# Identification of potential T-cell epitopes in factor VIII using peptide microarrays

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## OBJECTIVES

**Background:** The development of neutralizing anti-factor VIII (FVIII) antibodies ("inhibitors") can cause serious complications in hemophilia A patients. Inhibitor development requires stimulation of T cells by T-cell epitopes, which are short regions of FVIII that can bind effectively to MHC Class II receptors on antigen-presenting cells, and that are then recognized by effector T cells. Previous studies have identified T-cell epitopes that stimulate T cells from inhibitor subjects using overlapping synthetic peptides spanning the FVIII A2, A3, C1 and C2 domains (e.g.1-3). These systematic mapping experiments are time consuming and require large blood volumes

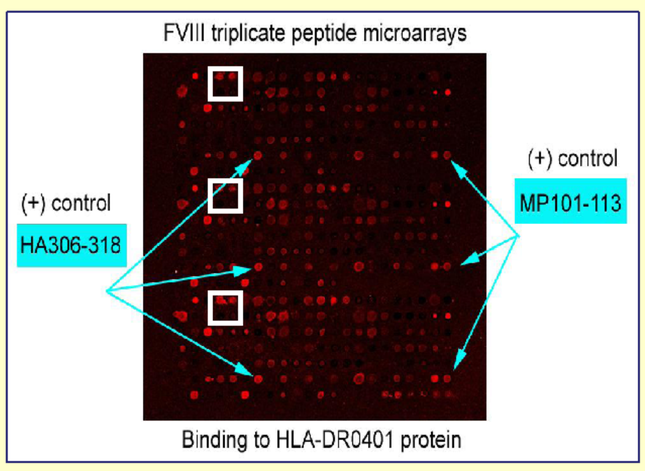
**Objectives:**

1. We present here an efficient method to identify specific amino acid sequences in FVIII that bind to recombinant MHC II-DR proteins corresponding to 10 common Class II-HLA-DRB1 alleles in the US population.
2. The experiments test the feasibility of screening for potential T-cell epitopes by detecting binding of HLA-DR proteins to synthetic, overlapping 20-mer peptides covalently immobilized on glass slides (arrays from JPT Technologies, Berlin, Germany).
3. Fluorescent intensities of peptide-bound HLA-DR proteins are compared with quantitative measurements of peptide-DR binding for a subset of peptides tested using a competition assay.
4. This information will be used to carry out more efficient assays to identify epitopes recognized by T cells in blood from inhibitor patients.

## METHODS

1. Peptide microarrays (5) consisting of 236 overlapping 20-mer peptides derived from human FVIII A1, A2, A3, C1 and C2 domains and other reference peptides were constructed.
2. The peptides were printed covalently in triplicate on glass slides via an activated epoxy linker attached to each peptide.
3. Soluble recombinant HLA-DR monomers were added to the slides, which were incubated and then washed.
4. Peptide-binding events were detected by addition of a fluorescent antibody that recognizes only the peptide-bound conformations of HLA-DR proteins.
5. Fluorescent "spots" with intensities significantly above background (calculated as ratios of foreground to background fluorescence on a log scale) are considered potential T-cell epitopes restricted to the corresponding HLA-DRB1 alleles.

## RESULTS




**Left:** The 2 dots enclosed in white boxes (shown in triplicate at left) show an example of overlapping peptides that may bind to a common epitope: FVIII residues 25-43: PVDARFPPRVPKSFPFNTSV and FVIII residues 33-51: RVPKSFPFNTSVVYKKTLFV.

The sequence in red font shows the overlap region. Possible epitopes here, in predicted order of affinity, are: VVYKKTLFV, FPFNTSVVY, FNTSVVYKK and VPKSFPFNT.

**Below:** Tetanus peptide TT503 elicited a DRB1*10:01-restricted T-cell response (4) but its binding affinity has not yet been measured. (+) and (-) control reference peptides are not yet available for HLA-DRB1*15:03. NA indicates "Not Available" for HLA-DRB1*15:03.

Negative control FVIII-M2238-1 peptide results for DRB1*15:01, DRB1*07:01 and DRB1*09:01 were measured in our laboratory.

| DR Protein | positive control reference peptide | | average fluorescence | SD |
|---|---|---|---|---|
| DRB1*0101 | HA 306-318 | PKYVKQNTLKLAT | 4.72 | 0.23 |
| DRB1*0401 | HA 306-318 | PKYVKQNTLKLAT | 3.74 | 0.21 |
| DRB1*1104 | HSV-2 VP16 34-44 | PLYATGRLSQA | 0.34 | 0.37 |
| DRB1*1501 | MBP 84-102 | NPVVHFFKNIVTPRTPPPS | 1.17 | 0.09 |
| DRB1*0301 | MYO 137-148 | LFRKDIAAKYKE | 2.41 | 0.25 |
| DRB1*1101 | HA 306-318 | PKYVKQNTLKLAT | 4.34 | 0.11 |
| DRB1*0701 | GAD 557I | NFIRMVISNPAAT | 0.40 | 0.06 |
| DRB1*0404 | GAD 557I | NFIRMVISNPAAT | 0.26 | 0.08 |
| DRB1*0901 | TT 503-515 | LNFNYSLDKIIVD | 0.04 | 0.05 |
| DRB1*1001 | TT 503-515 | LNFNYSLDKIIVD | 0.04 | 0.03 |
| DRB1*1503 | NA | NA | NA | NA |
| DRB1*1001 | CLIP 738-751 | ERRLFNLDVPESRR | 1.31 | 0.12 |

| DR Protein | negative control reference peptide | | average fluorescence | SD |
|---|---|---|---|---|
| DRB1*0101 | OspA 163-175, 165A | KSAVLEGTLTAEK | -0.37 | 0.08 |
| DRB1*0401 | OspA 163-175, 165A | KSAVLEGTLTAEK | -0.43 | 0.05 |
| DRB1*1104 | OspA 163-175 | KSYVLEGTLTAEK | 0.06 | 0.08 |
| DRB1*1501 | FVIII-M2238-1 | NNPKEWLQVDFQKTMKVTGV | 0.09 | 0.06 |
| DRB1*0301 | HA 306-318 | PKYVKQNTLKLAT | 1.57 | 0.20 |
| DRB1*1101 | OspA 163-175, 165A | KSAVLEGTLTAEK | 0.26 | 0.14 |
| DRB1*0701 | FVIII-M2238-1 | NNPKEWLQVDFQKTMKVTGV | -0.16 | 0.03 |
| DRB1*0404 | OspA 163-175, 165A | KSAVLEGTLTAEK | -0.10 | 0.01 |
| DRB1*0901 | FVIII-M2238-1 | NNPKEWLQVDFQKTMKVTGV | 0.09 | 0.14 |
| DRB1*1001 | vimentin 58-72 | GGVYATRSSAVRLRS | 0.00 | 0.10 |
| DRB1*1503 | NA | NA | NA | NA |

Assays were carried out to determine the binding of the following HLA-DR proteins to overlapping peptides spanning the FVIII sequence: HLA-DR0101, DR0401, DR0404, DR0301, DR0701, DR0901, DR1001, DR1101, DR1104 and DR1503.

1. The representative microarray shown at left illustrates binding of HLA-DR0401 protein to FVIII peptides imprinted on slides in a triplicate repeating pattern. The 3-fold symmetric fluorescent pattern indicates the high reproducibility of this binding assay.
2. 20-40% of the peptides, including several verified HLA-restricted (non-FVIII) T-cell epitopes included as positive controls (blue arrows), bound with significant affinity to each HLA-DR protein tested. Different HLA-DR proteins produced different patterns of spots (not shown).
3. Almost all of the reference (non-FVIII) peptides included as positive controls produced a fluorescent signal well above background when incubated with the relevant HLA-DR protein. Peptides containing known FVIII T-cell epitopes also produced the expected positive fluorescent signals.
4. HLA-specific peptide pools consisting of only peptides identified in these assays will be used for tetramer-guided epitope mapping. By eliminating non-MHC-binding peptides from these experiments, the requirement for blood is reduced by approximately half.

## CONCLUSIONS

MHC-peptide binding constitutes the principal basis for screening potential T-cell epitopes. Both computer prediction algorithms and peptide-binding experiments significantly over-predict T-cell epitopes, because binding of a peptide to an MHC Class II receptor is a necessary but not sufficient condition for subsequent T-cell activation. The experiments described here do not address the next step that occurs in inhibitor development, i.e. recognition of a peptide-MHC complex by effector T cells. Nevertheless, this approach, which characterizes the initial peptide-MHC binding event, reduces the complexity of the search for immunodominant T-cell epitopes, as there is no need to investigate T-cell responses to FVIII peptides that do not bind to the relevant MHC. Current experiments testing T-cell responses to FVIII are utilizing subsets of FVIII peptides that have been shown to bind the relevant HLA-DR protein(s). The use of peptide arrays to rule out a significant number of peptides as T-cell epitopes will allow us to make more efficient use of precious blood samples donated by research study subjects.

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