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FLEXIBLE PEPTIDE RECOGNITION BY HLA-DR TRIGGERS SPECIFIC AUTOIMMUNE T-CELL RESPONSES IN AUTOIMMUNE THYROIDITIS AND DIABETES

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Abstract

Autoimmune polyglandular syndrome 3 variant (APS3v) refers to the co-occurrence of autoimmune thyroiditis (AITD) and type 1 diabetes (T1D) within the same individual. HLA class II confers the strongest susceptibility to APS3v. We previously identified a unique amino acid signature of the HLA-DR pocket (designated APS3v HLA-DR pocket) that predisposes to APS3v. We hypothesized that both thyroid and islet peptides can be presented by the unique APS3v HLA-DR pocket, triggering AITD+T1D together. To test this hypothesis we screened islet and thyroid peptides for their ability to bind to the APS3v HLA-DR pocket. Virtual screen of all possible thyroglobulin (Tg), thyroid-stimulating hormone receptor (TSHR), thyroid peroxidase (TPO), insulin (Ins), and glutamic acid decarboxylase 65 (GAD65) peptides identified 36 peptides that bound to this unique pocket. *In vitro* binding assays using baculovirus-produced recombinant APS3v HLA-DR identified 11 thyroid/islet peptides (of the 36 predicted binders) that bound with high affinity. By immunizing humanized HLA-DR3 mice carrying the APS3v HLA-DR pocket we identified 4 peptides (Tg.1571, GAD.492, TPO.758, TPO.338) that were presented by antigen presenting cells and elicited T-cell response. We conclude that both thyroid and islet peptides can bind to this flexible APS3v HLA-DR pocket and induce thyroid and islet specific T-cell responses. These findings set the stage to developing specific inhibitors of the APS3v HLA-DR pocket as a precision medicine approach to treating or preventing APS3v in patients that carry this genetic HLA-DR pocket variant.

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Keywords

Autoimmune Thyroiditis; Hashimoto's disease; Graves' disease; type 1 diabetes; HLA

1. Introduction

Autoimmune endocrine disorders frequently occur in the same individual. This association is referred to as autoimmune polyglandular syndrome (APS) [1]. The most common type of APS consists of the co-occurrence of autoimmune thyroid disease (AITD), in which the target endocrine gland is the thyroid, and type 1 diabetes (T1D), in which the target endocrine gland is the pancreatic islets beta cells [2–5]. The clinical phenotype of AITD +T1D within the same individual is referred to as a variant of APS type 3 (APS3v). Indeed, AITD and T1D share common etiology and immunological abnormalities including infiltration of the target glands by lymphocytes reactive to self-antigens and production of antibodies directed at the target organs [1, 6]. While the prevalence of APS3v has not been calculated directly it can be estimated based on the prevalence of T1D in the US population (1.93 per 1000) [7] and the frequency of AITD in T1D patients (20–30%) [8]; based on these data the prevalence of APS3v in the US population is approximately 0.4 – 0.6 per 1000.

Family and population studies provide consistent evidence for the strong association of AITD and T1D. Whole genome linkage and Genome Wide Association Studies (GWAS) support a genetic etiology for this association [2] and identified five gene-loci that confer susceptibility to APS3v. They include the major histocompatibility (MHC) class II locus, cytotoxic T-lymphocyte antigen 4 (CTLA-4), protein tyrosine phosphatase non-receptor type 22 (PTPN22), G-protein-coupled receptor 103 (GPR103) and Forkhead box P3 (FOXP3) [9–12]. Among them, the MHC class II locus showed the strongest linkage and association with APS3v, and Human Leukocyte Antigen-DR3 (HLA-DR3) conferred most of the shared risk [8]. Our group has previously identified a pocket amino acid signature, DR β -Tyr26, DR β -Leu67, DR β -Gln70, DR β -Lys71 and DR β -Arg74 that is strongly associated with APS3v [9]. We hypothesized that this unique pocket predisposes to both AITD and T1D (APS3v) by enabling the presentation of thyroid and islet peptides within the same pocket structure.

To test this hypothesis we screened all potential peptides originating from the major AITD and T1D associated self-antigens (thyroglobulin [Tg], thyroid-stimulating hormone receptor [TSHR], thyroid peroxidase [TPO], insulin [Ins], and glutamic acid decarboxylase 65 [GAD65]) for binding to the APS3v HLA-DR pocket. We then tested whether peptides that showed strong binding to the APS3v-pocket induced T-cell responses in a humanized mouse model carrying the core APS3v pocket signature. Our data support the hypothesis that the APS3v HLA-DR pocket we identified enables the presentation of pathogenic thyroid and islet peptides to T-cells, thereby triggering both thyroid and islet T-cell responses and autoimmunity.

2. Materials and Methods

2.1 Production of recombinant APS3v HLA-DR

APS3v HLA-DR protein was produced using the baculovirus system using a similar strategy we described previously [13]. We designed 2 separate constructs for the α and β chains of the APS3v HLA-DR. The β chain construct contained the extracellular portion of the APS3v β chain fused to the coiled coil region of the basic Leucine zipper domain of JunB, and the α chain construct contained the extracellular portion of the α chain fused to the coiled coil region of the basic Leucine zipper domain of Fos. A tobacco etch virus (TEV) protease cutting site was introduced in each chain to allow removal of the dimerization motif. The JunB and Fos dimerization motifs allowed the protein to dimerize and form the final APS3v HLA-DR protein. The α chain has a His₆ tag while the β chain has a V5 epitope tag for purification purposes. (Figure 1). These constructs were used to produce the APS3v HLA-DR protein in a Baculovirus system using the Life Technologies Baculovirus protein production custom services (Carlsbad, CA, USA).

2.2 Construction of Model Structures and MD simulations

The structural models of the APS3v HLA-DR molecule were constructed based on the X-ray crystallographic structure of HLA-DR3 with a CLIP peptide complex (PDB ID: 1A6A) [14]. The amino acids at positions β 37, β 47 and β 86 were mutated using the VMD facility [15] N37->Y, F47->Y and V86->G. The structural models of the peptides in complex with APS3v were constructed by mutating the original sequence of the CLIP peptide using the VMD facility. The initial structures of the complexes of APS3v with the peptides were used to construct the system for simulations using the AMBER facility LEaP [16]. The system was placed in a truncated octahedron with walls 10Å from the solute, which was filled with water molecules and ions to create an ionic strength of 0.15 M. The systems consisted of approximately 70,000 atoms. The systems were minimized, heated and equilibrated with the solute positionally restrained while gradually removing the restraints. The equilibrated system was simulated under constant temperature and pressure (NPT) for 100 ns. The trajectories were recorded every 2 ps. After a short period (~1 ns) the systems usually stabilized showing a root mean square deviation from the initial structure of the order of 3Å. The first 10 ns of the trajectories were discarded and the remaining data were processed to remove the waters and ions. The resulting trajectories were used to calculate the binding energies with the MM-PBSA module in AMBER. Specifically, the residue contributions were calculated using the *decomp* option. The values discussed in the paper refer only to those associated with the peptide [17].

2.3 Virtual screening of thyroidal and islet peptides

We used the web-based server NetMHCIIpan-2.0 to predict binding of peptides to HLA alleles. The method is based on Artificial Neural Network trained on available binding data of peptides to HLA alleles [18]. The sequences of the antigen proteins (Tg [D1KKB3], TPO [P35419], TSHR [P16473], GAD65 [Q9UGI5] and Ins [A6XGL2]; all accession numbers are from uniprot.org) were used to perform the virtual screen on a selected DRB subtype DRB1*0301. Top ranked peptides from the list were selected and redundancies (i.e.,

proximal sequences) were removed based on a predicted common binding core of 9 residues. This resulted in the 36 predicted peptide binders listed in Table 1.

2.4 Peptide synthesis

The peptides used in this study were synthesized by Genscript (Piscataway, NJ). Based on the results of the virtual screen the following peptides were tested in vitro for binding to the APS3v HLA-DR: 5 thyroglobulin (Tg) peptides (Tg.2098, Tg.726, Tg.1951, Tg.1571 and Tg.202), 2 thyroid-stimulating hormone receptor (TSHR) peptides (TSHR.132 and TSHR.197), 6 insulin (Ins) peptides (Ins. B9-23, Ins.44, Ins.25, Ins.76, Ins.89, Ins.98), 10 glutamic acid decarboxylase 65 (GAD65) peptides (GAD.378, GAD.492, GAD.116, GAD.247, GAD.260, GAD.274, GAD.379, GAD.555, GAD.192, GAD.456) and 13 thyroid peroxidase (TPO) peptides (TPO.763, TPO.489, TPO.122, TPO.28, TPO.423, TPO.758, TPO.405, TPO.338, TPO.513, TPO.589, TPO.161, TPO.344 and TPO.351). The apo-peptide (APO), a peptide that was previously shown by us [13] and others [19] to be the best binder to this pocket, was used as a positive control, and a scrambled thyroglobulin peptide (scr2098) was used as a negative control. The sequences of the peptides are listed in Table 1.

2.5 In vitro screening of peptide binding to APS3v HLA-DR

The 36 peptides predicted by the virtual screen to bind the APS3v HLA-DR with high affinity were tested for binding to the APS3v HLA-DR pocket using a unique immunoassay we developed for this purpose. In order to test peptide binding to the APS3v HLA-DR pocket, we used the recombinant APS3v HLA-DR protein we generated. APO was used as a positive control, and scr2098 was used as a negative control. In this assay 0.012 mg/ml APS3v HLA-DR protein was incubated with 10 μ M of tested biotinylated peptide (Genscript), for 48 h at 37 °C in binding buffer (0.1% BSA/PBS with 0.05% Triton [PBST], Sigma Aldrich, St. Louis, MO). On the day before the immunoassay was performed, a 96-well DELFIA yellow plate (PerkinElmer, Waltham, MA) was coated overnight with 20 μ g/ml of L243 antibody (Hybridoma was purchased from American Type Culture Collection [ATCC], Catalog no. HB-55 and IgG was purified by QED Bioscience, San Diego, CA) in bicarbonate buffer pH 9.6 (Sigma-Aldrich). L243 is a monoclonal antibody that specifically recognizes the α chain of HLA-DR when it is properly folded and complexed with the β chain of HLA-DR [20]. The plate was then washed with DELFIA wash buffer (diluted 1:25 from DELFIA Wash Concentrate, PerkinElmer) to wash off the excess L243 antibody. Blocking was done using 2.5% BSA in PBS at room temperature for 1 h. After washing 4 times, 100 μ l of the pre-incubated complex (APS3v HLA-DR protein-peptide) were added to the plate and shaken at slow speed for 2 h at room temperature. After washing 4 times, DELFIA Eu-labeled Streptavidin (PerkinElmer) diluted in DELFIA assay buffer (PerkinElmer) was added for 30 min and shaken at slow speed at room temperature. After washing for 6 times, DELFIA Enhancement Solution was added for 1 h or until optimal signal was reached. Time-resolved fluorescence was measured using the BMG reader (BMG Labtech, Cary, NC). The experiment was performed in triplicates. As negative control peptide alone was added to the plate coated with L243. Fold increase was calculated as follows:

[(APS3v HLA- DR protein+peptide)/peptide alone].

Peptides binding with greater than 2 fold (>10 standard deviations over the average for peptide alone with no HLA) was considered as positive.

2.6 Mice

Mice transgenic for DRB1*0301 were originally generated by G.J Hämmerling and coworkers as previously described [21]. Briefly, the DR3 (DRA1*0101/DRB1*0301) transgenes were inserted into (C57BL/6 X DBA/2) F1 embryos and the progeny were back-crossed to B10.Q mice. The DR3 mice were crossed with I-A β knockouts (C57BL/6 x129) to obtain mice lacking murine MHC class II and expressing human DR3 [22]. The transgenic mouse line was maintained by intercrossing. The background non-MHC genes in the DR3 transgenic line was 50% C57BL/10 genes and 50% contribution from CBA, C57BL/6, and 129 mouse strains genes. These mice were then back-crossed into non-obese diabetic (NOD) background to produce the NOD-DR3 mice [23–25]. We have confirmed that these mice express the 5 critical amino acids of the APS3v HLA-DR pocket [26]. Mice were bred in a pathogen-free facility (Icahn School of Medicine at Mount Sinai, New York, NY and Albert Einstein College of Medicine, Bronx, NY), and expression of HLA-DR3 was tested by PCR using DR3-specific primers: forward primer 5' CGCTTCGACAGCGAC 3' and reverse primer 5' GACAAATCCACACTCCAC 3'. DR3 transgenic mice on NOD background known to be susceptible to Experimental Autoimmune Thyroiditis (EAT) [23] and to generate stronger autoimmune T-cell responses than DR3 mice on B6 background were used in this study.

2.7 Induction of T-cell responses in NOD-DR3 mice

Female NOD-DR3 mice, 4–6 weeks old, were injected subcutaneously with the peptides tested in this study in Complete Freund's Adjuvant (CFA, Sigma) to induce T-cell responses as previously described [27, 28]. Mice were immunized with peptides on day 0 and boosted on day 7, then sacrificed on day 21. Three mice were immunized with each peptide. As a control, two mice were immunized with PBS emulsified in CFA. We measured T-cell stimulation and interferon gamma secretion (described below). T-cell proliferation was calculated as stimulation index as follows:

Stimulation index=[% proliferating lymphocytes (peptide or mitogen- treated)]/[% proliferating lymphocytes (medium- treated)]

Stimulation index 1.5 was considered as a positive response as previously described [26].

2.8 Lymphocytes isolation

Spleen and draining lymph nodes were collected from mice upon sacrifice. The spleens and draining lymph nodes were harvested in complete RPMI (Corning, NY) supplemented with 10% FBS (Sigma-Aldrich) and 1 mM sodium pyruvate (Sigma-Aldrich). They were cut and pressed in circular motion using a plunger from a 10 ml syringe. The suspension was filtered through a 100 μ m cell strainer twice and centrifuged 200 $\times$ g for 10 min. The supernatant

was discarded. The pellet was washed with RPMI and centrifuged one more time. 5 ml Ammonium-Chloride-Potassium (ACK) lysis buffer was added to remove erythrocytes from the spleen. After 5-min incubation with ACK lysis buffer at room temperature with occasional shaking, cells were centrifuged at $200 \times g$ for 10 min. The pellet was resuspended in RPMI and the cells were counted and plated.

2.9 T-cell stimulation and CFSE analysis

Cells harvested from the spleen and lymph nodes were resuspended at 2×10^6 cells/ml in 0.1% BSA/PBS. 1×10^6 cells were labeled with 1.5 μ M carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Life Technologies). After incubating for 10 min at 37°C, the staining was terminated by the addition of 4 volumes of ice-cold RPMI/10% FBS. After 5 min of incubation on ice, the cells were washed 3 times with fresh RPMI and resuspended in fresh medium for counting.

The CFSE-labeled cells were plated at 2×10^5 cells per well in 100 μ l of medium (RPMI/10% FBS). The cells were treated with medium, the peptides tested in the study (20 μ g/ml), an unrelated negative control peptide (NC) (20 μ g/ml), or mouse CD3/CD28 beads (Life Technologies) as positive control. The cells were collected after 5 days for flow cytometry analysis. All experiments were performed in quadruplicates. The results were analyzed using Flowjo (Tree Star, Ashland, OR). The stimulation index was calculated by using the following formula:

Stimulation index = $[\% \text{ proliferating lymphocytes (peptide or mitogen- treated)}] / [\% \text{ proliferating lymphocytes (medium- treated)}]$

2.10 Cytokine assays

Milliplex mouse cytokines/chemokine magnetic panel (Catalog no. MCYTOMAG-70K, EMD Millipore Corporation, Billerica, MA) was used to assay the cytokines. Splenocytes were plated at 2×10^6 cells per well in 500 μ l of medium (RPMI/10% FBS). Supernatants from stimulated lymphocytes were collected 48 h after stimulation with peptides and stored in -80°C until the assay was performed. To begin the assay, the 96-well plate supplied in the kit was washed with the wash buffer supplied and the plate was shaken for 10 min at room temperature. Standards and quality controls were added, followed by the samples. The pre-mixed beads (IFN gamma, IL-2, IL-4, IL-10) were sonicated and vortexed, and then added to the wells. After shaking the plate overnight at 4 $^\circ\text{C}$, the plate was washed twice with wash buffer. Detection antibodies were added for 1 h at room temperature, and Streptavidin-Phycoerythrin was added for 30 min at room temperature. The plate was washed twice and sheath fluid was added to resuspend the beads for 5 min before reading in Luminex 200 with xPONENT software (Luminex, Austin, Texas).

2.11 Study approval

The project was approved by the Institutional Animal Care and Use Committees (IACUC) of the Icahn School of Medicine at Mount Sinai and Albert Einstein College of Medicine. All animal studies were carried out according to the guidelines of the IACUC of the Icahn School of Medicine at Mount Sinai and Albert Einstein College of Medicine.

2.12 Data analysis

We measured peptide binding, T-cell proliferation and interferon gamma secretion by T-cells. Peptide binding was calculated as fold increase as follows:

$$\text{Fold increase} = \frac{(\text{APS3v HLA- DR protein} + \text{peptide})}{\text{peptide alone}}$$

T-cell proliferation was calculated as stimulation index as follows:

$$\text{Stimulation index} = \frac{[\% \text{ proliferating lymphocytes (peptide or mitogen- treated)}]}{[\% \text{ proliferating lymphocytes (medium- treated)}]}$$

Interferon gamma was measured using Luminex. We determined the cutoffs for significance for these parameters (Figures 2, 4, and 5, respectively) as indicated in the text.

3. Results

3.1 Virtual screening results of thyroid and islet peptides binding to APS3v HLA-DR

The results from the virtual screen of antigens using the NetMHCIIpan method provide all the possible sequences from a particular antigen ranked by their predicted 'affinity' or %Rank. Sequences with predicted dissociation constant < 50 nM are termed Strong Binders (SB), those > 50 nM and < 500 nM are termed Weak Binders (WB) and those > 500 nM are not considered. Inspection of the results also shows that the core sequence of the 9 residues predicted to bind in the groove of the APS-specific HLA-DR3 are often degenerate in several sequences. For example the group of sequences from TSHR ranging from 520 as the initial residue to 525 as the initial residue have the same core MRLDRKIRL. To minimize the redundancy we chose from the list the sequences that passed the following criteria:

1. We used the degree of similarity to an optimal core sequence that was identified in our previous studies [9, 13] on thyroglobulin peptides. We considered the optimal core sequence in the Tg peptides as a good template because APS3v is very similar to DR3. This helped identify the most likely epitopes in the screens of the other antigens.
2. Analysis of the substitutions in the APS3v HLA-DR3 shows that Asn-β86 which defines part of the wall of P1 was substituted with Gly. This enlarges the pocket such that a Trp residue can fit now in P1 without distorting the structure of the protein. At the other end of the peptide-binding groove, Asn-β37 has been replaced with Tyr, substantially shrinking the size of the P9 pocket. Consequently, the preferred p9 residue in the peptide is small (e.g., Ala). The substitution of Phe-β47 for Tyr occurs in the vicinity of P7, which is usually a hydrophobic residue. In APS3v the presence of Tyr in position β47 creates a hydrogen bonding donor with a possibility of a polar or a charged residue in p7. These characteristics were added to the selection of the peptides from the screens of the antigens.

Using this strategy, in addition to the peptides we have identified from thyroglobulin, we also identified two peptides from TSHR, 5 peptides from insulin (in addition to Ins. B9-23, a

known T1D major epitope), 10 peptides from GAD65 and 13 peptides from thyroid peroxidase (TPO).

3.2 Peptides binding to APS3v HLA-DR in vitro

We screened the 36 peptides predicted by the virtual screen for binding to the recombinant APS3v HLA-DR protein. The sequences of these peptides are shown in Table 1. The predicted peptides were tested for binding to the APS3v HLA-DR utilizing the *in vitro* binding assay we developed (see methods). Peptides binding with greater than 2 fold (>10 standard deviations over the average for peptide alone with no HLA) were considered as positive. Our binding assay showed that 2 thyroglobulin peptides (Tg.1951, Tg.1571), 1 insulin peptide (Ins.76), 2 GAD65 peptides (GAD.378 and GAD.492) and 6 TPO peptides (TPO.489, TPO.758, TPO.338, TPO.589, TPO.161, TPO.344) bound positively to the APS3v HLA-DR protein, with fold increase greater than 2 (Figure 2). None of the predicted TSHR peptides bound to APS3v HLA-DR. APO was used as a positive control and, as expected, it bound to APS3v HLA-DR protein with > 25 fold, while scr2098 (a scrambled thyroglobulin peptide) that was used as a negative control had the fold increased binding of 1.

3.3 A molecular basis for peptide binding to APS3v

Examination of the sequences of the peptides that bind to APS3v does not offer a clear clue for a relation between sequence and the strength of binding. We therefore conducted MD simulations on 7 of the 11 peptides showing strong binding to the APS3v pocket to elucidate the role of specific residues as well as to understand which positions in the peptide contribute significantly to binding. We simulated 2 thyroglobulin peptides (Tg.1951 and Tg.1571), 2 GAD65 peptides (GAD.378 and GAD.492) and 3 TPO peptides (TPO.758, TPO.338 and TPO.161). The trajectories from the 100 ns simulations were clustered by 2D-rmsd resulting in 2 clusters – the major representing ~80% of configurations on the average, while the minor usually represented an equilibration phase of the trajectory. The MMGBSA analysis was decomposed into residue-based contributions and is shown in Table 2. The contribution of each residue to the interaction energy with the APS3v is expressed in kcal/mol relative to the separated molecules; thus the more negative numbers indicate a stronger interaction. Using the assumption that the overall interaction energy is simply a sum of the positional energies we can predict the best binder sequence based on these peptides by selecting the residues with the most negative values (see last column). If we include in the analysis the flanking residues the total predicted sequence is: PRYRW₁LFD₄CR₆LLY₉VW.

On inspection of the nature of the residues in the critical position responsible for peptide binding we note that p1 contains a hydrophobic residue with Trp having the largest contribution, consistent with the analysis of substitution in APS3v (see above). In p4 the preferred residue is polar and Asp makes the largest contribution because of its interaction with Arg-β74. The preferred residue in p6 is a positively charged group (e.g., Arg) because of a convergence of three negatively charged residues: Glu-α11, Asp-α66 and Glu-β9. In addition Asn-α62 positions its OD2 next to the Arg₆. Finally, p9 is highly variable and the best side chain is that of Tyr. As an example we show the binding of TPO.758, the strongest binder, to the APS3v in Figure 3.

To predict the potency of the optimal peptide we need a model to relate the particular contribution from a specific residue in its position in the sequence to the experimentally observed potency expressed as fold inhibition (see Figure 2). Since the peptides do not present an apparent sequence conservation, we decided to construct the model as a function of position rather than residue identity. Using multiple regression we obtain a good fit of the experimental data [29] expressed in terms of positional contributions $\{x_{ij}\}$. The following equation expresses the relation:

$$y_i = p_1 x_{i1} + p_4 x_{i4} + p_6 x_{i6} + p_9 x_{i9} + const$$

where p_j are the coefficients of position 1, 4, 6 and 9. The multiple regression fit has a *multiple R* of 0.9237, R^2 of 0.8532 and the values of the coefficients are: $p_1 = -2.45 \pm 2.04$; $p_4 = -2.32 \pm 1.02$; $p_6 = -0.91 \pm 0.56$; $p_9 = -3.93 \pm 1.42$; $const = -50.92 \pm 28.34$. Using this equation the predicted inhibitory potency of the optimal sequence shown in Table 2 is 394.4, which is 14 times more potent than TPO.758 – the most potent peptide binder (Figure 2).

It is useful to interpret the coefficients in the multiple regression. In the traditional interpretation the coefficient measures the change in the observable y upon change in a specific variable x_j while all the other variables are kept constants. As can be seen, all the coefficients are negative and since the interaction energies obtained from the decomposition are also negative (Table 2), increasing the strength of interaction between a residue and the APS3v pocket (making it more negative) will make the potency more positive, i.e., the inhibition will be stronger. This gives the rationale to search for the best binders by choosing the sequence that maximizes the interaction with the APS3v pocket. It is interesting to note that inclusion of other positions in the multiple regression yielded *positive* coefficients of positions 2, 3, 5, 7 and 8. According to our interpretation of the coefficients, increasing the interaction of these residues with the APS3v pocket reduces the potency of inhibition. Interestingly, these residues usually point outside the binding groove and are potentially involved in the interaction with the T-cell receptor (TCR).

3.4 Peptides inducing T-cell response in NOD-DR3 mice

3.4.1 T-cell proliferative responses—NOD-DR3 mice were used in this experiment because they are susceptible to EAT [28]. Each of the 11 peptides showing positive binding to the APS3v HLA-DR *in vitro* (2 Tg, 1 Ins, 2 GAD65 and 6 TPO) was used to immunize 3 female NOD-DR3 mice to induce T-cell responses to the peptide. After the second immunization (see methods), splenocytes of immunized mice were isolated and stimulated with: 1) peptide used for immunization; 2) negative control peptide (scr2098); 3) anti-mouse CD3/CD28 beads (positive control). Tg.1571, GAD.492, TPO.758 and TPO.338 induced T-cell proliferation with stimulation index greater than 1.5 (Figure 4A). Among them, TPO.758 showed the greatest response (stimulation index=4). As an immunization negative control, 2 female NOD-DR3 mice were immunized with PBS in CFA. As expected, the splenocytes of the PBS-CFA immunized mice did not show T-cell responses to the 4 positive peptides (Figure 4B), confirming the 4 peptides as true positive.

Based on our analysis of the binding as represented by the contribution of residues in individual positions, we wanted to examine the sequences of the four peptides that lead to T-cell response. As discussed above, optimization of binding in positions 2, 3, 5, 7 and 8 lead to a predicted reduction of the potency of binding of the peptides in our set of 7 peptides. Thus, these positions could be important in forming the complexes of APS3v•peptide with TCR. This would be consistent with the analysis based on several crystal structures of the ternary complexes of HLA, peptide and TCR [29]. The current structures of peptides bound to APS3v that result from the simulations do not contain the TCR, thus the residues in positions 2, 3, 5, 7 and 8 are possibly in the wrong conformation for interaction with TCR. In order for these to optimize their interaction with TCR they would have to undergo a conformational change. In an attempt to optimize the nature of the residue that will lead to activation, it is reasonable to select residues with the weakest interaction with the APS3v. Examining the decomposition energies of the four peptides that lead to activation we therefore predict the following residues as optimizing activation: p_2 = Thr, p_3 = Ser, p_5 = Glu, p_7 = His and p_8 = Gly.

3.4.2 Cytokine production—Interferon γ production was measured in splenocytes of mice immunized with the 11 peptides showing strong binding to APS3v HLA-DR. Splenocytes of mice immunized with Tg.1571, GAD.492, TPO.758 and TPO.338 (the 4 peptides that induced T-cell proliferative responses) also produced high levels of interferon γ (> 1000 pg/ml) when stimulated, while mice immunized with other peptides showed very low interferon γ production comparable to the negative control (< 50 pg/ml; Figure 5A). As expected, mice immunized with PBS-CFA did not produce significant levels of Interferon γ in response to the 4 peptides (Figure 5B).

4. Discussion

Our group has previously identified a unique HLA-DR pocket amino acid signature - DR β -Tyr26, DR β -Leu67, DR β -Gln70, DR β -Lys71, DR β -Arg74 - that predisposes to both autoimmune thyroiditis and diabetes (APS3v) [9]. An important question that arises from the identification of a unique HLA-DR pocket for APS3v is what is the mechanism by which this unique pocket triggers both AITD and T1D (APS3v) in the same individual? One potential mechanism is that the APS3v HLA-DR pocket presents peptides derived only from one target tissue (thyroid or islets) and a different HLA class II allele in tight linkage disequilibrium (LD) with the APS3v HLA-DR presents peptides derived from the other corresponding target tissue (Figure 6A). In fact, the AITD-associated allele (DRB1*0301) and T1D-associated allele (DQB1*0201) are in tight LD [8, 30]. The second potential mechanism is that the pocket that predisposes to AITD+T1D (APS3v) is a flexible pocket that can present both thyroid and islet antigens (Figure 6B). Our study strongly supports this second putative mechanism. We identified 11 peptides (2 Tg peptides, 1 insulin peptide, 2 GAD65 peptides, 6 TPO peptides) that were able to strongly bind to the APS3v HLA-DR. Moreover, when we immunized humanized DR3 mice (carrying the APS3v HLA-DR pocket) with these peptides we demonstrated that Tg.1571, GAD.492, TPO.758 and TPO.338 can be presented by antigen-presenting cells carrying the APS3v pocket and activate T-

cells. Taken together these data support the hypothesis that both thyroid and islet peptides can bind to the flexible APS3v HLA-DR pocket and elicit T-cell responses.

Our study is the first study to identify major antigenic epitopes that cause APS3v (AITD +T1D). Of 11 peptides that showed strong binding to the APS3v HLA-DR only 4 also stimulated T-cell responses, demonstrating that peptide binding to a unique HLA class II pocket does not necessarily indicate that it will activate pathogenic T-cell responses. Indeed, the majority of peptides with high binding affinity to the APS3v HLA-DR were not immunogenic in humanized mice carrying the APS3v HLA-DR. Our computational analysis leads to the conclusion that peptides with specific residues in position p1, p4, p6 and p9 contribute to binding. Position p1 prefers Trp, p4 Asp, p6 Arg and p9 Tyr. The remaining positions on the peptide (p2, p3, p5, p7 and p8) seem to reduce binding affinity, and consistent with crystal structures of the ternary complexes of MHC•peptide•TCR, they would engage in the interaction with the CDR loops of the TCR. However, the small number of activating peptides prevents us from predicting the nature of the activating residues with confidence. Of the 2 thyroglobulin peptides that bound to the APS3v HLA-DR only Tg.1571 stimulated T-cell responses suggesting that it is a major antigenic epitopes for APS3v. Interestingly, Tg.1571 was also identified as an important epitope in AITD [13, 28]. Hence, while some antigenic epitopes (e.g. Tg.1571) are common to AITD and APS3v, other T-cell epitopes are likely distinct for each phenotype.

We identified 6 TPO peptides that bound with high affinity *in vitro* to the APS3v HLA-DR; this was the highest number of antigen derived peptide-binders among the thyroid and islet antigens tested. Many other TPO peptides were identified as major T-cell epitopes in AITD [31–36]. For example, TPO 110-129, 211-230, 842-861, 882-901 were found to induce lymphocyte response from patients with AITD [34]. Another group found that peripheral blood mononuclear cells of 23–37% AITD or Graves' Disease (GD) patients were reactive to TPO 415-432, 439-457 and 463-481 [32]. A thyroid T-cell line derived from a GD patient was reactive to TPO.535-551 and 632-645 [35]. Other groups also reported different TPO epitopes in AITD using various strategies [31, 33, 36]. The fact that TPO-derived T-cell epitopes in AITD and APS3v are distributed over the whole TPO protein instead of concentrating at a particular domain suggests that responses to TPO are the result of epitope spreading. As in the case of the majority of AITD associated Tg epitopes [13] the TPO epitopes we identified in APS3v (TPO.758 and TPO.338) were distinct from those reported in AITD. These data suggest that the majority of epitopes causing AITD and APS3v are distinct and have different autoreactive T-cell repertoire. It is important to note that TPO.758 was found to be the strongest binder to the APS3v HLA-DR among all peptides tested (Figure 2), and similarly it also elicited the highest T-cell proliferative and cytokine responses (Figures 4A and 5A). The autoantigenicity of this TPO epitope is certainly worth further investigation in both AITD and APS3v. Intriguingly, none of the TSHR peptides that we tested was found to bind to the APS3v HLA-DR. Indeed, in most APS3v patients AITD manifests as HT and not GD [2] and this may be due to the lack of binding of TSHR peptides to the APS3v HLA-DR.

Among the ten GAD65 peptides we tested, GAD.378 and GAD.492 were found to bind to APS3v HLA-DR, although with different affinities. Interestingly, GAD.492 induced T-cell

response (both T-cell proliferation and cytokine response), but not GAD.378, even though GAD.378 showed much stronger binding to the APS3v HLA-DR than GAD.492. A potential explanation is that strong binding affinity to the APS3v HLA-DR could induce tolerance. Similar to TPO epitopes in AITD, GAD65 epitopes are very diverse in T1D [37], and a number of GAD65 epitopes were identified using different strategies [38–43]. This diversity of GAD65 epitopes identified in T1D is in contrast to APS3v where only one epitope, GAD.492 was identified. The fact that the Tg, TPO, and GAD65 epitopes identified in APS3v are more limited than those in AITD and T1D, respectively leads us to conclude that APS3v might have a more restricted autoreactive T-cell repertoire than AITD and T1D individually. This is strengthened by our observation that none out of the six insulin peptides we tested induce T-cell response.

We have shown that the major peptides triggering activation of autoreactive T-cells causing APS3v are derived from different antigens, namely Tg, TPO and GAD65. Therefore, an approach to target the HLA pocket predisposing to APS3v for therapeutic purpose would be more advantageous than targeting specific peptides. Non-peptidic small organic molecules targeting susceptible HLA alleles and blocking T-cell responses have recently shown promise as a potential new therapeutic approach in autoimmunity [28, 44–47]. Our lab has identified a small molecule inhibitor, Cepharanthine, that can block T-cell activation by Tg peptides within the HLA-DR β 1-Arg74 pocket [28]. For the first time we identified a small molecule that can block Tg peptide binding and presentation to T-cells in autoimmune thyroiditis. In view of our success in discovering a small molecule inhibitor in AITD, we believe that a similar strategy can be applied to screen for small molecule inhibitors that can block the APS3v HLA-DR pocket in order to treat or prevent APS3v.

In summary, the HLA-DR3 pocket confers the strongest susceptibility to APS3v. Our data support the hypothesis that this APS3v-specific HLA-DR pocket is a flexible pocket that can present both thyroid and islet peptides, thus conferring susceptibility to both AITD and T1D. Tg.1571, TPO.758, TPO.338 and GAD.492 are the major epitopes triggering APS3v. These data set the stage for development of novel treatment strategies in APS3v targeting the APS3v HLA-DR pocket by small molecule inhibitors or other molecules such as peptide aptamers.

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Highlights

- APS3v refers to the occurrence of AITD+T1D in the same individual
- A unique HLA-DR pocket predisposes to APS3v
- Both islet & thyroid peptides were presented to T-cells by the APS3v-DR pocket
- Flexible APS3v HLA-DR pocket triggers autoimmune T-cell responses in AITD+T1D
- The unique APS3v HLA-DR pocket is thus an attractive drug target

Fig. 1.

Diagram depicting the APS3v HLA-DR protein construct we produced. The α chain construct contained the DR α chain fused to the coiled coil region of the basic Leucine zipper domain of Fos. The β chain construct contained the coiled coil region of the basic Leucine zipper domain of JunB. The Jun and Fos dimerization motifs allowed the protein to dimerize to form the APS3v HLA-DR protein. The α chain has a His₆ tag while the β chain has a V5 epitope tag for purification purposes.

Fig. 2.

In vitro binding results of peptide binding to APS3v HLA-DR. 5 Tg, 2 TSHR, 6 Ins, 10 GAD65 and 13 TPO peptides were tested for binding to APS3v HLA-DR protein. APO was used as a positive control and scr2098 was used as a negative control. Peptides binding with greater than 2 fold (>10 standard deviations over the average for peptide alone with no HLA) were considered as positive and are indicated with black arrows.

Fig. 3.

A representative structure of the interaction between APS3v and TPO.758, the strongest binding peptide. The surface of the proximal residues to the peptide is colored by the electrostatic potential – red represent negative and blue positive potential. The binding pockets P1, P4, P6 and P9 are noted to highlight the mode of peptide binding. P1 is filled with the residue Leu and P9 with Tyr. Pockets P4, P5 and P6 create a polar arrangement characterized by positive potential from Arg-β74 near Ser₄ and opposing negative potential due to Glu-α11, Asp-α66 and Glu-β9 next to Arg₆.

Fig. 4. T-cell proliferative responses to stimulation of peptides. **(A)** Average proliferation indexes of T-cells to stimulation with 2 Tg peptides, 1 Ins peptide, 2 GAD65 peptides and 6 TPO peptides, when mice were immunized with the respective peptides. Stimulation with a scrambled Tg peptide was used as negative control (NC), and stimulation with CD3/CD28 beads was used as positive control. Proliferation index 1.5 was considered as positive response to the stimulant as previously described [26]. Black arrow indicates stimulation index 1.5. **(B)** Average proliferation indexes of T-cells to stimulation with Tg.1571, GAD.492, TPO.758 and TPO.338 (which were identified as positive peptides) when mice were immunized with PBS. Stimulation with a scrambled Tg peptide was used as negative control (NC), and stimulation with CD3/CD28 beads was used as positive control. Proliferation index 1.5 was considered as positive response to the stimulant.

Fig. 5.

Concentration of IFN- γ secreted by T-cells. **(A)** Concentration of IFN- γ secreted by T-cells of peptide-immunized mice in response to stimulation with the peptide immunized; NC, a scrambled Tg peptide; positive control, stimulation with CD3/CD28 beads. Four peptides showing the highest IFN- γ production (>1000 pg/ml, more than 2.5 standard deviations over the average of the negative control) were indicated by black arrows. **(B)** Concentration of IFN- γ secreted by T-cells of PBS-immunized mice in response to stimulation with the four positive peptides identified. NC, a scrambled Tg peptide; positive control, stimulation with CD3/CD28 beads.

Fig. 6.

Two potential explanations by which HLA class II molecules can predispose to both AITD and T1D. First hypothesis (**A**), alleles predisposing to AITD and T1D are distinct but in tight LD. Two distinct pocket structures present thyroid and islet peptides respectively and are expressed on APCs together. Second hypothesis (**B**), APS3v HLA-DR pocket on APCs is flexible to accommodate both thyroid and islet peptides and present them to T-cells for immune response.

Table 1

Sequences of peptides tested in vitro for binding to APS3v HLA-DR.

Peptide	Sequence
Tg.2098	LSSVVVDPSIRHFDV
Tg.726	CPTPCQLQAEQAFLRTV
Tg.1951	FRKKVILEDKVKNF
Tg.1571	EKVPESKVIFDANAPVAVRSKVPDSEF
Tg.202	VNTTDMMIFDLVHSYNRFPD
TSHR.132	GIFNTGLKMFDPDLTKVYST
TSHR.197	FNGTKLDAVYLNKNKYLTVI
Ins.B9-23	SHLVEALYLVCGERG
Ins.44	GERGFFYTPKTR
Ins.25	FVNQHLCGSHLVEA
Ins.76	PGAGSLQPLALEGSLQKRG
Ins.89	RGIVEQCCTSICSL
Ins.98	SICSLYQLENYCN
GAD.378	SRKHKWKLSGVERANSVTW
GAD.492	REGYEMVFDGKQHTNVCF
GAD.116	QDVMNILLQYVVKSFDRST
GAD.247	NMYAMMIARFKMFPEVKEKG
GAD.260	PEVKEKGMAALPRLIAFTSE
GAD.274	IAFTSEHSHFSLK
GAD.379	WKLSGVERANSV
GAD.555	NFFRMVISNPAAT
GAD.192	AADWLTSTANTNMFT
GAD.456	WLMWRAKGTTGFEAH
TPO.763	SGRRVLVYSCRHGYELQGR
TPO.489	AAFRFGHATHPLVRRLDASFQEH
TPO.122	ALSEDLLSIANMSGCLPY
TPO.28	SRGKELLWGKPEESRVSSV
TPO.423	ALNAHWSADAVYQEARKVV
TPO.758	ESGRRVLVYSCRHGYELQG
TPO.405	ALHTLWLREHNRLAAALKA
TPO.338	RQLRNWTSAEGLLRVHARL
TPO.513	DLPGLWLHQAFFSPWTLLR
TPO.589	PGYNEWREFCGLPRLETPA
TPO.161	RDHPRWGASNTALARWLPP
TPO.344	TSAEGLLRVHARLRDGRA
TPO.351	RVHARLRDGRAYLPFVPP
APO (positive control)	IPDNLFLKSDGRIKYTLNK
scr2098 (negative control)	HDLFSRIDSSVVVVP

Table 2

Decomposition analysis of the core sequence (Seq.) of peptides that bind to APS3v. Numerical values for each residue are interaction energies (Int. E) between the residue and the APS3v protein (in kcal/mol).

P	Peptides that bind and activate										Peptides that bind only									
	Tg.1571		GAD.492		TPO.758		TPO.338		GAD.378		TPO.161		Tg.1951		Predicted Optimal Binding Sequence					
	Seq.	Int. E	Seq.	Int. E	Seq.	Int. E	Seq.	Int. E	Seq.	Int. E	Seq.	Int. E	Seq.	Int. E	Seq.	Int. E	Seq.	Int. E	Seq.	Int. E
1	VAL	-6.65	MET	-7.93	LEU	-8.06	TRP	-10.91	TRP	-9.95	TRP	-9.97	ILE	-9.32	TRP		TRP		TRP	-10.91
2	ILE	-5.09	VAL	-4.45	VAL	-4.93	THR	-2.78	LYS	-1.90	GLY	-1.60	LEU	-5.17	LEU		LEU		LEU	-5.17
3	PHE	-5.81	PHE	-5.27	TYR	-3.89	SER	-0.02	LEU	-4.25	ALA	-3.88	GLU	-0.08	PHE		PHE		PHE	-5.81
4	ASP	-7.77	ASP	-2.19	SER	-5.28	ALA	-1.60	SER	-4.75	SER	-8.16	ASP	-8.69	ASP		ASP		ASP	-8.69
5	ALA	-2.53	GLY	-0.02	CYS	-7.27	GLU	0.59	GLY	-1.08	ASN	-7.17	LYS	-0.53	CYS		CYS		CYS	-7.27
6	ASN	-2.05	LYS	-11.23	ARG	-12.32	GLY	0.07	VAL	-5.95	THR	-5.15	VAL	-3.84	ARG		ARG		ARG	-12.32
7	ALA	-4.66	PRO	-4.35	HIS	-2.51	LEU	-5.00	GLU	-2.56	ALA	-3.80	LYS	-0.02	LEU		LEU		LEU	-5.00
8	PRO	-3.84	GLN	-1.62	GLY	-0.36	LEU	-4.14	ARG	-1.29	LEU	-4.56	ASN	-3.50	LEU		LEU		LEU	-4.56
9	VAL	-6.52	HIS	-5.35	TYR	-9.05	ARG	-6.91	ALA	-4.42	ALA	-2.86	PHE	-3.94	TYR		TYR		TYR	-9.05