The Journal of Immunology

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J Immunol published online 17 June 2009
http://www.jimmunol.org/content/early/2009/06/17/jimmunol.0801473

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Published June 17, 2009, doi:10.4049/jimmunol.0801473

Evidence for the Specificity for Platelet HPA-1a Alloepitope and the Presenting HLA-DR52a of Diverse Antigen-Specific Helper T Cell Clones from Alloimmunized Mothers[1]

Rachel Rayment,*‡ Taco W. Kooij,* Wei Zhang,*† Christian Siebold,§ Mike F. Murphy,* Dave Allen,* Nick Willcox,[2†] and David J. Roberts[2*]

Maternal alloantibodies against the human platelet Ag (HPA)-1a allotype of the platelet β_3 integrin GpIIb/IIIa can cause severe fetal or neonatal hemorrhage. Almost all anti-HPA-1a-immune mothers are homozygous for HPA-1b and carry HLA-DR52a (DRB3*0101). The single Pro^{33} →Leu substitution (HPA-1b→HPA-1a) was previously predicted to create a binding motif for HLA-DR52a that can lead to alloimmunization. We have isolated six CD4$^+$ T cell clones from three such mothers, which all respond to intact HPA-1a$^+$, but not HPA-1b$^+$, platelets. We used them to define the "core" and "anchor" residues of this natural T cell epitope. Molecular modeling based on a recently published crystal structure can explain the preferential presentation of the Leu^{33} (but not Pro^{33} variant) by HLA-DR52a rather than the linked HLA-DR3 or the allelic DR52b. The modeling also predicts efficient anchoring at position 33 by several alternative hydrophobic α-amino acids; indeed, a recently identified variant with Val^{33} is presented well to two clones, and is therefore potentially alloimmunogenic. Finally, these HPA-1a-specific T cell clones use a variety of T cell receptors, but all have a "Th1" (IFN-γ-producing) profile and are suitable for testing selective immunotherapies that might be applicable in vivo. ***The Journal of Immunology,*** **2009, 183: 677–686.**

Alloimmune responses by mothers against fetal Ags have been studied much less than autoimmune reactions, although the original alloantigens are easier to identify. There have been some reports on maternal T cell responses to rhesus Ags (1), but few on fetomaternal alloimmune thrombocytopenia (FMAIT)[3] (2), although much is known about its immunogenetics (2, 3).

FMAIT causes considerable fetal and neonatal morbidity and mortality. The overall incidence of FMAIT is estimated to be 1 in 1163 live births (86 per 100,000), the incidence of severe thrombocytopenia (platelet count $<50 \times 109$/L) is 1 in 1695 (or 59 per 100,000), and intracranial hemorrhage occurs in the range of 10–20%, most of which occur antenatally (4–6). Most of the deaths are associated with intracranial hemorrhage; the mortality has been estimated in some studies as >10% (7), although recent studies suggest this is an overestimate (5, 6). Unfortunately, unlike in hemolytic disease of the newborn, there is as yet no reliable laboratory method to predict pregnancies at high risk of severe FMAIT and intracranial hemorrhage (8, 9).

FMAIT frequently affects first pregnancies (unlike hemolytic disease of the newborn), and neonates typically present unexpectedly with skin, mucosal, or more severe hemorrhage due to thrombocytopenia. Subsequent pregnancies are at greater risk of severe hemorrhage. There has been significant progress in the antenatal management of FMAIT in the last 20 years, but the management itself is not without significant risk to the fetus (5, 6, 8, 9). Greater knowledge of the maternal alloimmune response should improve monitoring and prediction of the severity of FMAIT and might lead to development of safer, more selective therapies.

In most cases of FMAIT, the mothers' alloantibodies recognize human platelet Ag (HPA)-1a on the platelet glycoprotein IIb/IIIa, an $\alpha_{IIb}\beta_3$ integrin that is their main fibrinogen receptor (10). Most FMAIT mothers are homozygous for its rare HPA-1b allelic variant (11), and they make alloantibodies against their babies' HPA-1a$^+$ platelets, which differ only by having a Leu^{33} instead of Pro^{33} of HPA-1b in its β_3-chain (12). In such cases, the mothers almost always have HLA-DR52a (DRB3*0101) (13).

Gorski and colleagues showed that the Leu^{33} in HPA-1a enables its peptide 23–38 to bind to HLA-DR52a, unlike its Pro^{33} counterpart (14), and to stimulate a specific T cell response from an alloimmune mother (2). Sometimes linked to HLA-DR13,

*Nuffield Department of Clinical Laboratory Sciences and National Blood Service Oxford Centre, and †Neurosciences Group, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom; ‡Department of Haematology, Arthur Bloom Haemophilia Centre, University Hospital of Wales, Cardiff, United Kingdom; and §Division of Structural Biology, University of Oxford, Oxford, United Kingdom

Received for publication May 30, 2008. Accepted for publication March 27, 2009.

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[1] This work was supported by the Muscular Dystrophy Campaign, Myasthenia Gravis Association, and National Health Service Blood and Transplant and benefited from funding by the United Kingdom National Health Service R&D Directorate, the National Institute for Health Research Centre Programme, and the Howard Hughes Medical Institute.

R.R., D.J.R., M.M., and D.A. conceived and planned the study; N.W. also helped to design it, and provided clone KB-D1; R.R. performed the T cell experiments; W.Z. helped in the ELISPOT assays and T cell cloning. C.S. did the molecular modeling; T.K. did the TCR identification. R.R. and N.W. prepared the first draft of the paper, and all authors helped to finalize it.

[2] Address correspondence and reprint requests to Dr. David J. Roberts, Nuffield Department of Clinical Laboratory Sciences and National Blood Service Oxford Centre, John Radcliffe Hospital, Oxford OX3 9BQ, U.K. E-mail address: david.roberts@ndcls.ox.ac.uk or Dr. Nick Willcox, Neurosciences Group, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, U.K. E-mail address: nick.willcox@imm.ox.ac.uk

[3] Abbreviations used in this paper: FMAIT, fetomaternal/neonatal alloimmune thrombocytopenia; AChR, acetylcholine receptor; DC, dendritic cell; HPA, human platelet Ag; IvIg, intravenous immunoglobulin; MG, myasthenia gravis; PBMCx, irradiated peripheral blood mononuclear cell; rh, recombinant human; RH5, RPMI with 5% heat-inactivated human HPA-1b plasma.

Table I. *Patient characteristics for donors of T cell clones*

No. Affected Children	No. with Intracranial Hemorrhage	No. with Platelet Count <30 ($\times 10^9$/L)	Timing of Sample at Attempted T Cell Cloning (months postpartum)	Clone	Age
1	0	1	7	ANN 6[a]	26
2	0	2	6	BEV 3.6[b] BEV 3.9[b] BEV 3.10[b]	28
2	1 at 33/40	1	5	CAZ 1.4[a] CAZ 3.8[b] CAZ 3.10[b]	34

[a] Clones were derived from 21–38a-stimulated lines.
[b] Clones were derived from 23–45a-stimulated lines.
All mothers were HPA-1b homozygous and DR52a$^+$; their partners were all HPA-1a homozygous.

HLA-DR52a more often resides in the HLA-DQ2-DR3-B8 haplotype that also predisposes to several highly specific autoimmune disorders, including myasthenia gravis (MG) (15, 16), where it presents at least two epitopes from the target acetylcholine receptor (AChR) to potentially pathogenic T cells (17, 18). In contrast, the somewhat similar DQ2-DR3-B18 haplotype includes DR52b (DRB3*0202), which is negatively associated with MG (15, 16) and does not predispose to FMAIT.

Here, we characterize T cell clones derived from several FMAIT mothers, confirm that they respond even to small numbers of HPA-1a$^+$ platelets, and define their restricting class II molecule and epitope "core". Molecular modeling based on a recent crystal structure of this epitope in DR52a (19) provides firm explanations for their restrictions and specificities. Finally, we demonstrate that HPA-1a-specific T cell clones are suitable for testing immunotherapeutic strategies.

Materials and Methods

Clinical cases and controls

The FMAIT mothers all had at least one child with documented FMAIT due to HPA-1a alloimmunization, and they carried HLA-DRB3*0101 (DR52a) (Histocompatibility and Immunology Laboratory, National Health Service, Blood and Transplant (NHSBT), Bristol, U.K.) (Table I). PBMCs were obtained from HLA-DR-typed volunteer donors as a source of APCs and dendritic cells (DCs). All samples were taken with informed consent, local and national Ethical Committee approval, and were used in compliance with the guidelines of the Declaration of Helsinki (World Medical Association, 2000).

Platelet preparations

Platelets were obtained by platelet pheresis from homozygous HPA-1a or -1b donors and stored in 10% DMSO, 90% HPA-1b serum (National Blood Service) at 5–10 $\times$ 10^8/ml at −80°C. After thawing, they were washed three times in RPMI 1640, resuspended at 1 $\times$ 10^9/ml, and used the same day at 1 $\times$ 10^8/ml. PBMCs were separated from heparinized blood on Lymphoprep (Nycomed), washed, and resuspended in RPMI 1640 with 5% heat-inactivated human HPA-1b plasma (RH5) obtained from platelet pheresis donations. When required, they were irradiated with 30 Gy from a ^{137}Cs source.

Preparation of peptides

The native peptide sequences (residues 21–45) are ^{21}PMCAWCSDEAL PLGSPRCDLKENLL^{45} (HPA-1a) and ^{21}PMCAWCSDEALPPGSPRC DLKENLL^{45} (HPA-1b). Peptides used for cloning T cells (21–38a, Leu^{33}; 21–38b, Pro^{33}; 24–45a, Leu^{33}; or 24–45b, Pro^{33}) and truncated variants were synthesized by Southampton Polypeptides (University of Southampton, Southampton, U.K.), and variants (with Cys^{38} or Ser^{38} or Val^{33}) by Dr. K. di Gleria (Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, U.K.), by solid phase synthesis, using f-moc chemistry; their >95% purity was confirmed by HPLC, and their identity was confirmed by mass spectrometry. All peptides were dissolved in H_2O at 1 mg/ml, filtered, frozen immediately at −20°C, and used at 10 μg/ml unless otherwise stated.

Generation of Ag-specific T cell lines

Fresh PBMCs were cultured in 48-well plates at 3.0 $\times$ 10^6/ml in RPMI/5% autologous plasma with HPA1-a or HPA-1b peptides plus recombinant human (rh)IL-7 (25 ng/ml; PeproTech). We added rhIL-2 (100 U/ml; PeproTech) on day 3 and at ~3 daily intervals for ~18 days, when we checked for Ag specificity in IL-4 or IFN-γ ELISPOT assays with fresh autologous PBMCs as APCs. A long initial culture period was necessary, as we always failed to detect proliferative responses by day 14, even after CD8 depletion or using autologous DCs as APCs (except against purified mycobacterial protein derivates). For ELISPOT assays, PBMCs were used at 2.5 $\times$ 10^5/ml or 5 $\times$ 10^4/ml, and washed T cells at 1, 2.5, and 5 $\times$ 10^5/ml. Lines showing a significant, specific IFN-γ response, more than twice background, were cloned; since three of three reactive T cell lines showed no detectable IL-4 responses, we subsequently tested only for IFN-γ.

Cloning HPA-1a-specific Th cell lines

Cloning was performed by limiting dilution in 96-well round-bottom plates, using irradiated PBMCs (PBMCx) from three separate donors as APCs (1 $\times$ 10^5/well), plus PHA (2 μg/ml); 100 U rhIL-2 were added on day 3, and the clones expanded with rhIL-2 for 21 days, and then tested in IFN-γ ELISPOT assays (see below). Those responding specifically were expanded in RH5 plus rhIL-2, and restimulated every 14 days with fresh PBMCx; PHA and IL-2 were added on day 0 and IL-2 was added every ~3 days thereafter.

Surface immunophenotyping of clones

Clones were phenotyped by indirect immunofluorescence/flow cytometry using the following mAbs: CD3-PE, clone UCHT1; CD4-FITC, clone RPA-T4; CD8-FITC, clone LT8; TCR$\alpha\beta$-PE, clone BMA-031; TCR$\gamma\delta$-FITC, clone 5A6E9; CD45RA-PE, clone F8-11-13; CD45RO-PE, clone UCHL1; isotype control IgG2a-FITC or -PE, clone OX34; and IgG1-FITC or -PE, clone W3/25 (Serotec). Cells were incubated with mAbs for 15 min on ice in PBS/0.1% FCS, washed twice, and analyzed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

Cytokine secretion of clones

Five $\times$ 10^5 cloned T cells were stimulated in 24-well plates using CD3/CD28 Dynabeads. Culture supernatant was removed on day 3 for use in IFN-γ and IL-4 Quantikine ELISAs according to the manufacturer's instructions (R&D Systems). Supernatants were assayed (in quadruplicate) at 1:2 for IFN-γ and neat for IL-4.

B cell lines as APCs

The B cell lines L0081785 (HLA-DR3, DR52b (DRB3*0202), B18), HHKB (DR13, DR52a, B7) (20), and NW (DR3, DR52a, B8/DR14, B27) were maintained in RPMI 1640/10% FCS (RF10) for at least 5 days. Before use as stimulators in proliferation assays, they were incubated for 1 h at 37°C with mitomycin C (50 μg/ml; Sigma-Aldrich), washed three times, and resuspended at 5 $\times$ 10^6/ml in RH5. They were then incubated with 10 μg/ml peptide, 2 μg/ml PHA, or in medium alone (negative control) for 3 h at 37°C, washed, and resuspended at 1 $\times$ 10^6/ml.

Assays for T cell responses
ELISPOT assays

IFN-γ and IL-4 ELISPOT kits (Mabtech) were used according to the manufacturer's instructions. In brief, 96-well plates with 0.45 μm hydrophobic

high protein-binding Immobilon-P membrane (Millipore) were incubated with anti-cytokine "capture" mAb (anti-IFN-γ or IL-4) overnight at 4°C. After washing and blocking with RH5, we added 3×10^4 T cells/well plus 4×10^4 autologous PBMCs (preincubated with test Ag for 3 h and washed). After 16 h at 37°C, and washing between each step, the wells were incubated for 2 h with biotin-labeled "detector" anti-IFN-γ or anti-IL-4 (1/1000 in PBS) at 20°C, then with streptavidin-AP conjugate (1/1000), followed by an AP substrate kit (Bio-Rad). Spots were counted using the AID ELISPOT reader and software (Cadana Medical).

Proliferation assay

Ag-pulsed PBMCx or B cells (10^5) were cocultured with 3×10^4 T cells in 96-well round-bottom plates for 48 h. The cells were then pulsed with 1 μCi of [^{3}H]thymidine (Amersham International) for 18 h. Plates were frozen, thawed, and DNA was harvested onto filtermats using an LKB Wallac harvester and counted on a Betaplate flatbed liquid scintillation counter (Wallac). A significant response was considered to be a stimulation index >2.

Generation of DCs from PBMCs

Monocytes were isolated from HLA-DR52a$^+$ donor NW using CD14 microbeads and a magnetic separator (Miltenyi Biotec) and then cultured (1.5×10^6 cells in 3.0 ml) in 6-well plates with GM-CSF (80 ng/ml) and IL-4 (50 ng/ml). On day 6, they were incubated with test Ag for 6 h, and then LPS was added (100 ng/ml) for 16 h before washing and analysis by FACS or culture with T cell clones.

Phenotyping of cultured DCs

Cultured DCs were immunophenotyped using mouse mAbs: HLA class I (clone W6/32; Dr. A. J. McMichael, Weatherall Institute of Molecular Medicine, Oxford, U.K.), HLA class II (clone CR3/43; Dako), CD3 (clone OKT3; American Type Culture Collection), CD4 (clone RPA-T4), CD8 (clone LT8), CD14 (clone UCHM1), CD19 (clone LT19), CD40 (clone B-B20; Diaclone Research), CD80 (clone MEM223), CD83 (clone HB15e), CD86 (clone BU63), IgG1 isotype control (clone DAK-G01; Dako), and IgG2a isotype control (clone OX34) (Abs from Serotec unless otherwise stated). After washing and staining with goat anti-mouse IgG-FITC (Sigma-Aldrich), they were washed and analyzed as described above.

TCR identification

Total RNA was isolated from 1×10^6 clonal T cells using TRIzol reagent (Invitrogen) and depleted of DNA with DNA-free (Ambion). cDNA was prepared using the StrataScript first strand synthesis system (Stratagene), and it was further purified using ProbeQuant G-50 Micro columns (Amersham Biosciences), following the manufacturers' instructions. Poly-G tails were added to the 5′ ends of the transcripts using recombinant TdT (Invitrogen). TCRα and TCRβ RNAs were amplified from five clones using a poly-C primer (T004, 5′-GAATTGAGACCCCCCCCCCCCCC-3′) to anneal to their 5′ ends and generic primers for the C regions of the TCRα (T006, 5′-TGTTGTTGAAGGCGTTTGCACATGCAAA-3′) or TCRβ (T007, 5′-GGAGATCTCTGCTTCTGATGGCTC-3′). DNA was amplified using Platinum *Taq*DNA Polymerase High Fidelity (Invitrogen), with 2 mM $MgSO_4$, 0.2 mM of each dNTP (Amersham Biosciences), 0.5 μM each primer and 4 μl of purified cDNA (on an MJ Research DNA Engine Dyad Peltier thermal cycler). Annealing and extension were conducted at 68°C. PCR products were purified from 1.5% Tris-borate EDTA agarose gel using the High Pure PCR product purification kit (Roche) according to the manufacturer's instructions.

Cloning and sequencing of TCRα and TCRβ V region

Nested C region primers (TCRα: T001, 5′-TGTTGAAGGCGTTTGCAC-3′; TCRβ: T002, 5′-ATCTCTGCTTCTGATGGC-3′) and V region-specific primers were used to amplify the five TCRα and five TCRβ transcripts, as previously described (21), before sequencing to determine TCR Vα and Vβ usage. Reactions were conducted as above but in 50 μl with 1.5 U Platinum *Taq*DNA polymerase, 0.3 μM each of the primers, and 2 μl of purified cDNA and 30 s annealing at 60°C and 60 s extension at 68°C in each cycle. The transcripts were immediately cloned using the TOPO TA cloning kit for sequencing (Invitrogen) according to the manufacturer's instructions. The Vα primer panel included no primer specific for the sequence from EVE3.10, so one was designed (T020, 5′-GAGCCCTCAGTTTCTAAGC-3′).

A complete panel of V region primers, including our new primer T020, was used to amplify the remaining two TCR Vαs (BEV3.6 and DOT3.9) and two TCR Vβs (BEV3.6 and GIA3.10). Reactions were conducted as above but in 25 μl with 0.5 U *Taq*DNA polymerase (Invitrogen), containing 1.5 mM $MgCl_2$, 0.4 μM each of the primers, and 0.5 μl of purified cDNA and 30 amplification cycles. The two TCRα and two TCRβ transcripts were amplified, cloned, and sequenced as described above.

Structural modeling

The program O described by Otwinowski and Minor (22) was used to substitute (in silico) residues from DR3 and DR52b for those in the crystal structures of DR52a complexed with the HPA-1a epitope (Research Collaboratory for Structural Bioinformatics Protein Data Bank are available under accession code 2Q6W) (19) and DR3:CLIP (Protein Data Bank accession code 1A6A) (23). The volumes of pockets available to specific side-chains at certain positions were calculated by the program VOLUMES (24). It takes the solvent-accessible volume (probe radius 1.4 Å) around the HLA class II molecule without the peptide, selecting the region closest to the peptide side-chain of interest, which is delimited at the bottom and either side by the solvent-accessible HLA surface and at the top by the main chain atoms of the peptide. Fig. 5 was prepared with the program PyMOL (www.pymol.org).

Results

Derivation of clones and Th1 profiles

Four of the FMAIT mothers were studied at 5–8 mo postpartum (Table I); 3 wk after their PBMCs were stimulated in vitro with HPA-1a peptide, three mothers yielded specifically responsive lines from each of which we isolated specific clones. The fifth mother was 20 wk pregnant with an affected baby. Although her line responded strongly on day 21 (using autologous plasma), it yielded no specific clones.

Once established, the clones were all CD3$^+$, CD4$^+$, CD8$^-$, CD45RA$^-$, CD45RO$^+$, and HLA-DR$^+$. In response to a maximal stimulus (10 μg/ml peptide 21–38a), they all produced >1000 pg/ml IFN-γ (as expected after their ELISPOT screening) and no detectable IL-4; in parallel, the "myasthenic" "Th0" clone (KB-D1) (responding to peptide α147–160 from the AChR[11]) produced 500–700 pg/ml IL-4. Thus, the present clones were clearly Th1 in phenotype.

Ag specificity of clones

Responses to HPA-1a 21–45 peptides from the 21–45 region

All clones showed strong responses to APCs pulsed with the HPA-1a peptide 21–38a, but not to the allelic HPA-1b sequence, which differs only by its Leu^{33}→Pro substitution (Fig. 1*A* and data not shown). All gave weaker responses to 24–45a, even where it had been used to stimulate the original line (Fig. 1*A*). Peptide titrations showed half-maximal responses to the 21–38a peptide at $<10^{-6}$ M (2 μg/ml; Fig. 1*B*), so we routinely used it at 10 μg/ml subsequently as the positive control. The one clone tested also responded well when we replaced the Cys^{38} with a Ser in 21–38a (Fig. 1*A*) to prevent the peptide from forming the disulfide bridges previously described by Maslanka and colleagues (2).

Responses to HPA-1a platelets

Recognition by this clone of independently synthesized HPA-1a peptides (Fig. 1*A*) already implies specificity for the true sequence. That is unequivocally confirmed by the maximal responses of the clones to whole platelets from HPA-1a$^+$ but not HPA-1b$^+$ donors (Fig. 1*C*). Titrations again show high sensitivity (Fig. 1*D*); at threshold doses, and assuming 10^5 molecules per platelet, we estimate a maximum input of 10^{-10} M HPA-1a epitope, although its processing efficiency from whole platelets is very unlikely to approach 100%. Thus, these clones were 100- to 1000-fold more sensitive to the epitope in natural rather than synthetic form, which suggests that they could have been primed by native Ag in the donor.

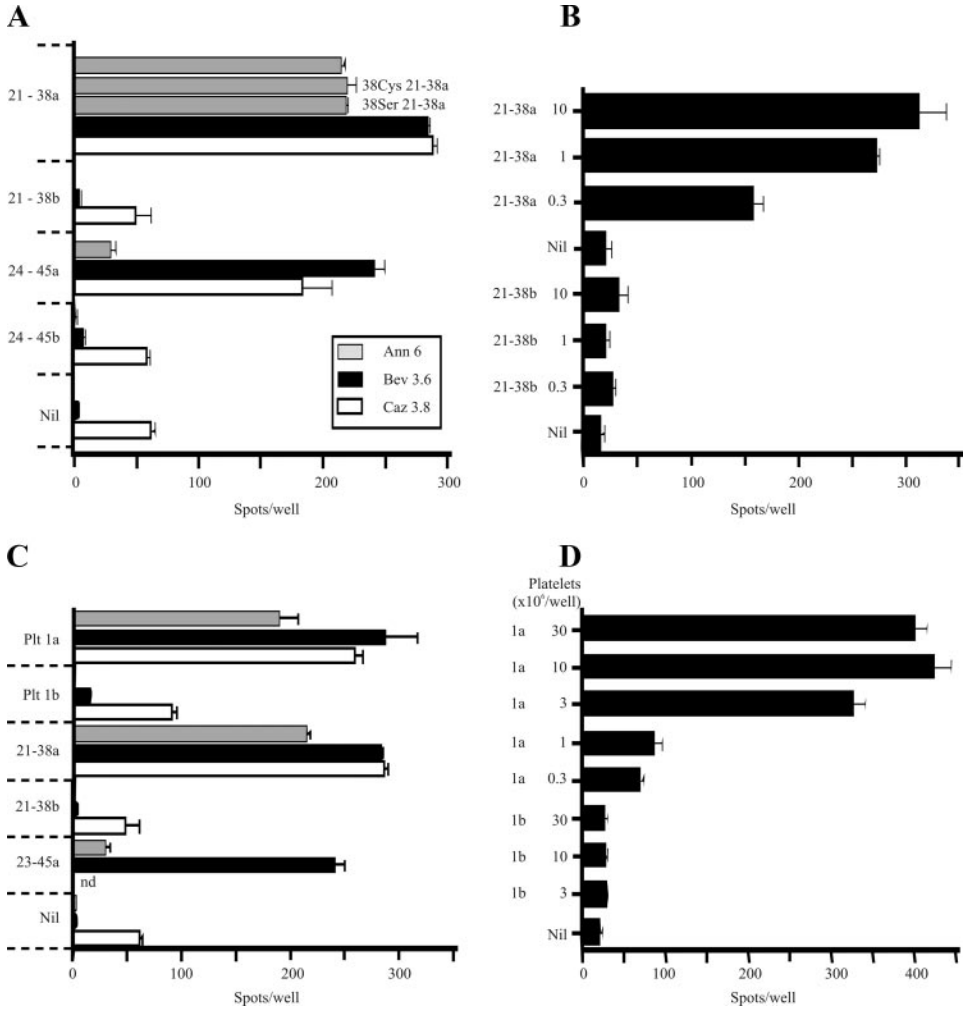


FIGURE 1. Responses of FMAIT Th clones to the 21–38 or 24–45 HPA-1a and HPA-1b peptides and platelets. ELISPOT responses for IFN-γ of clone ANN6 (gray bars), clone BEV3.6 (black bars), and CAZ3.8 (white bars) against (*A*) the 21–38 or 24–45 HPA-1a and HPA-1b peptides (10 μg/ml); for clone ANN6, responses are shown for the Cys^{38} and Ser^{38} peptides, synthesized independently of the others (in Oxford); (*B*) peptide dose (μg/ml) response assay; in each case, the APCs were autologous PBMCx and were prepulsed with the indicated peptide before washing and coculture with the T cells; (*C*) platelets (Plt), present continuously, either at 2×10^7/well or (*D*) in a dose response assay. Clone ANN6 (gray bars) was raised against 21–38a; clones BEV3.6 (black bars) and CAZ3.8 (white bars) were raised against 24–45a.

Background stimulation by APCs without added Ag

From here onward, we concentrate on clones ANN6 and BEV3.6, since they consistently showed the lowest backgrounds with allogeneic APCs in the absence of added Ag. For all clones, backgrounds were almost always low with autologous PBMCx. With some clones, however, they varied substantially (Fig. 1, *A* and *C*), both with different allogeneic APCs (see Fig. 2*B*, HHKB vs NW) and between experiments with the same APCs, even though we always used HPA-1b serum in the cultures. They were sometimes so high that they masked Ag stimulations (Fig. 2*B*, HHKB) that were clearly positive with autologous APCs tested in parallel. These background responses were unpredictable, but occurred with APCs derived from donors who were HPA-1a-negative, HLA-DR52a-positive or -negative, and with B cell lines (e.g., HHKB) that failed to stain for CD61 (the β_3 integrin). Despite much further work, we cannot explain these backgrounds and their variability.

The HLA-class II restriction of the clones

We first identified the restricting class II isotype; responses were clearly blocked by mAbs to HLA-DR (by 44.0–99.0%; median, 96.0%; SD, 21.1) but not to -DQ or -DP (0.0–26.0%; median, 7.0%; SD, 7.5).

To identify the restricting allele(s), we tested HLA-sharing B cell lines and PBMCx as APCs (Fig. 2). The FMAIT-susceptibility allele HLA-DR52a (DRB3*0101) resides in the HLA-DR3-B8 haplotype, as in donor NW, but it can also be linked to DR13, as in the HHKB B cell line (20). As shown in Fig. 2, APCs with DR52a presented the 21–38a epitope maximally, whereas those with DR3 and DR52b instead consistently failed to present detectably to either clone (Fig. 2; L0081875); these latter alleles reside in the related DR3-B18 haplotype, which does not predispose to FMAIT. Results were similar when we instead used PBMCx with one or other of these haplotypes as APCs (data not shown).

Definition of the epitope

Wu and colleagues (14) predicted that the HPA-1a epitope core is the 25–33 sequence (Fig. 3) and is "anchored" into HLA-DR52a by its Trp^{25} at peptide "p1", its Asp^{28} at p4, and its Leu^{33} at p9. To test these predictions, we first synthesized peptides from the 21–38a region with serial truncations at either end (Fig. 3). For both of the clones tested, residue 33 proved to be essential for recognition, but not aa 34–38. There was more variability near the N terminus; clone ANN6 required residues from 23 onward for maximal stimulation (arrowed in Fig. 3*A*), whereas aa 24 onward were sufficient for clone BEV3.6 (arrowed in Fig. 3*B*).

We next substituted a nondescript Ala residue for each of the proposed p1, p4, and p9 anchor residues (Fig. 4). The p1 and p4 Ala substituents were not recognized detectably by any of the four clones tested, even at 30 μg/ml. Their responses to the Ala^{33} substituent at p9 were more variable; for BEV3.6 (Fig. 4*A*) and BEV3.9 (data not shown), they were ~60% of maximum at 10 μg/ml and ~70% at 30 μg/ml, whereas they were undetectable at any dose for BEV3.10 and ANN6 (Fig. 4). The responses of ANN6 and BEV3.6 were also strong to Val^{33}, but not to Gly^{33} or Ser^{33}

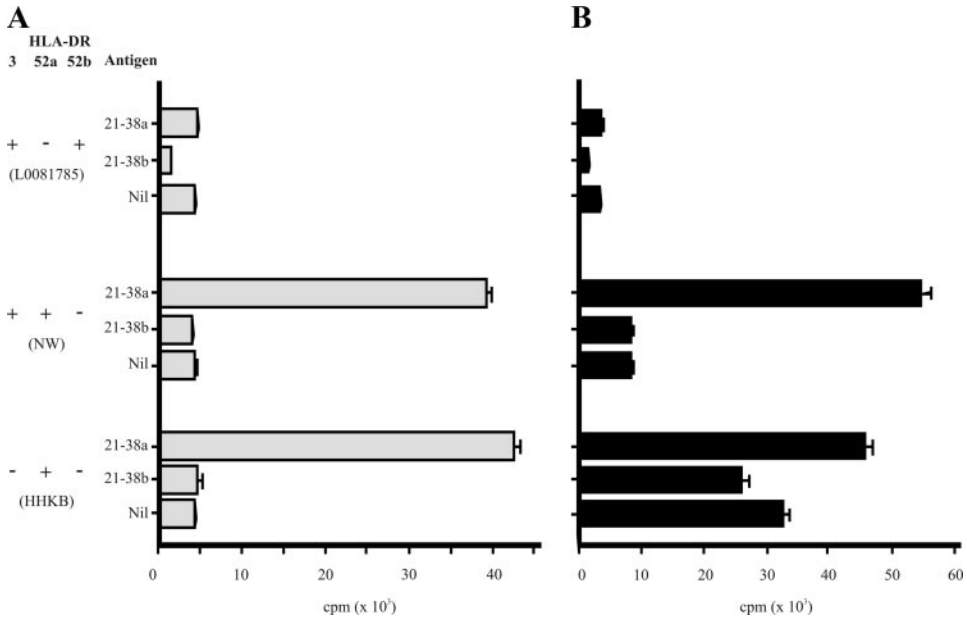


FIGURE 2. The presenting HLA-DR molecule for the indicated FMAIT clones. Proliferative responses for (*A*) clone ANN6 (gray bars) and (*B*) clone BEV3.6 (black bars) against the 21–38 HPA-1a and HPA-1b peptides (10 μg/ml) presented by the indicated B cell lines; NW also has DR14, and HHKB has DR1301. We confirmed the costimulatory activity of L0081785 by using PHA as stimulus (not shown). Responses were assayed by [^{3}H]thymidine uptake.

(Fig. 4*A* and data not shown). Therefore, the clones vary significantly in fine specificity, with clone BEV3.6 being especially tolerant of truncations of aa 24 (Fig. 3) or substitutions of Leu^{33} (Fig. 4). These results provide experimental, functional evidence for the epitope core and anchors predicted by Wu and colleagues (14) and by Parry and colleagues (19).

Complementary structural and T cell response data explain the singular ability of DR52a to present the HPA-1a epitope

We used program "O" (22) to model presentation of the HPA-1a epitope by DR52a, based on the coordinates recently published for DR52a complexed with Cys^{26}→Arg modified HPA-1a ^{24}AWRS DEALPLGS^{35} peptide (19) (overview in Fig. 5).

While HLA-DR52a shows several similarities to DR3 and DR52b, its apparently unique ability to present the HPA-1a epitope also reflects distinctive residues in or near pockets 1 and 4 (see below) and particularly also pocket 9 (Fig. 5).

Effects of Leu^{33}→Pro in HPA-1 on anchoring in pocket 9

The consistently undetectable responses of the clones to HPA-1b variant peptides (Figs. 1 and 2) agree well with their poor binding to DR52a (14). These poor responses are a reflection of not only

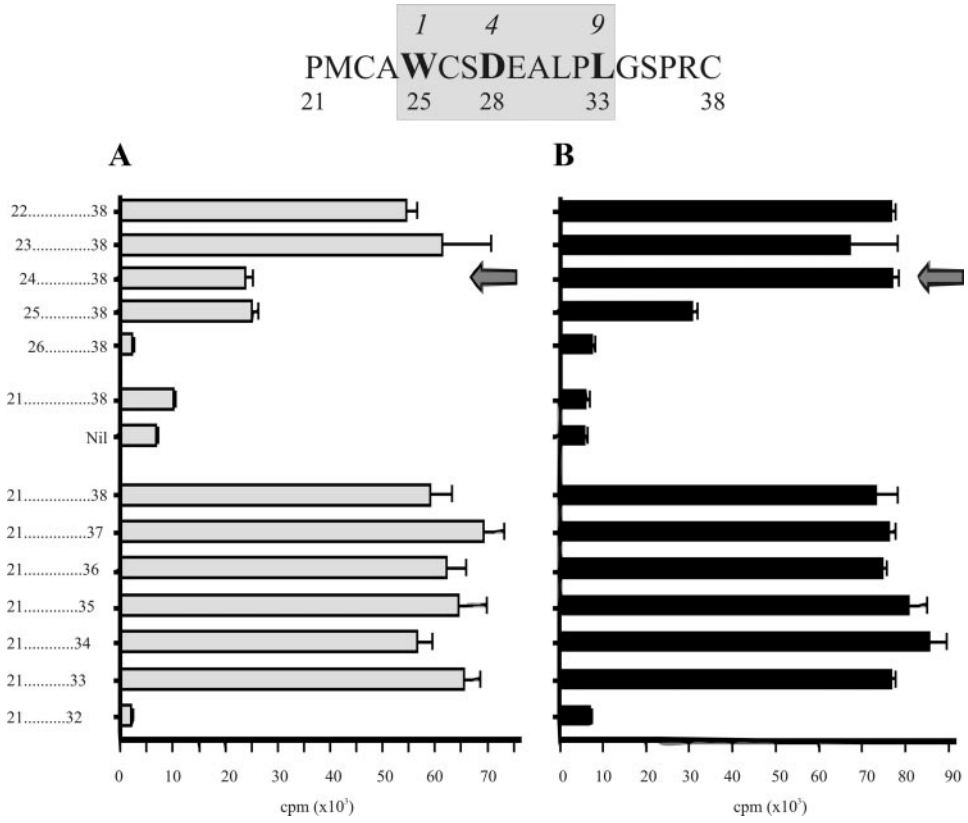


FIGURE 3. The epitope "core" in the HPA-1a 23–38 sequence as defined by serial truncations from either end. Proliferative responses for (*A*) clone ANN6 (gray bars) and (*B*) clone BEV3.6 (black bars) against the serially truncated HPA-1a peptide (10 μg/ml) as indicated. Responses were assayed by [^{3}H]thymidine uptake. *Upper panel*, Predicted "anchor" residues for DR52a (14), numbered above as p1, p4, and p9. The APCs were autologous PBMCx. The arrows mark the single difference in perception by these two clones.

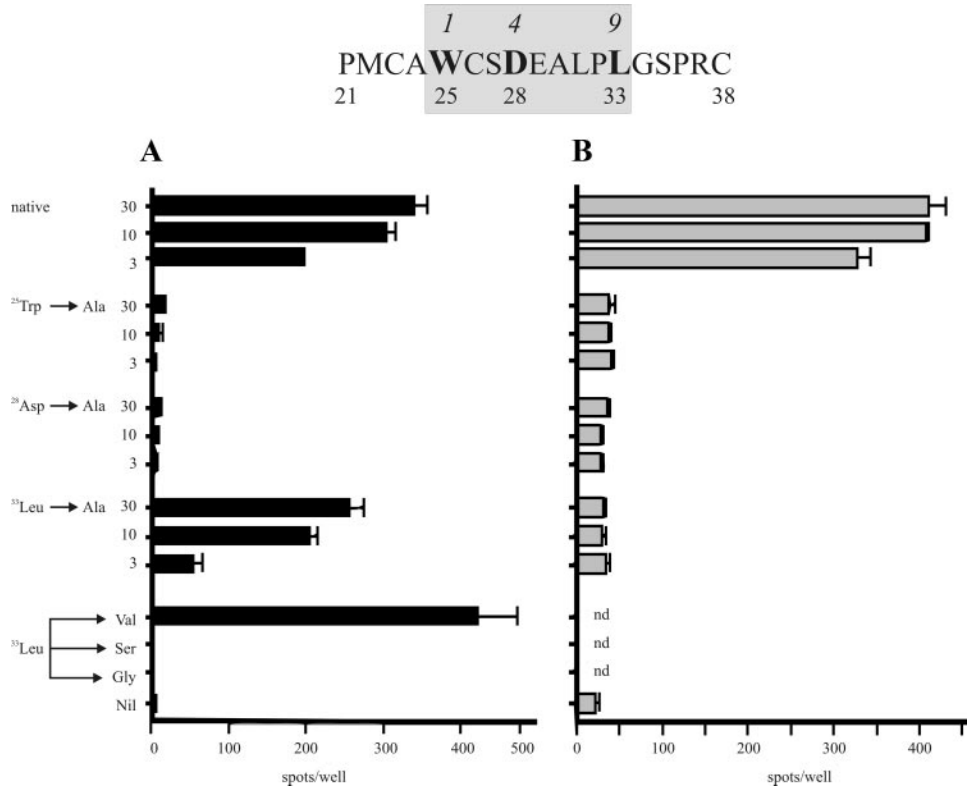


FIGURE 4. Using peptide substitutions to confirm the anchoring roles of p1, p4, and p9 in HPA-1a 23–38 peptide. Proliferative responses for (*A*) clone BEV3.6 (filled bars) and (*B*) clone BEV3.10 (gray bars) against HPA-1a peptide substituents (10 μg/ml) as indicated. The APCs were autologous PBMCx, and responses were assayed by [^{3}H]thymidine uptake. *Upper panel*, Predicted "anchor" residues for DR52a (14), numbered above as p1, p4, and p9.

the predicted/observed steric clashes between their Pro^{33} and Asn^{69} in the (invariant) DRα (19), but also the inability of Pro^{33} (which has no α-NH group) to form the highly conserved H bond

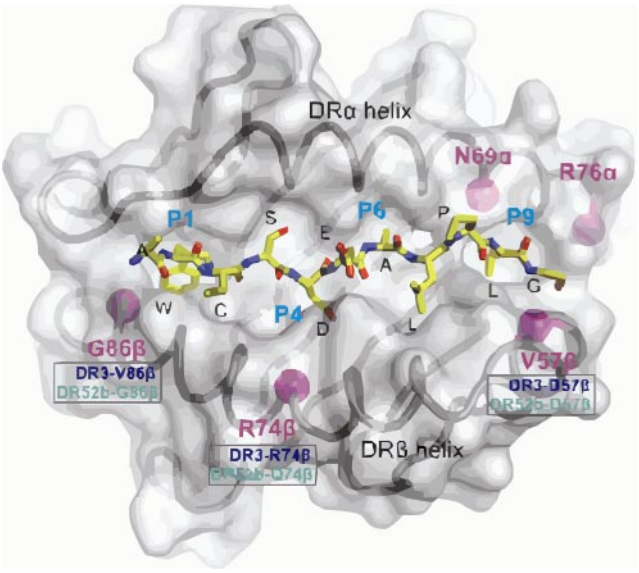


FIGURE 5. Modeling the structure of the HPA1a:HLA-DR52a complex. Overview from the TCR's perspective of the peptide-binding groove of the HPA-1a:HLA-DR52a complex modeled on the published crystal structure (19) (Research Collaboratory for Structural Bioinformatics Protein Data Bank, accession code 2Q6W). Solvent-accessible surfaces are colored in gray. The polypeptide backbone of the modeled DR52a molecule is shown in dark gray. Selected residues are highlighted as Cα spheres and colored in magenta for DR52a, and any replacements in DR3 (blue) or DR52b (green) in the boxes beneath. The residues of the peptide are depicted as sticks in atomic coloring (HPA-1a peptide carbon, yellow; nitrogen, blue; oxygen, red; sulfur, orange). Pockets P1, P4, P6, and P9 are labeled in blue.

that otherwise connects Leu^{33}'s α-NH group to the same Asn^{69}. Our structural modeling predicts that other α-amino acids should also anchor efficiently at p9 provided that they have hydrophobic side-chains. Indeed, variants with Leu, Val, or even Ala could clearly be presented well, unlike those with Gly or Ser (Fig. 4*A*). Interestingly, the variant with Val^{33} has recently been reported to be a naturally recognized serological epitope (25).

In pocket 9, intimate docking is favored by its more hydrophobic Phe^{37}-Leu^{38} (instead of the Asn^{37}-Val^{38} of DR3) in the DRβ floor (19); particularly also by the distinctive hydrophobic Val^{57} nearby in the α-helix of DR52a. In contrast, about two-thirds of DR alleles, including DR3 and DR52b, have Asp^{57} instead (26). If so, its acidic side-chain forms a salt bridge across to the Arg^{76} in the (invariant) α-helix of DRα that runs deep to the bound peptides (23) as in HLA-DQ6 (27); its absence in DR52a opens the hydrophobic pocket 9 and so allows more intimate docking of the epitope's C-terminal Leu^{33} than in DR3 or DR52b (19). That also releases the side-chain of Arg^{76} in DRα so that it can interact with the peptide backbone at p8–9 (19).

Overhanging pocket 4 is the unusual Arg^{74} of DR52a and DR3 (in the middle of the DRβ helix), whereas DR52b has Gln^{74} (26). That Arg^{74} was predicted (17, 28) (and our own modeling) to coordinate the Asp that is highly prevalent at p4 in the peptides bound by DR52a (14) and DR3 (23) and is clearly critical for recognition of the HPA-1a epitope by each of our clones (Fig. 4). Indeed, the Arg^{74}'s ε-NH group does form an H bond to the carboxyl group of this Asp^{28} (19). It also coordinates the side-chain of the adjacent Gln^{70} in DRβ, which, in turn, H bonds to the Glu^{29} at p5 in the peptide (19); together, these three side-chains form a likely central focus for TCR recognition.

Parry and colleagues also noted unexpected H bonds between the Asp^{28} and the guanidine group of the almost unique Arg^{11} in the floor of the DR52a groove (vs Ser^{11} in DR3 or Leu^{11} in

Table II. *TCR gene usage of HPA-1a-specific T cell clones*[a]

Clone Line	V	CDR3	J
TCRα			
BEV3.6	36, 25		
BEV3.9			
BEV3.10	27	PDRYNFNKF	21
ANN6	12.2	YFGNEKL	48
CAZ1.4	36	YTGTASKL	44
CAZ3.8	40	KAAGNKL	17
CAZ3.10	13		
TCRβ			
BEV3.6	5.1		
BEV3.9	9	SVLAGITDTQ	2.3
BEV3.10	11.3	RHALRGLGTQ	2.3
ANN6	7.7	SLRQGVYEQ	2.7
CAZ1.4	20.1	SGVTGTYEQ	2.7
CAZ3.8	9	SVLAGITDTQ	2.3
CAZ3.10			

[a] cDNA was made from RNA isolated from 1×10^6 clonal T cells. The table shows TCRα and TCRβ V region usage, the CDR3 sequence, and J region usage.

DR52b) (19). That is possible in DR52a because the Arg^{11} side-chain has been displaced to the "left" (i.e., along the floor of pocket 6) by the adjacent Tyr^{30} (to its "right"). The consequently shallower pocket 6 is suited well for the small Ala^{30} at peptide p6 (19).

In pocket 1, DR52a (like DR52b) again differs crucially from DR3 in its small/permissive Gly^{86} (Fig. 5). As expected, the bulkier Val^{86} of DR3 protrudes into this pocket (19, 23), and so prevents docking of such large p1 anchor residues as Trp^{25}, which is neatly accommodated in DR52a (19) as in other alleles (29, 30). From modeling studies, we calculate that pocket 1 has a ~50% greater volume in DR52a (198 $Å^3$) than in DR3 (137 $Å^3$). This latter should still accommodate less bulky anchors such as Phe, Ile, Val, or Met. Indeed, in some DR52a-restricted peptides, merely changing the Trp at p1 to a Phe is sufficient to allow significant presentation to some clones by DR3 as well as DR52a (17, 18). However, the equivalent $Trp^{25}\rightarrow$Phe, Ile, or Val variants of 21–38a were still not presented detectably to clone BEV3.6 by DR3 (or DR52b), but only by DR52a (80–100% of its maximal response). Despite adequate anchoring in pocket 1, they may still not dock recognizably in pockets 4 and 9 because of the contrasting absence of Arg^{11} and presence of Asp^{57} and its salt bridge in DR3 (see *Discussion*).

T cell receptors

The clones all used different TCR Vα and Vβ genes, apart from BEV3.9 and CAZ3.8, which used the same TRBV9*01.03 gene (Table II).

Strategies for specifically inducing unresponsiveness

Our first strategy was to engage these clones' TCRs without optimal costimulation. Because most human T cell clones express HLA-class II, they can bind and present peptides to each other, but they usually costimulate poorly, which leads to unresponsiveness and even apoptosis in our myasthenic clones (31).

We preincubated two FMAIT clones with peptide overnight without APCs and then challenged them with an optimal stimulus of peptide plus APCs the next day. Clone BEV3.6 was clearly inhibited almost completely by preexposure to the HPA-1a but not the HPA-1b peptide (Fig. 6). In contrast, clone ANN6 perceived the preexposure by itself as a maximal stimulus (not shown), suggesting that it has unusually strong costimulatory activity/responsiveness or distinctive cytokine reactivity.

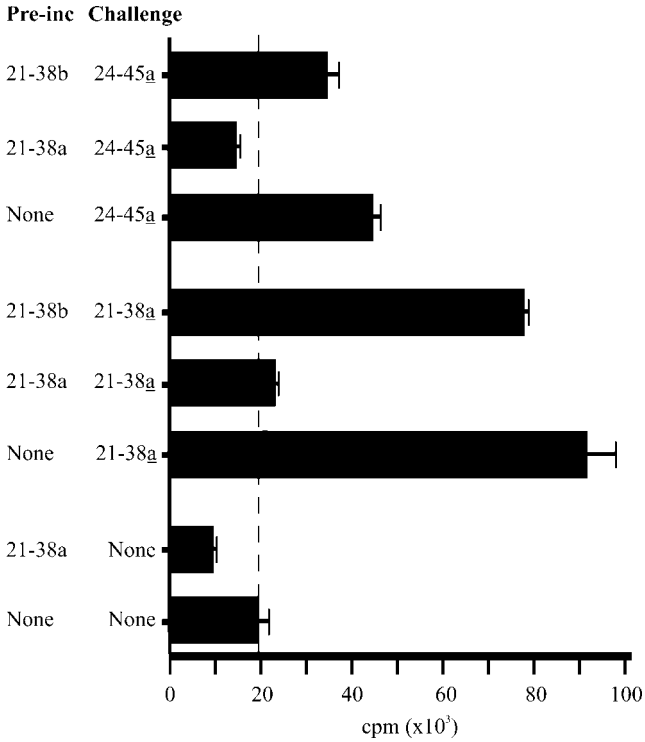


FIGURE 6. Inducing unresponsiveness specifically by preexposing clonal Th cells to free peptide. Clonal Th cells were preincubated overnight in the absence of APCs with the indicated peptide (pre-inc). The next day, they were washed, counted, and "challenged" with an optimal stimulus (the $DR52a^+DR3^+$ B cell line from NW prepulsed with the indicated HPA-1 peptide (10 μg/ml)); responses were assayed by [^{3}H]thymidine uptake. Dotted line indicates control with no Ag exposure.

Alternatively, Ag-presenting activity can be attenuated by pretreating APCs with corticosteroids (32) or IL-10 (33); since the therapeutic actions of intravenous immunoglobulin (IvIg) preparations in FMAIT are not understood, we also tested one of them in parallel. We preexposed $DR52a^+$ monocyte-derived DCs to IvIg (or human serum albumin as a control), dexamethasone, or IL-10 in the presence of peptide. Notably, the IvIg markedly down-modulated surface MHC and costimulatory molecules,

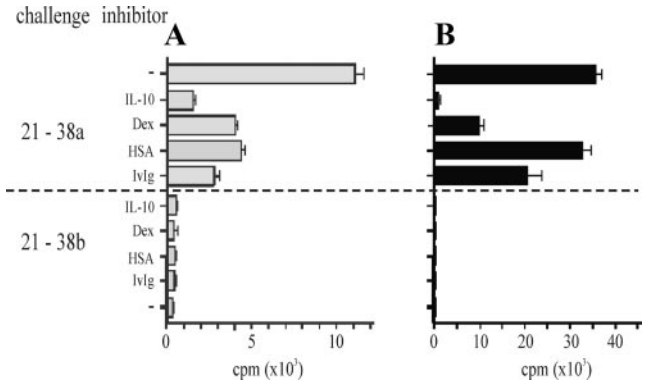


FIGURE 7. Testing potential immunotherapeutics on FMAIT T cell clones. We cultured DCs from NW ($DR52a^+DR3^+$) for 5 days and then pretreated them with/without the indicated therapeutic ("inhibitor") (for a further 24 h) with/without LPS (for the final 6 h) with/without the indicated peptide stimulus (for the final 3 h) before washing and coculturing with the indicated T cell clone. Results were almost identical with 21–38b and no peptide at all (not shown) as negative controls. *A*, Clone ANN6. *B*, Clone BEV3.6. Dex, dexamethasone; HSA, human serum albumin.

whereas IL-10 had less effect on CD40, and dexamethasone induced only minor changes (data not shown). Effects on Ag presentation to T cells were strikingly different. While dexamethasone and IL-10 consistently inhibited strongly (Fig. 7), effects were more variable with IvIg and were sometimes significant even with human serum albumin (Fig. 7).

Discussion

Here, for the first time, we have isolated and characterized six HPA-1a-specific $CD4^+$ T cell clones from alloimmunized women with FMAIT. They are all highly sensitive to the 25–33a epitope (with Leu^{33} instead of Pro^{33}), and particularly to whole HPA-$1a^+$ platelets. Our results from both serial truncations and Ala substitutions unequivocally demonstrate the anchor residues of this epitope for the HLA-DR52a molecule to which the clones are clearly restricted (14). Furthermore, structural modeling can explain both the undetectable presentation of 25–33a by the linked DR3 and the allelic DR52b class II molecules and the failure of the corresponding 25–33b to anchor in pocket 9 of DR52a. Taken together, these results strongly suggest that these clones' precursors had been primed in vivo in the donor FMAIT mothers and were potentially pathogenic. Thus, they appear suitable for testing generic or selective immunotherapeutic strategies such as pretreating the APCs with dexamethasone or IL-10, or engaging their T cell receptors without optimal costimulation.

Detecting specific T cells in FMAIT mothers

The first attempt to define the T cell alloimmune response in FMAIT used a circularized HPA-1a peptide spanning residues 26–45 to stimulate PBMCs cultured in bulk from an alloimmunized woman (2). The resulting short-term line responded to a linear 24–45a but not 26–45a peptide, which is consistent with the essential anchoring role (14) of Trp^{25} that our results now confirm. This mapping was supported by a report of ex vivo T cell responses to 15-mer peptides spanning the 19–33 region in HPA-1b/1b alloimmune women carrying HPA-1a fetuses. Responses were not significant to the corresponding HPA-1b peptides, or in unimmunized women or controls. HLA-DR52a was significantly overrepresented in these alloimmunized responders (3), but T cells were not cloned. Jackson et al. have recently shown responses to HPA-1a peptides 24–45 and 20–39 in bulk PBMC cultures in 21% of alloimmunized mothers, but also to the 1b peptide or the 1a and the 1b peptides simultaneously (34).

We succeeded in isolating clones from three mothers, who were between 5 and 8 mo postpartum. These clones all respond to HPA-$1a^+$ platelets, which clearly depends on DR52a^+ APCs and therefore on processing of their epitope from the platelets. It seems highly unlikely that these cloned T cells, with their sharply defined peptide specificities, were also responding to some other Ag coincidentally present on/in platelets with HPA-1a donors but not HPA-1b donors. Remarkably, the platelets delivered a much more potent stimulus (on a molar basis) to our clones than did the peptides, perhaps implying superefficient uptake/processing by HPA-1a-specific B cells and/or influences from the rest of the GpIIb/IIIa molecule/platelet. Indeed, their sensitivity to whole platelets is consistent with their being part of the response in vivo.

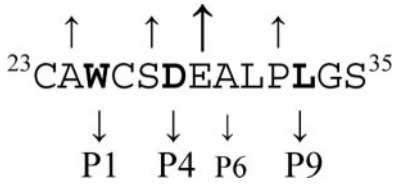
The ability to stimulate T cells with synthetic peptides was a major advantage for us. In our previous experience in MG patients, by contrast, almost all the T cell lines we initiated by peptide stimulation proved to be specific for unnatural AChR epitopes or even for synthetic errors, unlike those evoked by longer recombinant Ags (17, 35). That may reflect natural tolerance to most epitopes in the AChR autoantigen that would not be expected with the present alloepitope. Indeed, together with the previous reports (2, 3), the broadly similar specificities of the present clones for peptide and DR52a (and possibly their Th1 behavior) suggest that they are representative of the pathogenic response in the alloimmune mothers.

In fact, since ~30% of potential responder (i.e., HLA-DR52a^+/HPA-$1a^-$) mothers with an HPA-$1a^+$ fetus do respond, often in the first pregnancy, this alloantigen must somehow deliver a very potent priming stimulus. That could reflect the unexpected presentation by cell types such as trophoblast (36) or DCs, the ability of platelets themselves to express CD40L and costimulate T cells directly (37, 38), and/or the prevailing conditions during priming of the T cells in pregnancy that might alter the cytokine balance and so explain a Th1 response.

The background "responsiveness" of some clones to allogeneic APCs, despite their clear specificity for the 25–33a alloepitope presented by autologous APCs, was a persistent and inconvenient finding. In theory, it might reflect prior loading of the stimulator APC's DR52a by the HPA-1a epitope, or possibly by some cross-reactive sequence (as high backgrounds did not always correlate with HPA-1a expression by the APCs). However, other integrin sequences are so different that they seem very unlikely to engage the same TCRs or even to bind to HLA-DR molecules. A simpler explanation may be that some clones are more sensitive to cytokines released by the APCs.

The HPA-1a epitope and its presentation by DR52a

There is compelling evidence that DR52a confers particularly high responsiveness (12, 13), apparently by presenting the HPA-1a epitope (14). Moreover, our evidence from T cell recognition strongly supports the predictions of Wu and colleagues (14) concerning both the anchor residues in the peptides presented by DR52a and the core of the HPA-1a epitope, which have now been confirmed in a crystal structure (19). Taking these together with our own previous findings (17), we summarize the main anchor residues (↓) and T cell contact sites (↑) thus:



There is now a clear structural basis for the selective presentation of the HPA-1a variant by DR52a, reflecting the efficient anchoring of its Leu^{33} in the singularly receptive hydrophobic pocket 9 of DR52a, docking that seems almost impossible with the Pro^{33} of HPA-1b (19). Indeed, Pro is not a recognized anchor at p9 in epitopes restricted to any HLA-DR molecules nor in peptides eluted from them (39). In sharp contrast, provided that they are hydrophobic, several other α-amino acids can clearly anchor efficiently there, even including Ala^{33} for some of our T cells (Fig. 4) (as in the human AChR (17)), although other clones prefer a larger hydrophobic side-chain. The strong presentation of the variant with Val^{33} (Fig. 4) is interesting because its natural counterpart has recently been identified; although it reacts variably with anti-HPA-1a alloantisera (25), it was clearly recognized by clone BEV3.6 and could therefore be immunogenic. The individual preferences of some clones must reflect the differences in their TCRs, though any complete explanation requires detailed co-crystal structures.

In the case of DR3, an obvious reason for its failure to present HPA-1a 25–33 epitopes is that its Val^{86} restricts access to pocket 1 of such bulky p1 anchor residues as the Trp^{25} (17, 18). As in many DR alleles, that alone would preclude optimal docking in

DR3, as predicted by modeling and by the crystallographic structure of DR52a (19). In contrast, the smaller Gly^{86} of DR52a (and other alleles) is more permissive, even for a Trp at p1 (17). Previously, we could readily adapt two different DR52a-restricted AChR epitopes for presentation by DR3 to some T cells (although not all) merely by substituting their Trp at p1 with a less bulky anchor residue (17, 18). Our failure to achieve that here with equivalent replacements in the 25–33a epitope, even with a concomitant Leu^{33}→Ala at p9, suggests overriding importance of optimal docking in pocket 9 (and possibly pocket 4, too) for this epitope. Indeed, the very origins of our clones depend on their ability to distinguish Leu^{33} from Pro^{33} at p9. However well we may have adapted these variants for docking in pocket 1, their recognition nearer their C termini is evidently prevented by the DR3↔ DR52a differences in pockets 4 and especially 9. These result from 1) the reportedly unique availability of Arg^{11} in pocket 4 (19), 2) the unusually intimate docking of Leu^{33} into pocket 9 of DR52a, and 3) the absence in DR52a of the salt bridge (in both DR3 and DR52b) that would otherwise cross from Asp^{57} in DRβ to the Arg^{76} in DRα, which now becomes free to H bond to the peptide backbone at Gly^{34}-Ser^{36} (19) (Fig. 5). By contrast, our AChR-α-specific clones apparently focus more on the N terminus of their epitope (17); while some of them are less sensitive to these differences in pockets 4 and 9 in DR52a, another is greatly influenced by interactions in DR4 between pockets 1, 4, and 9 (40). Considerations 1–3 apply almost equally to DR52b, which has an additional Arg^{74}→Gln substitution overhanging pocket 4.

Thus, these critical effects of the replacements of Asp^{57}↔Val, Val^{86}↔Gly, and possibly Ser^{11} (or Leu^{11})→Arg^{11} from DR3 (or DR52b)→DR52a can neatly explain the striking predisposition to FMAIT by DR52a, rather than by DR52b (or DR3) or indeed by other common MHC alleles. These considerations preclude presentation of the HPA-1a epitope by all other common HLA-DR alleles. In theory, there could be a second essential MHC allele that happens to be linked to DR52a but not DR52b, like those near TNF or HLA-B8 that predispose to MG (41), or presentation by a much less common HLA-DR allele.

The evident focus of the present T cell clones on pocket 9, the C terminus of the epitope, with its critical Leu^{33}, may also explain the weaker response to the 23–45 peptide, even by clones first raised against it (Fig. 1). That may reflect cyclization (2), altered peptide conformation, or hindrance caused by a longer C-terminal overhang, as noted in mouse IE (42) and proposed previously for this epitope (3); presumably, that is avoided during processing of the natural epitope from whole platelets in responder subjects.

These T cells also vary quantitatively in their requirements for an N-terminal Ala^{24} in the peptides (Fig. 3). Such variation has been noted previously (e.g., Ref. 17); a more complete understanding would be important for the design and evaluation of antagonist peptides for immunotherapy.

Prospects for immunotherapy

Finally, with its high degree of specificity both for this single Leu^{33}→Pro substitution and for DR52a, the pathogenic response in FMAIT should be eminently suitable for selective therapy (e.g., with antagonist peptides). Indeed, because of their subtly different fine specificities, these T cell clones may prove useful both for tailoring such peptides to suit the pathogenic repertoire(s) and for testing their efficacy.

Other immunotherapies may also be suitable in FMAIT, as suggested by our initial experiments with DCs preexposed to dexamethasone or IL-10, or with peptide-MHC presented without optimal costimulation (Fig. 7). Interestingly, the effects of IvIg were less complete and consistent, despite recent reports of inhibition of Ag-specific T cell responses with IvIg in vitro (43). The inconsistency we observed may reflect its variable efficacy in clinical practice and/or differences between batches (5). Comparisons of the effects of IvIg, IL-10, and dexamethasone should increase understanding of their molecular modes of action. Furthermore, it should be possible to detect any adverse enhancement of specific T cell responses or Ab titers that any of these treatments might unexpectedly cause. Indeed, FMAIT offers a safe context in which to test such therapies, since the mothers themselves are unaffected.

In conclusion, we have analyzed the molecular specificity of HPA-1a-alloimmune T cell clones in FMAIT. Our results clearly confirm the predicted anchor residues and core of their epitope. Molecular modeling neatly explains the highly selective association with HLA-DR52a in NAIT, which correlates with the much more favorable docking specifically of the 25–33a epitope in DR52a than in DR3 or DR52b. The clones show some heterogeneity in fine peptide specificity and in the repertoire of TCRs they use. Clearly, the least diverse component of this trimolecular recognition is the peptide-HLA-DR52a complex. It therefore seems the most promising for use as a specific therapeutic (44), for which our clones should be a suitable test bed.

Acknowledgments

We thank the staff in the Histocompatibility and Immunogenetics Laboratory, National Blood Service Bristol for HLA typing, Dr. R. E. Esnouf for giving us access to the VOLUMES program, Dr. K. Di Gleria for peptides, and all the donors for blood samples.

Disclosures

The authors have no financial conflicts of interest.

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