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Epitope Recognition in HLA-DR3 Transgenic Mice Immunized to TSH-R Protein or Peptides

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1 **Title**

2 EPI TOPE RECOGNITION IN HLA-DR3 TRANSGENIC MICE IMMUNIZED TO TSH-R
3 PROTEIN OR PEPTIDES

4

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14 **Abbreviated title**

15 TSH-R epitope in HLA-DR3 transgenic mice

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19 **Key words**

20 epitope, autoimmune thyroid disease, thyrotropin receptor, HLA-DR3

21 **Disclosure Statement**

22 The authors declare that no competing financial interests exist.

1 **Abstract**

2 Development of autoimmune thyroid disease (AITD) including Graves' disease (GD) and
3 Hashimoto's thyroiditis (HT) is related to expression of HLA-DRB1*0301(DR3). The
4 extracellular domain (ECD) of human TSH receptor (hTSH-R) is a crucial antigen in GD. hTSH-
5 R peptide 37 (AA 78-94) is an important immunogenic peptide in DR3 transgenic mice.

6 In the current study, DR3 transgenic mice were immunized to recombinant hTSH-R-ECD
7 protein or peptides. Serum anti hTSH-R protein antibody, or anti hTSH-R peptide antibodies were
8 titrated by ELISA. Further, a mutant hTSH-R 37 (ISRIYVSIDATLSQLES: 37m), in which DR3
9 binding motif position 5 was mutated V>A, and position 8 Q>S, was synthesized. 37m was
10 predicted to bind to HLA-DR3, but not bind to T-cell receptors. DR3 transgenic mice were
11 immunized to hTSH-R 37 and 37m.

12 Mice immunized to hTSH-R-ECD protein developed strong anti hTSH-R antibody, and
13 the antisera reacted strongly with hTSH-R peptides 1 to 5 (AA 20-94), sequences recognized as B-
14 cell epitopes that reacted with human TRAb. In addition, the antisera recognized hTSH-R
15 peptides 21(258-277), 41(283-297), 36(376-389), and 31(399-418). Strikingly, antisera raised to
16 hTSH-R peptide 37 bound hTSH-R peptides 1-7(AA 20-112), 10(132-50), 33(137-150), 41,
17 23(286-305), 24(301-320), 36, and 31, as well as to hTSH-R-ECD protein. Both antibody titers to
18 hTSH-R 37, and reaction of splenocytes to hTSH-R 37, were significantly reduced in mice
19 immunized to hTSH-R 37 plus 37m, compare to mice immunized to hTSH-R 37 alone.

20 Binding of anti hTSH-R antibodies to the amino-terminal end of the ECD was confirmed in
21 our DR3 transgenic mice. The ability of immunization to a single peptide to induce antibodies that
22 bind hTSH-R-ECD protein, as well as multiple unrelated peptides, is a unique observation. The
23 mechanism may relate to TSH-R epitope spreading. Immunogenic reaction to hTSH-R peptide 37
24 could be partially suppressed by 37m, and this might contribute to immunotherapy of AITD.

1 **Introduction**

2 Autoimmune thyroid disease (AITD) is an organ specific autoimmune disease. Graves'
3 disease (GD) and Hashimoto's thyroiditis (HT) are the major components of AITD. Thyrotropin
4 receptor (TSH-R) is one of the well-known target antigens in GD, and also often serves as antigen
5 in HT(1). Ectodomain of TSH-R (TSH-R-ECD) mediates various immune responses in the
6 development of GD (2), and multiple epitopes are thought to exist(3-6).

7 Shed TSH-R-ECD is endocytosed into antigen presenting cells (APCs), and processed to be
8 presented on the surface of APCs as HLA-DR-epitope complexes. These complexes
9 are recognized by CD4⁺ T-cells and positive signals for B-cell proliferation are transmitted.
10 Subsequently, anti-TSH-R antibody is secreted by B-cells. Hyperthyroidism is induced by anti
11 TSH-R antibody that stimulates TSH-R. HLA-DRB1*0301(DR3)(7), CTLA-4(8), TSH-R(9),
12 FCRL3(10), PTPN22(11), and other genes were found to be associated with GD. In addition, HD
13 is also related to inheritance of HLA-DR3(7).

14 In our previous study, hTSH-R peptide 37 (AA78-94: ISRIYVSIDVTLQQLES) was
15 demonstrated to be one of the strongest T-cell epitopes in HLA-DR3 transgenic mice, and was an
16 important epitope in the early phase of the immunogenic reaction (5), however, little information
17 is known about B-cell epitopes using DR3 transgenic mice immunized to hTSH-R-ECD protein or
18 peptides.

19 In an HLA molecule, the anchoring side chains of the peptides (positions 1, 4, 6, 7, and 9)
20 bind in the HLA-DR binding groove. Side chains of amino acids in positions 2, 3, 5, and 8 are
21 believed to contact the T cell receptor (TCR), activate CD4⁺ T-cells, and eventually B-cells are
22 driven to produce antibodies (12).

23 Many studies showed immune suppression by using altered ligand peptides (ALP) in
24 autoimmune diseases. For example, mutated MBP peptides were found to antagonize T-cell

1 reactions in Multiple sclerosis (13). To induce tolerance of CD4⁺T-cells by attenuating binding to
2 TCR, we designed a mutated hTSH-R peptide 37 (ISRIYVSIDATLSQLES: 37m) using a computer
3 algorithm. In 37m, amino acids in position 5 and 8 were altered from those in hTSH-R peptide 37
4 (ISRIYVSIDVTLQQLES)(6). 37m was expected to bind to HLA-DR3 firmly, but not bind well
5 to TCR.

6 In the current study, 1) we demonstrate distributions and characteristics of anti-hTSH-R-
7 ECD antibodies in DR3 transgenic mice immunized to hTSH-R-ECD protein or peptides, 2) and we
8 demonstrate inhibitory effects of mutant hTSH-R peptide to B-cell and T-cell reactions. We seek
9 the possibility of an immunological treatment for GD, based on epitope recognition in DR3
10 transgenic mice.

1 **Materials and Methods**

2 Mice transgenic for HLA-DR3

3 Mice transgenic for HLA-DR3 were generated by Dr. Chella David of the Mayo Clinic (14
4), and supplied through his courtesy. The mice have approximately 75% of Black/6, and 10% of
5 CBA and Black 10 genes. Mice PBMC were stained with FITC-labeled anti-HLA-DR antibody
6 (BD Bioscience, San Jose, CA). Mice expressing HLA-DR on more than 20% on their PBMC were
7 used for studies. All studies were performed under a protocol approved by the Institutional
8 Animal Care Committee (IACUC)

9 Peptide synthesis

10 Forty-one 14-20mer hTSH-R-ECD peptides were synthesized as previously described (6).
11 Peptides 32-41 were predicted to have high binding affinity to HLA-DR3 or multiple HLA-DRs,
12 based on EpiMatrix program analysis(4,6). In addition, a mutated hTSH-R-ECD peptide 37,
13 named 37m (ISRIYVSIDATLSQLES) was generated. This peptide has two sites of mutation
14 (peptide-DR3 binding position 5 :V to A,and 8: Q to S) intended to diminish the peptide binding
15 affinity to TCR(Table 1). The sequences of all peptides and purity were confirmed by reverse
16 phase HPLC, and purity was 90-95%.

17 Preparation of human recombinant hTSH-R-ECD protein

18 hTSH-R-ECD protein was generated from recombinant baculovirus (5)(Chesapeake
19 Protein Expression and Recovery Labs, Savage, MD). hTSH-R 19-417 cDNA was sequenced, and
20 cloned into baculovirus protein expression system. Trichoplusia ni (cabbage looper caterpillar)
21 were infected with the recombinant virus. After 96 hours (in 26C and 70% humidity), larvae were
22 harvested, and recombinant hTSH-R-ECD was purified through immobilized nickel affinity
23 chromatography, and dialyzed against phosphate-buffered saline. Protein purity was analyzed by
24 SDS-PAGE electrophoresis as a single 55kD band. Average yield was 0.5 mg/dl in PBS. Protein

1 concentration was measured by using BCA protein assay kit (Thermo Scientific, Rockford, IL).

2 Immunization of DR3 transgenic mice to hTSH-R-ECD protein / peptides

3 For immunization with hTSH-R-ECD protein (N=11), 25 ug of protein was emulsified in
4 100ul of complete Freund's adjuvant (CFA, Sigma, St. Louis, MO), and given at tail base on day 0,
5 followed by three sc injections of protein in 100ul of incomplete Freund's adjuvant (IFA) on days 7,
6 14, and 21 in groin. hTSH-R-ECD peptide immunization was performed using 25 ug of each
7 peptides in the same intervals and methods. Mice were killed on day 28 (5). hTSH-R peptide
8 37(high immunogenicity, N=6), or peptide 32(AA105-118)(low immunogenicity, N=4)(4) was
9 used for immunization, and the same amount of PBS emulsified in CFA or IFA (N=7) was used as a
10 control immunization in the same intervals and methods.

11 Inhibition of B-cell and T-cell immune response by mutant peptide

12 DR3 transgenic mice were divided into 5 groups as follows. Each group consisted of 4
13 mice. The mice were immunized at the same intervals as in the experiment above. First
14 immunization was injected at tail base, others sc in groin. First dose was emulsified in 100 ul of
15 CFA, and others in the same amounts of IFA: Group A), Immunize mice with 25ug of hTSH-R-
16 ECD protein: Group B), Immunize mice with 25ug of hTSH-R-ECD protein + 25ug of 37m: Group
17 C), Immunize mice with 25ug of hTSH-R peptide 37: Group D), Immunize mice with 25ug of
18 hTSH-R peptide 37 + 25ug of 37m: Group E), Immunize mice with 25ug of 37m.

19 Cell proliferation assay

20 10^5 splenocytes were incubated in each well of round bottom microplates with or without
21 10 ug/ml antigens. 1uCi of [3 H]-thymidine was added to each well. After 72 hours incubation,
22 [3 H]-thymidine incorporation was measured by liquid scintillation counting. Results were
23 expressed as a stimulation index; the ratio of [3 H]-thymidine uptake in samples with antigen, to
24 samples incubated without antigen. Various concentration of hTSH-R 37 peptide were added to

1 observe response of splenocytes from immunized mice. All assays were done in triplicate for each
2 epitope studied. Data presented in figures are average stimulation index (SI) of all mice in the
3 group for each epitope. Experiments were repeated 3 times, and representative studies are
4 presented.

5 Thyroid function tests

6 Serum total T4 and TSH concentrations were measured as previously described (15). Briefly,
7 Serum total T4 concentration were measured with radioimmunoassay(RIA)(Diagnostic Products,
8 Los Angeles, CA) using 25µl mouse serum. Serum TSH concentration was assayed using a double
9 antibody precipitation RIA in 50 µl serum.

10 ELISA of mouse sera

11 Forty-one peptides covering the entire hTSH-R-ECD were used to coat 96 well plates using
12 1 ul of 5 ug/ml solution diluted to 50 ul in PBS-0.1% TritonX-100, each in triplicate (4-6).
13 hTSH-R-ECD protein was diluted in the same buffer as hTSH-R-ECD peptides and used to coat 96
14 well plates (250 ng/50 ul/ well) overnight at 4C. After washing with washing buffer (PBS-0.05%
15 Tween 20), the plates were blocked with 5% BSA in PBS at RT. Sera were diluted to 1:1000 in
16 PBS, 0.05% Tween 20, 3% BSA, and 1mM EDTA, and 50ul of aliquots were added to each well.
17 After incubation for 3 hour at RT, the plates were washed with washing buffer, and 50 ul of a
18 1:1000 dilution of peroxidase-labeled goat anti-mouse Fc were added to each well. After 1 hour at
19 RT, the wells were washed, and 75 ug o-phenylenediamine dihydrochloride-H₂O₂ solution was
20 added as substrate. The reactions were stopped with 50ul of 1M H₂SO₄, and absorbance at 450nm
21 was determined.

22 For peptide inhibition assays, 1 ul aliquots from 5mg/ml stock of each peptide was added
23 to the diluted sera during incubation on the antigen-coated plate. The same amount of hTSH-R-
24 ECD protein was used in the blocking experiments. As a control, PBS with no antigen was used to

1 coat the ELISA plate (Fig.2).

2 Statistical analysis

3 For analysis of comparison with each experiment in ELISA (Fig1, Fig.2) or T-cell
4 stimulation test (Fig.3), Student's t-test was conducted. In each test, p-value was examined, and p-
5 value <0.05 was indicated as a significant value.

1 **Results**

2 DR3 transgenic mice immunized to recombinant hTSH-R-ECD protein or peptides were
3 generated as an AITD model. Serum anti hTSH-R-ECD protein antibody, or anti hTSH-R-ECD
4 peptide antibodies were titrated by ELISA to recognize B cell epitopes. Serum T4 and TSH value
5 were measured as thyroid function tests for these mice. To evaluate inhibition of T-cell response
6 induced by 37m, mice splenocytes were prepared, and cultured with peptide antigens in vitro.

7 Anti-hTSH-R-ECD antibodies from DR3 transgenic mice immunized to hTSH-R-ECD protein

8 DR3 transgenic mice were immunized to hTSH-R-ECD protein, to induce immunity to
9 hTSH-R antigen (Fig.1A). After 4 times immunization in CFA or IFA, 63.6% of mice developed
10 anti-hTSH-R-ECD antibody measured on ELISA, although thyroid function was mostly normal
11 (Table 2). Average serum TSH value (73.09+-55.05 mIU/l, normal range: 18-180) was not
12 suppressed, and T4 concentration (4.77+-0.83 ug/dl, normal range: 3.5-5.5) was not elevated.

13 Antisera from the mice reacted strongly with hTSH-R-ECD peptides 1 to 5, covering amino
14 acid residues 20-94, as shown in (Fig.1A). In addition, hTSH-R-ECD peptides 21 (258-277), 41
15 (283-297), 36 (376-389), and 31 (399-418) bound the antisera. Inhibition of antibody binding to
16 peptides by hTSH-R-ECD protein, the immunizing protein, was not obtained for binding in the
17 amino-terminal peptides, perhaps because of relatively insufficient amounts of hTSH-R-ECD were
18 used to compete with strong antibody binding. In contrast, added hTSH-R-ECD protein
19 significantly inhibited binding of antisera to the carboxy-terminal peptides 36(376-389) and 31(399-
20 418). Binding of antisera to hTSH-R peptide 21(258-277) was not inhibited by immunizing antigen.
21 As expected, adding the same peptide used for coating the wells effectively inhibited binding of the
22 antibody to all peptides.

23 Anti-hTSH-R-ECD antibodies from DR3 transgenic mice immunized to hTSH-R-ECD peptides

24

1 Then HLA-DR3 transgenic mice were immunized to hTSH-R peptide 37, 50% of mice
2 developed anti-hTSH-R-ECD antibody measured on ELISA. Serum TSH value (47.0+-5.29 mIU/l)
3 and T4 concentration (4.19+-0.22 ug/dl) were within normal ranges. Antisera raised to hTSH-R
4 peptide 37 (AA 78-94) provided striking results (Fig.1B), with strong binding to the same peptides
5 to which hTSH-R-ECD protein antisera bound, and in addition to a more extensive set of peptides.
6 The antisera bound to hTSH-R peptides 1-7 with AA 20-112, peptide 10 (132-150), 33 (137-150),
7 41 (283-297), 23 (286-305), 24 (301-320), 29 (376-389), and 31 (399-418), as well as to hTSH-R-
8 ECD. hTSH-R-ECD protein inhibited the responses significantly (30-70%). Binding to peptides
9 was mildly inhibited by adding the same peptide to the wells, except for complete inhibition of
10 binding to hTSH-R peptide 37.

11 When DR3 transgenic mice were immunized to hTSH-R peptide 32, a less immunogenic
12 peptide, 50% of mice developed anti-hTSH-R-ECD antibody measured on ELISA (Table 2).
13 Serum TSH values (66.5+-19.84 mIU/l) and T4 concentrations (3.54+-0.76 ug/dl) were normal. The
14 mice developed similar but weaker responses to multiple peptides (1, 2, 3, 4, 5, 32, 13, 21, 34, 35,
15 and 36), many the same as with hTSH-R peptide 37 (Fig.1C).

16 We also checked antibody response by using non-hTSH-R peptides, and none of the
17 peptides bound anti hTSH-R peptides or protein antibodies. Further, cross reaction (one peptide
18 inhibiting binding to a different peptide) also was not seen. In PBS immunized mice, antisera did
19 not react with hTSH-R-ECD protein or peptides (Fig.1D). Serum TSH value (96.42+-52.35 mIU/l)
20 and T4 concentration (4.82+-0.31 ug/dl) were normal in these mice.

21 We compared the number of sequence differences between mice / human peptides to
22 ELISA OD450nm values, and no correlation was found in any group of mice (data not shown).

23 Effect of peptide 37m on immunity induced by hTSH-R-ECD or hTSH-R 37

24 hTSH-R peptide 37 (AA 78-94) is an important immunogenic peptide in DR3 transgenic

1 mice (4-6). Based on an informatics analysis we have identified the 9-mer frame at amino acid
2 position 83 as the most likely restricting element. In order to test our theory we designed a mutant
3 peptide which retains its specificity for DR3 but which lacks the outward facing contour recognized
4 by T cells reacting to hTSH-R peptide 37. The mutant peptide (ISRIYVSIDATLSQLES: 37m) has
5 been modified at positions 87 (V87A) and 90 (Q90S); TCR facing positions 5 and 8 within the
6 restricting 9-mer. In both cases the native amino acid was replaced by another amino acid having
7 similar chemical properties but less bulk.

8 37m was predicted to bind to HLA-DR3, but not bind to TCR. To confirm binding
9 affinity, EpiMatrix computer algorithm predicted a binding z-score of 37m ($z=2.05$) to DR3, the
10 same as hTSH-R peptide 37. Further, in vitro peptide binding assay using our standard method (4
11), showed IC_{50} of 37m to DR3 was 0.3 μ M and similar to IC_{50} of hTSH-R peptide 37.

12 Anti hTSH-R protein antibody and anti hTSH-R peptide 37 antibodies were measured with
13 ELISA (Fig.2). Co immunization of 37m with hTSH-R peptide 37 did not inhibit development of
14 antibodies reacting to hTSH-R-ECD). Mice immunized to hTSH-R peptide 37 produced anti
15 hTSH-R peptide 37 antibody, however, mice immunized with hTSH-R peptide 37 and 37m did not
16 develop anti hTSH-R peptide 37 antibody ($P<0.01$). No mouse in any group, including mice
17 immunized only to 37m, developed antibody for 37m.

18 We also evaluated T-cell responses in splenocytes of DR3 transgenic mice immunized with
19 hTSH-R-ECD protein or hTSH-R-ECD peptides by proliferation assays (Fig.3). Reaction of
20 splenocyte to hTSH-R peptide 37 was not inhibited in mice immunized to hTSH-R-ECD protein
21 together with 37m, or mice immunized to hTSH-R-ECD (NS). Reaction of splenocytes to hTSH-
22 R peptide 37 was significantly reduced in mice co-immunized to hTSH-R peptide 37 plus 37m,
23 compare to mice immunized to hTSH-R peptide 37 alone, when using 10 μ g of antigen (Fig.3C)(SI:
24 $P<0.01$). Mice immunized to 37m did not produce significant reaction.

1 **Discussion**

2 The etiologies of AITD including GD and HT have been extensively explored, and the
3 relationship to HLA-DR and TSH-R has been identified (1,7). HLA-DR3 has an important
4 immunological role predisposing to both GD and HT (7). Although many studies have been done
5 on conformational and linear epitopes of anti-hTSH-R antibody (16-24), no studies have identified
6 B-cell epitopes of TSH-R in relation to HLA-DR3. In this study, we have examined the B-cell
7 immune response of HLA-DR3 transgenic mice immunized to hTSH-R-ECD or peptides. B-cell
8 epitopes appearing predominantly on N-terminus of hTSH-R in the mice were quite similar to those
9 of non-DR transgenic mice. Further, a mutated hTSH-R peptide 37 was generated, and by using
10 the mutated peptide together with immunonegic hTSH-R peptide 37, a partially effective immuno
11 suppression of B-cell and T-cell responses was obtained.

12 Thyroid function tests

13 Based on the results of TSH and T4 assays, thyroid function was not changed in any group
14 (Table 2), and TSH levels were not correlated with anti-hTSH-R antibody titers.

15 Immunological response of DR3 mice to hTSH-R-ECD protein or peptides

16 Our results using DR3 transgenic mice are confirmatory of anti-TSH-R antibody
17 preferentially binding to the N-terminus of hTSH-R (16-18)(Fig.1A-C). We previously reported
18 development of TSAb in mice immunized to hTSH-R-ECD peptides (19). Endo, et al. also
19 confirmed production of TSAb in rabbits immunized to N-terminus of hTSH-R peptide (16).
20 Recently, Chazenbalk reported that hTSH-R A subunit (17-228), not whole hTSH-R, is
21 preferentially recognized by TSAb in GD patients (20). In some mouse models of GD,
22 immunization with hTSH-R A subunit effectively induce hyperthyroidism (21). These reports
23 indicate that hTSH-R A subunit, especially N-terminus region, contains immunogenic sequences for
24 production of TSAb. In the current study, DR3 transgenic mice immunized to hTSH-R-ECD protein

1 produced anti-hTSH-R antibody preferentially bound to hTSH-R amino acids 20-94(Fig.1A),
2 reported as B-cell epitopes that react with TSAAb (16,17), however, thyroid function was normal
3 (Table 2). This suggested that the antibodies were primarily binding but not stimulatory. hTSH-R
4 peptide 21 (22), and peptides in the carboxy-terminal portion of the ECD (23,24) were other B-cell
5 epitopes in these mice, containing the sequences of binding site forTSBAb/TSH.

6 hTSH-R-ECD protein, the immunizing protein, did not inhibit binding of anti hTSH-R-
7 ECD peptides antibodies for binding in the amino-terminal peptides, perhaps because of low cross-
8 reaction to the antibodies against each peptide, or an insufficient amount of the competing antigen.
9 In contrast, for reasons unknown, added hTSH-R-ECD protein strongly inhibited binding of anti-
10 hTSH-R peptides in carboxy-terminal antibody in antisera from hTSH-R-ECD immunized mice,
11 and binding of the antibody to hTSH-R peptide 21(258-277) was increased in the presence of
12 adding hTSH-R-ECD protein. Biological significance of antisera binding is shown by the fact that
13 all responses to epitopes were strongly inhibited by adding the same peptide to the wells (Fig.1A).

14 Antisera raised to hTSH-R peptide 37 showed binding to the same peptides to which hTSH-
15 R-ECD protein antisera bound, and also unrelated hTSH-R peptides (Fig.1B). Comparing
16 antisera for mice immunized to hTSH-R-ECD, to those immunized to hTSH-R peptide 37, (Fig.1B),
17 antibody to hTSH-R peptide 21 was seen only in hTSH-R-ECD protein immunized mice. hTSH-
18 R-ECD protein inhibited the responses significantly (approximately 30-70%), indicating reaction of
19 anti-peptide antibody to hTSH-R protein. Binding to peptides by anti hTSH-R peptide 37 antisera
20 was not inhibited by adding the same peptide to the wells, except for complete inhibition of binding
21 to hTSH-R peptide 37. This probably relates to differences in affinities of the antisera to the
22 individual peptide linear antigens.

23 It is difficult to explain with certainty why hTSH-R peptide 37 induced antibodies reacting
24 with multiple non-related TSH-R peptides. We hypothesize that the immunization induced

1 antibodies reacting with host (mouse) TSH-R, which then led to antisera binding to a broad
2 spectrum of mTSH-R epitopes, which overlapped binding with anti-hTSH-R-ECD responses.
3 Otherwise it is difficult to understand how the mice could have developed responses to TSH-R
4 peptides to which the animals were not exposed. Antisera of mice immunized to hTSH-R peptide
5 32 reacted weakly to multiple hTSH-R peptides(Fig.1C), supporting the idea that hTSH-R peptide
6 32 is a low immunogenic peptide(4-6).

7 hTSH-R 317-366 is reported to be excised during secretion of hTSH-R A subunit into
8 circulation (18,21). None of mice in the the different immunization groups produced antibodies to
9 this region, perhaps because of low immunogenicity of the section.

10 The amino acid residue homology between mouse TSH-R and human TSH-R is 86.25%.
11 Among our synthesized hTSH-R peptides, peptide 13, 19, 21, 22, 23, 34, 40, and 41 have identical
12 amino acid sequences to mouse TSH-R. Other hTSH-R peptides have one or more differences
13 from mouse TSH-R sequences (Table 1). We compared the number of differences between mice /
14 human peptides to ELISA OD450nm values, and no correlation was found in any mice, suggesting
15 no relationship between the difference in species and the ability to produce antibodies (data not
16 shown).

17 Flynn et al. found that HLA-DR3 transgenic mice became hyperthyroid and showed
18 thyroiditis after injection of hTSH-R-ECD DNA (14). Pichurin et al., showed that hTSH-R
19 amino acids 142-161 possibly include a major T cell epitope in DR3 transgenic mice immunized to
20 adenovirus coding hTSH-R A-subunit (25). In our study, hTSH-R 37 (AA 78-94) was indicated
21 as one of the strongest epitopes. The reason for differences of T-cell response among studies is
22 not known. Other reports show a change of thyroid function due to transition of stimulation to
23 blocking anti TSH-R antibodies (26), as is seen clinically in patients with AITD. Thus, thyroid
24 function in these mice may change in the course in our study.

1 Effect of peptide 37m on immunological responses induced by hTSH-R peptide 37

2 In the experiment using 37m, production of anti hTSH-R antibody in the mice immunized
3 to hTSH-R-ECD protein was not suppressed by the presence of 37m (Fig.2). However, anti-
4 hTSH-R peptide 37 antibody induced by hTSH-R peptide 37 immunization was significantly
5 inhibited when immunized together with 37m (Fig.2). This suggested that B-cell function induced
6 with hTSH-R peptide 37 was partially suppressed, by competitive binding of 37m to HLA-DR3,
7 and thus reducing presentation of peptide 37 to TCR.

8 In our current study, splenocytes from mice immunized to hTSH-R-ECD reacted to hTSH-
9 R-ECD, and the reaction was not inhibited with addition of 37m (Fig.3). In contrast, splenocyte
10 reaction to hTSH-R peptide 37 from mice immunized to hTSH-R peptide 37 was significantly
11 reduced with addition of 37m (Fig.3). The reason for this is considered to be an effect of
12 competitive binding inhibition of 37m described above. This fact supports another study using
13 mutant peptides having inhibitory effect for cell proliferation (13).

14 In our previous report on AITD, hTSHR-ECD induced T-cell response strongly to hTSH-R
15 peptide 37 and weakly to hTSH-R peptide 41 by 2 weeks (5). Further, 4 weeks after
16 immunization, B-cell produced antibodies to multiple hTSH-R peptides (Fig.1A), while T-cells
17 responded preferentially to hTSH-R peptide 37 (5). This suggests that B-cell epitope spreading
18 progressed more rapidly than T-cell epitope spreading. Similarly, hTSH-R peptide 37
19 immunization induced T-cell response only to hTSH-R peptide 37 in 4 weeks(5), but drove B-cell
20 to produce antibodies to multiple peptides (Fig.1B). This also supports the idea that B-cell epitope
21 spreading occurs earlier than T-cell epitope spreading in this study.

22 Usually linear B-cell epitopes are quite distinct from conformational epitope (27). We
23 have demonstrated only linear B-cell epitope for hTSH-R-ECD protein and peptides, so further
24 studies are required to elucidate conformational and functional B-cell epitopes.

1 In thymus, T-cells are selected through positive selection and negative selection (28).
2 Then progenitor T cells grow acquiring cell surface markers (CD4, CD8, or CD25), and TCR are
3 rearranged. Once naïve T-cell meets epitope, by interaction of TCR+TSH-R epitope+HLA-DR
4 complex, it begins to proliferate. Then effector cells (Th1, Th2, or Th17), or regulatory T cells
5 differentiate. Our results suggest that in the early phase of immunological reaction, mutant hTSH-
6 R-ECD peptide may bind to HLA-DR, effectively inhibiting T-cell signals. In other words, when
7 certain HLA-DR is saturated with bound mutant peptides, programmed (epitope-sensitized)
8 immunogenic T-cell cannot receive positive signals. Taken together, administration of the various
9 mutant hTSH-R-ECD peptides designed for various HLA-DR could in theory be effective for
10 various HLA-DR carriers.

11 In conclusion, binding of anti hTSH-R antibodies to the amino-terminal end of the ECD
12 was confirmed in our DR3 transgenic mice. In addition, the ability of immunization to a single
13 peptide to induce antibodies that bind whole TSH-R protein, as well as multiple unrelated peptides,
14 is a unique observation. The mechanism may relate to developing immunity to host TSH-R, and
15 epitope spreading along this antigen. Finally, immunogenic reaction to hTSH-R peptide 37 could
16 be partially suppressed by competing peptide 37m, and in theory this might contribute to
17 immunotherapy of AITD.

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3 T4 and TSH in mouse serum.

1 **Figure legends**

2 Fig.1A: Antisera from DR3 transgenic mice immunized to hTSH-R-ECD protein, were
3 used to measure antibodies to individual hTSH-R peptides, indicated as OD 450nm value on
4 ELISA. Individual hTSH-R-ECD peptides are set in order of sequence from N-terminus of hTSH-
5 R-ECD. Solid bar represents result of ELISA without inhibition (peptide only), gray bar shows
6 results with added hTSH-R-ECD (inhibited by hTSH-R-ECD). White bar indicates experiment
7 with adding the same peptide as coated on the assay plate (Inhibited by the same pep). Error bars
8 represent data + SD.

9 Fig.1B: Antisera from DR3 transgenic mice immunized to hTSH-R-ECD peptide 37 were
10 used to measure antibody for each anti-hTSH-R peptide, reported as OD 450nm on ELISA, and
11 with the same binding inhibition studies as in 1A.

12 Fig.1C: DR3 transgenic mice immunized to hTSH-R-ECD peptide 32. ELISA was
13 performed in the same methods as above.

14 Fig.1D: DR3 transgenic mice immunized to PBS.

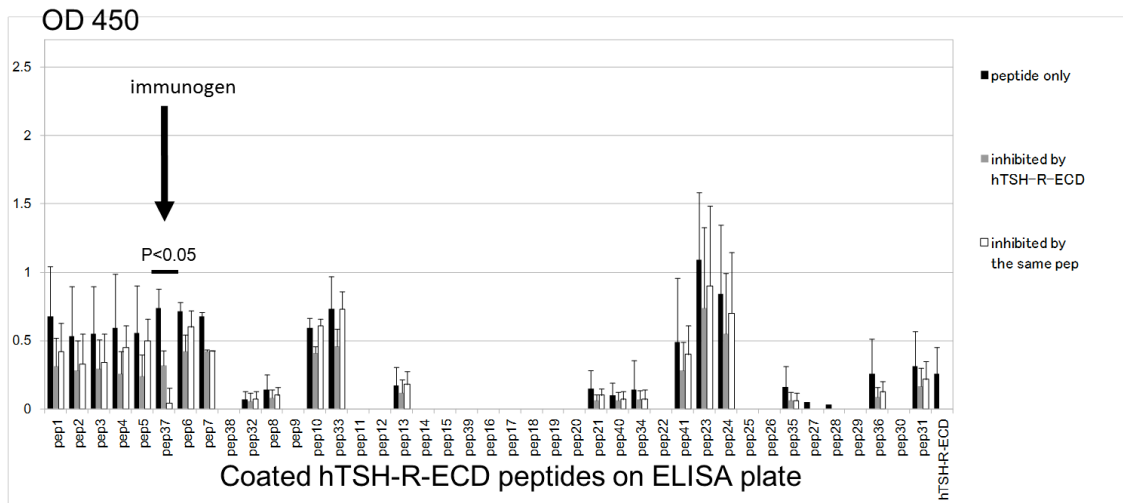
15 Fig.2: ELISA on antisera from DR3 transgenic mice immunized to hTSH-R-ECD protein
16 and peptide 37 with/without 37m. Data are represented with SD as error bars. Anti hTSH-R-ECD
17 protein or peptide antibody titer is represented as OD 450nm. As a control, PBS with no antigen
18 was used to coat the ELISA plate. NS: not significant

19 Fig.3: Responses of DR3 transgenic mice splenocytes immunized to hTSH-R-ECD protein
20 and peptide 37 w/wo 37m are shown. ³H incorporation value (cpm) for splenocytes from mice
21 immunized to PBS, incubated without antigen is selected as control. SI (stimulation index) is
22 calculated as measured value/control value. Data are presented with SD as error bars.

23 Table 1: HLA-DR binding affinities of hTSH-R-ECD derived peptides (IC₅₀ in μM) and
24 binding predictions are shown. Underline denotes predicted amino acid sequences of the included

- 1 motif giving its best binding to HLA-DR3.
- 2 *1 position shows amino acid position.
- 3 *2 DR3 indicates IC₅₀ binding of each hTSH-R peptide to HLA-DR3 (6)
- 4 *3 DR3z indicates EpiMatrix predicting binding score to HLA-DR3, and data with above 1.64
- 5 means moderate binding prediction (4).
- 6 *4 Position Z represents predicted binding site to HLA-DR3.
- 7 *5 M/H difference shows the number of difference in TSH-R between mouse / human.
- 8 Table 2: DR3 transgenic mice serum data for average TSH values \pm SD, average T4
- 9 values \pm SD, average ELISA OD450nm values for anti-hTSH-R-ECD antibody \pm SD, and
- 10 percentage of mice having ELISA OD450nm value > 0.200 are shown.

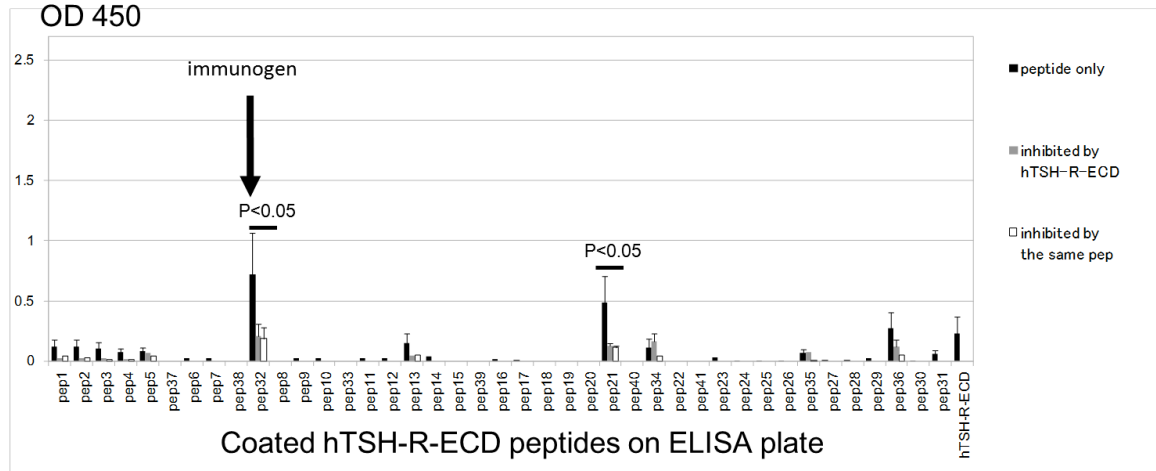
Fig.1B



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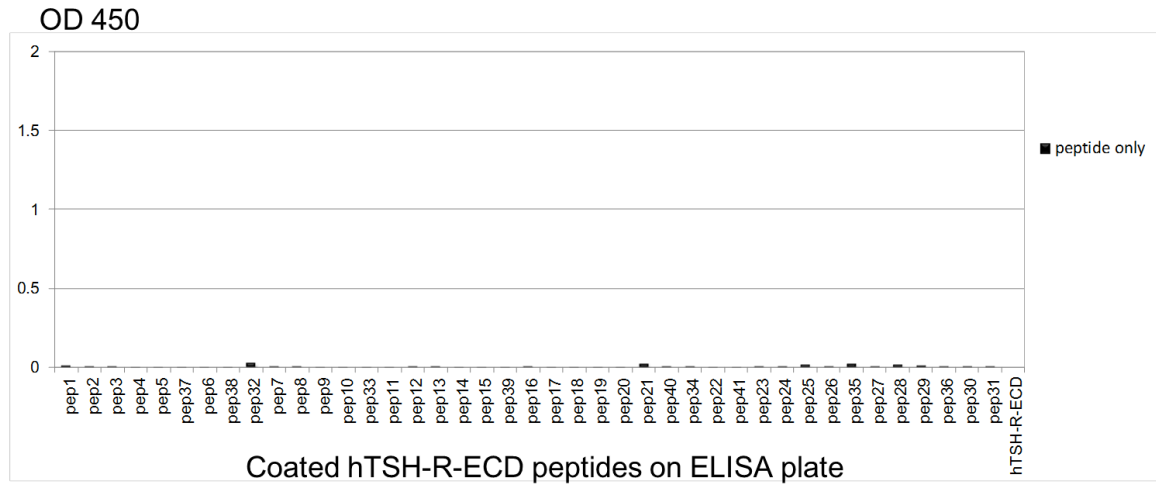
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Fig.1C



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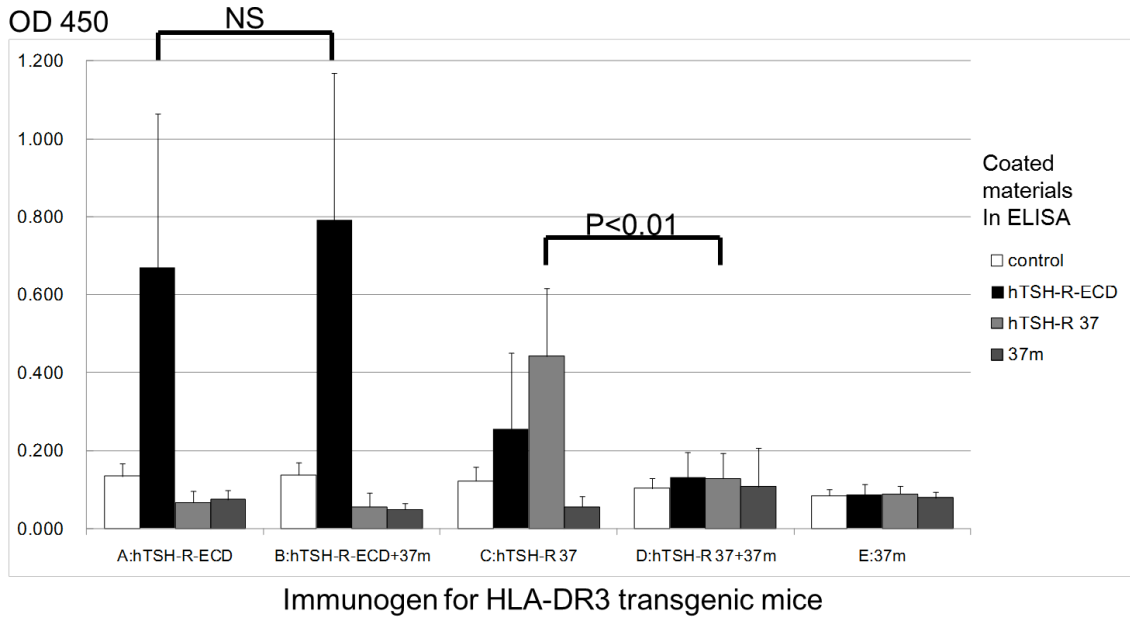
Fig.1D



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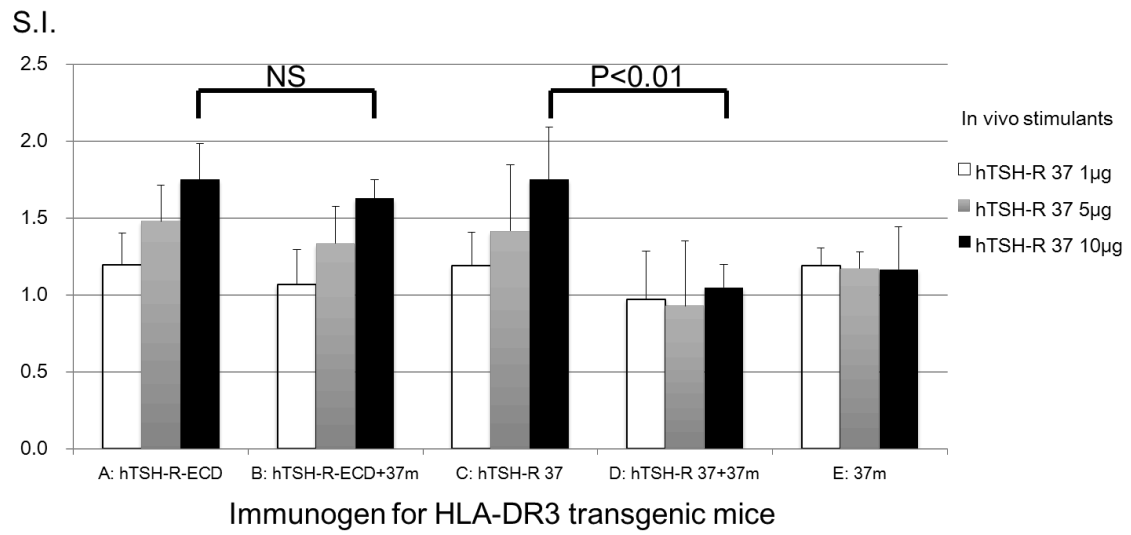
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Fig.2



- 1
- 2
- 3

Fig.3



1
2

1 Table 1

	position ^{1*}	Amino Acid Sequence	DR3 ^{2*}	DR3z ^{3*}	Position Z ^{4*}	M/H difference ^{5*}
1	20-35	GGMGCSSPPCECHQEE	>100	1	22-30	5
2	30-49	ECHQEEDFRV TCKDIQRIPS	>100	1.3	33-41	4
3	44-62	IQRIPSLPPSTQTLKLIET	>100	0.8	44-52	2
4	57-75	LKLIETHLR TIPSHA FSNL	>100	1.7	57-65	3
5	70-88	HAFSNLPNISRIYV SIDVT	29	1	70-78	4
6	83-102	VSIDVTLQQLESHSFYNLSK	41.3	2.1	83-91	4
7	97-112	FYNLSKVTHIERNTR	42	2	104-112	1
8	109-124	RNTRNLTYIDPDALKE	5.5	0.9	115-123	2
9	119-137	PDALKE LPL LKFLGIFNTG	>100	0.8	124-132	1
10	132-150	GIFNTGLKMF PDLTKVYST	0.64	2.3	140-148	3
11	145-163	TKVYSTDIFFILETDNPY	19.3	1.3	154-162	1
12	158-176	ITDNPYMTSIPVNAFQGLC	50	1.2	163-171	2
13	172-186	FQGLCNETLTKLYN	18.3	1.2	172-180	0
14	183-198	KLYNNGFTSVQGYAFN	>100	1.1	185-193	1
15	195-210	YAFNGTKLDAVYLNKN	46.6	1.6	196-204	1
16	207-222	LNKNKYLTVIDKDAFG	40	1.6	214-222	2
17	217-232	DKDAFGGVYSGPSLLD	90	0.9	224-232	2
18	227-242	GPSLLDV SQTSVTALP	45	1.4	229-237	2
19	237-252	SVTALPSKGLEHLKEL	>100	0.7	237-245	0
20	248-263	HLKELIARNTWTLKKL	40	1	250-258	2
21	258-277	WTLKKLPLSLSFLHLTRADL	16.7	2	269-277	0
22	272-291	LTRADLSYPSHCCAFKNQKK	14.2	1.8	273-281	0
23	286-305	FKNQKKIRGILESLMCNESS	60	2.6	286-294	0
24	301-320	CNESSMQSLRQRKSVNALNS	46.7	1.2	309-317	6
25	315-334	VNALNSPLHQEY EENLGDSI	33.8	1.4	318-326	8
26	329-348	NLGDSVGYKEKSKFQDTHN	80	1.6	329-337	10
27	343-362	FQDTHNNAHYVFFEEQEDE	20	1.6	354-362	5
28	357-376	EEQEDEIIGFGQELKNPQEE	45	1.8	366-374	2
29	371-390	KNPQEETLQAFDSHYDY TIC	18.3	2.5	379-387	2
30	385-404	YDY TICG DSEDMVCTPKSDE	13	1.3	389-397	2
31	399-418	TPKSDEFNPCEDIMGYKFLR	>100	1.7	410-418	1
32(like8)	105-118	HIEIRNTRNLTYID	10	1.7	108-116	1
33(like10)	137-150	GLKMF PDLTKVYST	0.2	2.3	140-148	3
34(21-22)	267-282	LSFLHLTRADLSYPSHC	27.6	2	269-277	0
35(26-27)	339-352	EKSKFQDTHNNAHY	37.5	1.7	342-350	7
36(like29)	376-389	ETLQAFDSHYDYTI	25	2.5	379-387	2
37(5-6)	78-94	ISRIYV SIDVTLQQLES	0.3	2.1	83-91	4
37m	78-94*	ISRIYV SIDA TL SQLES	0.3	2.1	83-91	3
38(7-8)	103-119	VTHIERNTRNLTYIDP	17.5	2	104-112	2
39(15-16)	201-217	KLDAVYLNKNKYLTVID	>100	2.2	204-212	2
40(21-22)	266-284	SLSFLHLTRADLSYPSHCC	5	2	269-277	0
41(22-23)	283-297	CCA FKNQKKIRGILE	>100	2.6	286-294	0
ex1	HSP3-13	KITAYDEEARR	0.3	2.7	5-13	

2

1 Table 2

	immunization	TSH mIU/l	T4 ug/dl	ELISA OD	ELISAOD450>0.200
N=11	hTSH-R-ECD	73.09 +- 55.05	4.77+-0.83	0.671+-0.384	63.6%
N=6	hTSH-R 37	47.0 +- 5.29	4.19+-0.22	0.255+-0.195	50%
N=4	hTSH-R 32	66.5 +- 19.84	3.54+-0.76	0.232+-0.134	50%
N=7	control	96.42 +- 52.35	4.82+-0.31	0.096 +- 0.076	0%

