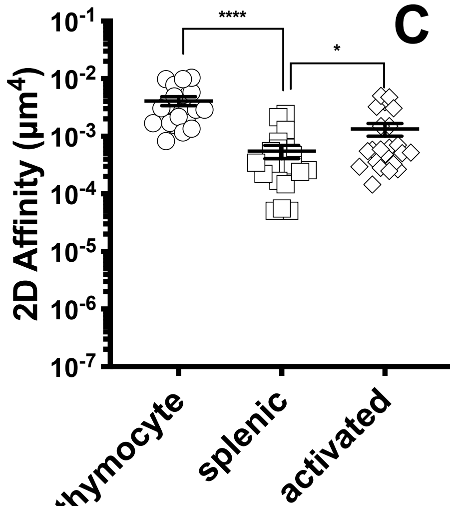
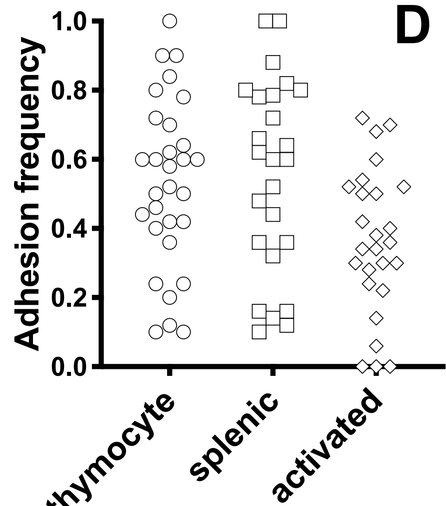
10-2
10-3
10-4
10-5
10-6
10-7
preselection thymocyte
splenic
activated


BDC2.5 vs HIP2.5
B
Adhesion frequency
1.0
0.8
0.6
0.4
0.2
0.0
preselection thymocyte
splenic
activated


BDC2.5 vs Mim1
C
****
*
2D Affinity (μm4)
10-1
10-2
10-3
10-4
10-5
10-6
10-7
preselection thymocyte
splenic
activated


BDC2.5 vs Mim1
D
Adhesion frequency
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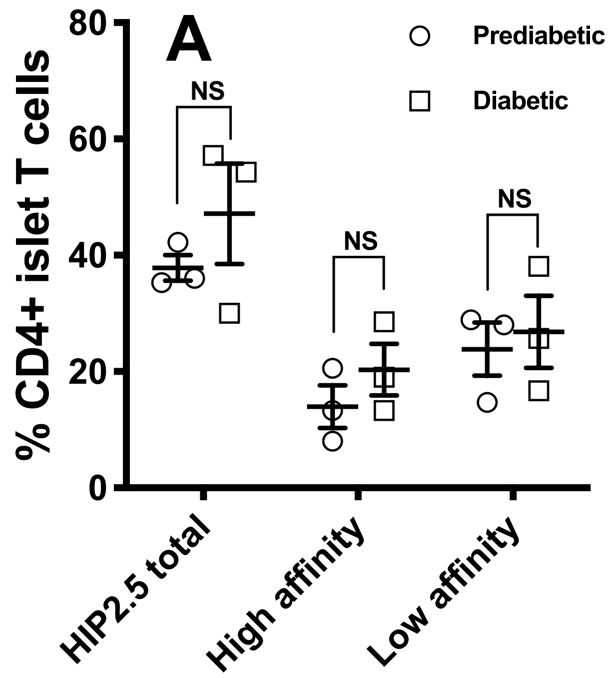
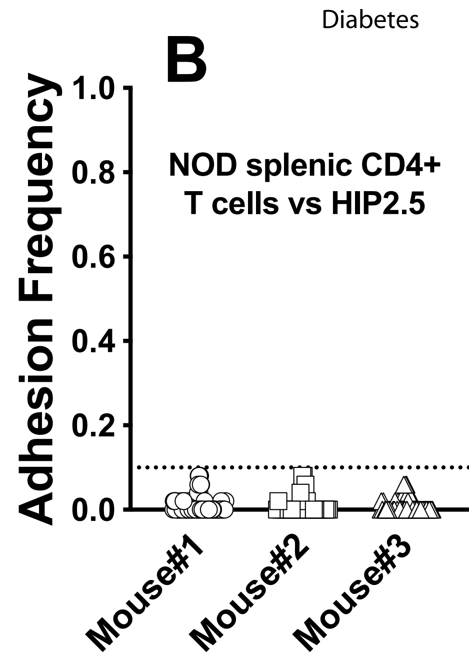
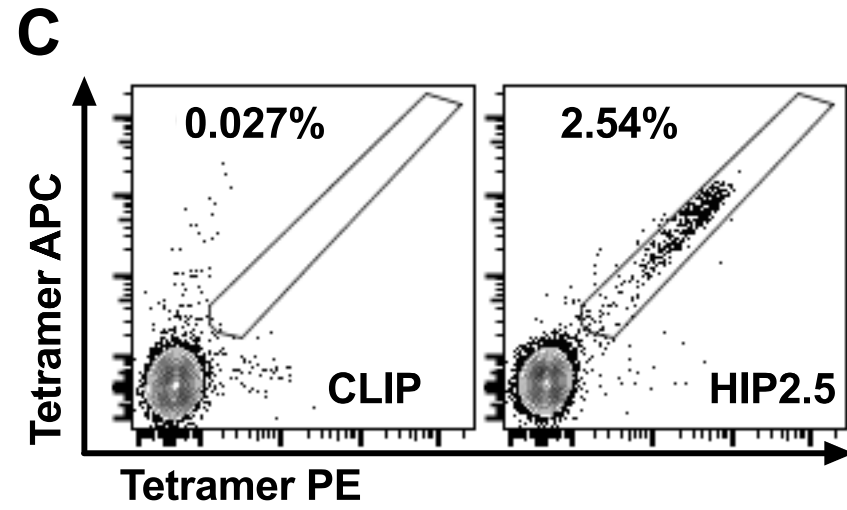
0.6
0.4
0.2
0.0
preselection thymocyte
splenic
activated


A
Prediabetic
Diabetic
NS
NS
NS
% CD4+ islet T cells
80
60
40
20
0
HIP2.5 total
High affinity
Low affinity


B
Adhesion Frequency
1.0
0.8
0.6
0.4
0.2
0.0
NOD splenic CD4+
T cells vs HIP2.5
Mouse#1
Mouse#2
Mouse#3


C
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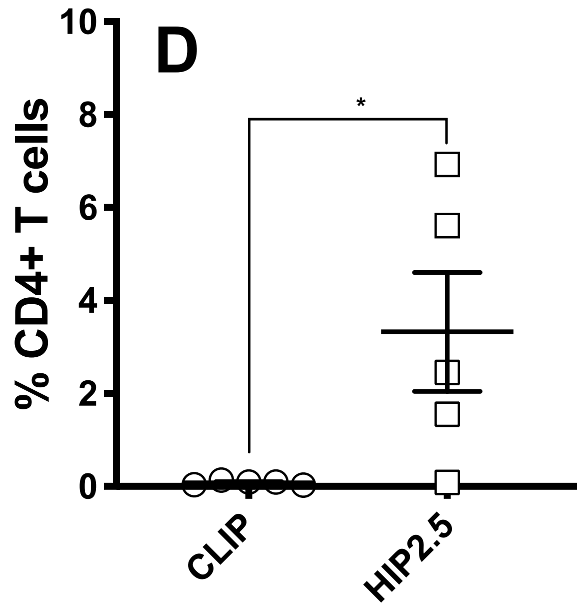
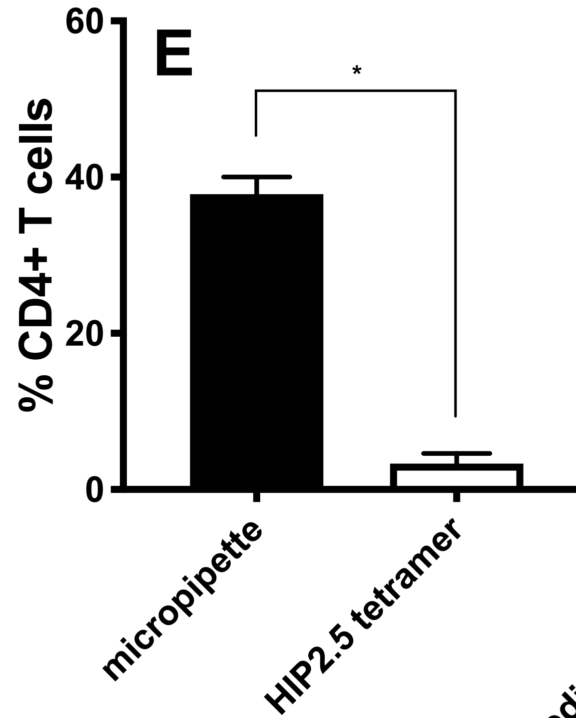
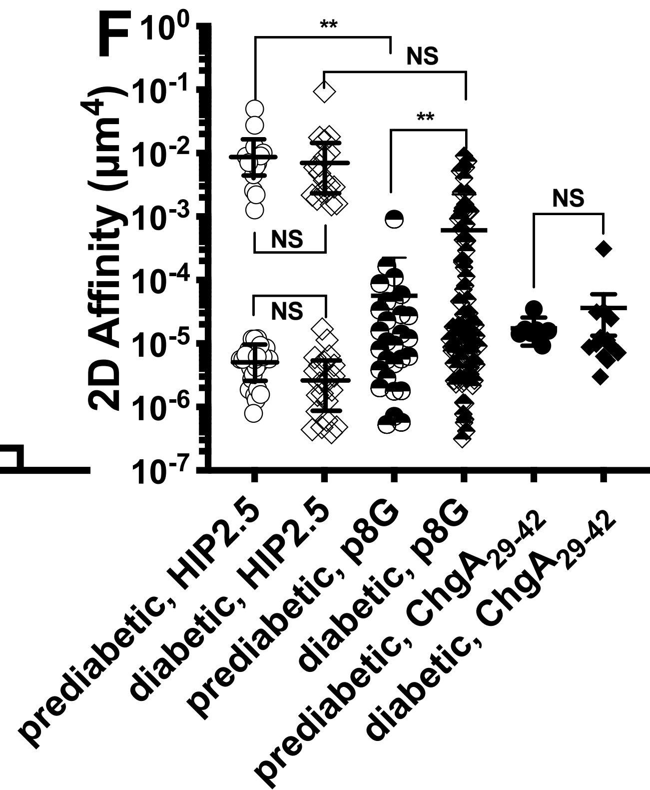
2.54%
CLIP
HIP2.5
Tetramer APC
Tetramer PE


D
% CD4+ T cells
10
8
6
4
2
0
*
CLIP
HIP2.5


E
% CD4+ T cells
60
40
20
0
*
micropipette
HIP2.5 tetramer


F
2D Affinity (μm⁴)
10⁰
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
10⁻⁷
**
NS
**
NS
NS
NS
prediabetic, HIP2.5
diabetic, HIP2.5
prediabetic, p8G
diabetic, p8G
prediabetic, ChgA29-42
diabetic, ChgA29-42