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Insights into HLA-Restricted T Cell Responses in a Novel Mouse Model of Dengue Virus Infection Point toward New Implications for Vaccine Design

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The frequency of dengue virus (DENV) infection has increased dramatically in the last few decades, and the lack of a vaccine has led to significant morbidity and mortality worldwide. To date, a convenient murine system to study human T cell responses to DENV has not been available. Mouse transgenic for HLA are widely used to model human immune responses, and it has been shown that mouse-passaged DENV is able to replicate to significant levels in IFN-α/βR−/− mice. To cover a wide range of HLA phenotypes, we backcrossed IFN-α/βR−/− mice with HLA A*0201, A*0101, A*1101, B*0702, and DRB1*0101-transgenic mice. A DENV proteome-wide screen identified a total of 42 epitopes across all HLA-transgenic IFN-α/βR−/− strains tested. In contrast, only eight of these elicited responses in the corresponding IFN-α/βR−/− mice. We were able to identify T cell epitopes from 9 out of the 10 DENV proteins. However, the majority of responses were derived from the highly conserved nonstructural proteins NS3 and NS5. The relevance of this model is further demonstrated by the fact that most of the epitopes identified in our murine system are also recognized by PBMC from DENV-exposed human donors, and a dominance of HLA B*0702-restricted responses has been detected in both systems. Our results provide new insights into HLA-restricted T cell responses against DENV, and we describe in this study a novel murine model that allows the investigation of T cell-mediated immune mechanisms relevant to vaccine design.

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Abbreviations used in this article: APL, altered peptide ligand; DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; E, envelope; GE, genomic equivalent; NCBi, National Center for Biotechnology Information; NS, nonstructural; SFC, spot-forming cell.

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tion, resulting in higher viral loads and more severe disease via a phenomenon known as Ab-dependent enhancement (17, 18). Another hypothesis postulates that T cells raised against the original infecting serotype dominate during a secondary heterologous infection in a phenomenon termed “original antigenic sin” (19, 20). In sequential DENV infections, due to the 67–75% sequence homology between different serotypes (21), memory T cells from a primary DENV infection with one serotype respond to a secondary infection by a different serotype. However, these memory cells have a lower threshold for activation than naïve cells (22) and possibly a lower affinity to Ags from the current infection that might lead to an altered response, including delayed viral clearance and immunopathology (19).

This suspected dual role of T cells in protection and pathogenesis is difficult to study in humans because in most donor cohorts, the time point of initial infection and, in the case of secondary infections, the sequence of infection, are unknown and thus do not allow direct correlations with T cell responses. A mouse model, which allows the investigation of adaptive immune responses restricted by human MHC molecules to DENV infection, would greatly contribute to shed light on the role of T cells in protection and/or pathogenesis. Mice transgenic for HLA are widely used to study T cell responses restricted by human MHC molecules, and studies in other viral systems have shown the valuable impact of HLA-transgenic mice in epitope identification (23–25). The host specificity of DENV is extremely narrow, and thus DENV does not replicate in wild-type mice. However, it has been shown that mice lacking the IFN-α/β receptor support a productive infection and allow the study of T cell responses after DENV infection (8, 9, 26). To cover a wide range of HLA phenotypes, we backcrossed IFN-α/βR−/− mice with HLA, A*0201, A*0101, A*1101, B*0701, and DRB1*0101-transgenic mice and determined the T cell response against infection with DENV. We applied bioinformatics predictions on HLA affinity to identify T cell targets in the DENV proteome.

By combining the bioinformatics approach and the HLA-transgenic mouse model, we were able to identify 42 T cell epitopes derived from 9 out of the 10 DENV proteins. The majority of responses, however, were directed against epitopes from the nonstructural proteins NS3 and NS5; 80% of the epitopes identified in HLA-transgenic mice were also able to elicit a T cell response in human donors, previously exposed to DENV. Thus, the mouse model described in this study reflects the response patterns observed in humans, making it a valuable model to study epitopes of human relevance during infection with DENV.

Materials and Methods

Viral stocks
S221 is a plaque-purified DENV2 strain, which was derived from the clinical isolate PL046 (27), by passing through IFN-α/βR−/−; IFN-γR−/− and mosquito cells as described previously (9, 28). Viral stocks were amplified in C6/36 mosquito cells and purified over a sucrose gradient, as described previously (29). Infectious doses were determined based on genome amplification in C6/36 mosquito cells and plaque assay on baby hamster kidney cells as described previously (30).

Mice and infections
HLA-A*0201/Kb, A*1101/Kb, A*0101, B*0702, and DRB1*0101-transgenic mice were bred at the La Jolla Institute for Allergy and Immunology facility (La Jolla, CA) as previously described (23, 25, 31, 32). IFN-α/βR−/− mice on the C57BL/6 background were originally obtained from Dr. W. Yokoyama (Washington University, St. Louis, MO) and subsequently bred and maintained in-house. All transgenic mouse strains were subsequently backcrossed with the IFN-α/βR−/− mice at the animal facility at the La Jolla Institute for Allergy and Immunology. Mice were used between 6 and 10 wk of age. For all experiments, mice were infected retro-orbitally with 1010-GE S221 (∼2 × 104 PFU) in 100 μl PBS. On day 7 postinfection, the mice were sacrificed, and splenic CD8+ or CD4+ T cells were used in mouse IFN-γ ELISPOT assays. All mouse experiments were performed following Institutional Animal Care and Use Committee-approved animal protocols.

Bioinformatic analyses
All 9- and 10-mer peptides encoded in the DENV2 proteome were predicted for their binding affinity to MHC class I alleles A*0201, A*0101, A*1101, and B*0702. Binding predictions were performed using the command-line version of the MHC class I consensus prediction tool available on the Immune Epitope Database (http://www.iedb.org) Web site (33). Peptides were selected if they were in the top 1% of binders in a given strain. For MHC class II binding predictions, all 15-mer peptides were predicted for their binding affinity to the DRB1*0101 allele using the consensus approach as previously described (34). The top 2% of predicted binders were then selected for synthesis. All peptides tested in this study were derived from the DENV2 virus strain S221, which was also used as the infectious agent in this study, as described above.

For the conservancy analysis, full-length DENV polyprotein sequences were retrieved for each serotype from the National Center for Biotechnology Information (NCBI) Protein database using the following query: txd11053 AND 3000:5000[plen]. The number of isolates from any one country was limited to 10 to eliminate geographical bias. Sequences were considered unique if they varied by at least 1 aa from all other sequences. In summary, we retrieved 162 DENV1, 171 DENV2, 169 DENV3, and 53 DENV4 sequences from the NCBI Protein database and investigated the conservancy of the identified epitopes within the sequences of the respective serotypes.

Peptide synthesis
All peptides used in this study were synthesized by Mimotopes (Victoria, Australia). A total of 431 9-mer and 10-mer peptides were identified by MHC class I predictions, as described above, and made as crude material. Peptides were combined into pools of 10 individual peptides, according to their predicted HLA restriction. MHC class II predictions resulted in the synthesis of 12 15-mer peptides, which were tested individually.

MHC peptide-binding and restriction assays
Purification of HLA A*0201, A*0101, A*1101, B*0702, and DRB1*0101 MHC molecules and quantitative assays to measure the binding affinity of peptides to purified MHC were performed as described elsewhere (35, 36). Binding assays are based on competitive inhibition of binding of a radio-labeled standard peptide. Briefly, after a 2-d incubation, inhibition of binding of the radiolabeled probe peptide to the corresponding MHC molecule, in the presence of various doses of a pest peptide, was determined by capturing MHC/peptide complexes on Greiner Lumintrace 600 microplates (Greiner Bio-One, Monroe, NC) coated with either the W6/32 (HLA class I-specific) or L243 (HLA DR-specific) mAbs. Bound cpm were then measured using the Topcount microscintillation counter (Packard Instrument, Meriden, CT). The concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe peptide (IC50) was then calculated.

The tumor cell line 721.221 (37), which lacks expression of HLA-A, -B, and -C class I genes, was transfected with the HLA-A*0201/Kb or HLA-A*1101 chimeric genes and used as APCs in the restriction assays. The nontransfected cell line was used as a negative control.

Human blood samples
Peripheral blood samples were obtained from healthy adult blood donors from the National Blood Center, Ministry of Health, Colombo, Sri Lanka, in an anonymous fashion. Donors are of both sexes and between 18 and 60 yr of age. PBMC were purified by density gradient centrifugation (Ficoll-Paque Premium; GE Healthcare Biosciences, Kowloon, Hong Kong). Cells were suspended in FBS (Gemini Bio-products, Sacramento, CA) containing 10% DMSO and cryo-preserved in liquid nitrogen. DENV seropositivity was determined by dengue IgG ELISA as previously described (38). Flow cytometry-based neutralization assays were performed for further characterization of seropositive donors, as previously described (39).

Genomic DNA isolated from PBMC of the study subjects by standard techniques (QiAmp; Qiagen, Valencia, CA) was used for HLA typing. High-resolution Luminex-based typing for HLA class I and II was used according to the manufacturer’s protocol (Sequence-Specific Oligonucleotides typing; One Lambda, Canoga Park, CA). Where needed, PCR-based
methods were used to provide high-resolution subtyping (Sequence-Specific Primer typing; One Lambda). Based on the HLA phenotype, 15 DENV-seropositive and 5 DENV-seronegative donors have been selected for the validation experiments described in this study.

**IFN-γ ELISPOT assay**

For all murine experiments, splenic CD4+ or CD8+ T cells were isolated by magnetic bead positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany) 7 d postinfection with the S221 strain of DENV2. A total of 2 × 10^5 T cells were stimulated with 1 × 10^5 uninfected splenocytes as APCs and 10 μg/ml individual DENV peptides in 96-well flat-bottom plates (Immobilon-P; Millipore, Bedford, MA) coated with anti-IFN-γ mAb (clone 1-D1K, Mabtech, Stockholm, Sweden). Each peptide was tested in triplicates. Following a 20-h incubation at 37°C, the wells were washed with PBS/0.05% Tween 20 and then incubated with biotinylated IFN-γ mAb (clone R4+6A2; Mabtech) for 2 h. The spots were developed using Vectastain ABC peroxidase (Vector Laboratories, Burlingame, CA) and 3-amin-9-ethylcarbazole (Sigma-Aldrich, St. Louis, MO) and counted by computer-assisted image analysis (KS-ELISPOT reader; Zeiss, Munich, Germany). Responses against peptides were considered positive if the net spot-forming cells (SFC) per 10^6 were ≥20, had a stimulation index of ≥2, and a p value <0.05 in a t test comparing replicates with those from the negative control.

For ELISPOT with human cells, 2 × 10^5 PBMC/ml was stimulated in the presence of 1 μg/ml individual peptide for 7 d. Cells were cultured at 37°C, 5% CO2, and recombinant IL-2 (10 U/ml; eBioscience, San Diego, CA) was added 3 d after antigenic stimulation. After 7 d, PBMC were harvested, and 1 × 10^5 cells were added to each well of a 96-well flat-bottom plate (Millipore). The IFN-γ ELISPOT assay was performed as described above, except the mAb 1-D1K (Mabtech) and mAb 7-B6-1 (Mabtech) were used as coating and biotinylated secondary Ab, respectively. All epitopes identified in the murine study have been tested in all of the donors and subsequently assigned to the HLA-matched or non-HLA-matched group according to the actual HLA phenotype of the donors. To be considered positive, IFN-γ responses needed to exceed the threshold set as the mean responses of HLA nonmatched and DENV-seronegative donors plus three times the SD.

**Results**

**A novel system to identify DENV-specific HLA A*0201 epitopes**

Mice transgenic for HLA are widely used to study T cell responses restricted by MHC molecules, and it has been shown that mouse-passaged DENV is able to replicate to significant levels in HLA-α/β⁻/⁻ mice. In this study, we backcrossed HLA A*0201-transgenic and IFN-α/β⁻/⁻ mice strains as a model system to study DENV-specific HLA-restricted T cell responses. These mice were then infected with the mouse adapted DENV2 strain S221, and purified splenic T cells were used to study the anti-DENV CD8+ T cell responses.

To this end, a panel of 116 predicted A*0201-binding peptides were generated using bioinformatics predictions previously used to define HLA-restricted CD8+ responses in the vaccinia virus system (40). Predicted HLA A*0201-binding peptides were combined into pools of 10 individual peptides and tested in an IFN-γ ELISPOT assay using CD8+ T cells from HLA-transgenic IFN-α/β⁻/⁻ and IFN-α/β⁻/⁺, DENV2-infected mice, respectively (data not shown). Positive pools were deconvoluted, and the individual peptides were tested in two independent experiments. Using this approach, we identified a single peptide in the HLA A*0201 IFN-α/β⁻/⁺ mice (NS5_{5955–6060} , Fig. 1A, white bars), whereas screening in HLA A*0201 IFN-α/β⁻/⁺ mice lead to identification of this epitope and 10 additional epitopes (Fig. 1A, black bars). The CD8+ T cell responses were higher in the knockout mice than the wild-type mice. Even increasing the infectious dose 10-fold did not lead to an increase in magnitude or repertoire in the HLA A*0201 IFN-α/β⁻/⁺ mice (data not shown). These results demonstrate that the use of HLA A*0201-transgenic IFN-α/β⁻/⁻ mice allows for the discovery of a stronger and broader T cell response.

**Broad population coverage by additional HLA-transgenic IFN-α/β⁻/⁻ mouse strains**

We next addressed whether similar observations could be made by measuring T cell responses in other HLA-transgenic IFN-α/β⁻/⁻ and IFN-α/β⁻/⁺ mice. For this purpose, we backcrossed IFN-α/β⁻/⁻ mice with HLA A*0101, A*1101, and B*0702-transgenic mice. These alleles were chosen as representatives of three additional HLA class I supertypes (A1, A3, and B7, respectively).

Screening in HLA A*0101 and A*1101-transgenic IFN-α/β⁻/⁻ mice revealed 9 HLA A*0101-restricted (Fig. 1B, black bars) and 16 A*1101-restricted epitopes (Fig. 1C, black bars), respectively. In the case of the HLA A*0101-transgenic wild-type mice, no epitope could be detected, whereas the HLA A*1101-transgenic mice showed an overlap of five epitopes with the corresponding IFN-α/β⁻/⁻ strain (M111-120, NS3_{1608–1617}, NS4B_{2286–2296}, NS4B_{2315–2323}, and NS3_{3112–3121}). Two of these epitopes were able to elicit a stronger response in the HLA A*1101 IFN-α/β⁻/⁺ mice compared with the IFN-α/β⁻/⁻ strain (M111-120 and NS4B_{2287–2296}). All other responses observed were more robust in the IFN-α/β⁻/⁻/+ mice. It should be noted that for several epitopes in NS5, inconsistent responses were observed in the HLA A*1101 IFN-α/β⁻/⁻/+ mice. In these cases, positive responses were noted only in one out of two independent experiments, as also reflected by the large SD. Our stringent criteria of positivity requires consistent responses in two out of two independent experiments, as described in the figure legend. For the same reason, we have excluded some of the inconsistent responses observed in the IFN-α/β⁻/⁻/+ mice (Fig. 1A, NS4A_{2150–2159} ff., 1B, NS3_{1607–2087}). In our experience, the application of these stringent criteria is important for defining the most robust and thereby most relevant epitopes.

To extend our observations to mice transgenic for an HLA B allele, we infected HLA B*0702-transgenic IFN-α/β⁻/⁻ and IFN-α/β⁻/⁺ mice and compared epitope recognition between the two strains. We identified 15 B*0702-restricted epitopes in the IFN-α/β⁻/⁻ strain (Fig. 1D, black bars), with one of these also being detected in the corresponding IFN-α/β⁻/⁺ mice (NS4B_{2280–2296}; Fig. 1D, white bars). Similar to the other HLA-transgenic mouse strains, the responses observed in the HLA B*0702-transgenic IFN-α/β⁻/⁻/+ mice were not only broader but also >10-fold higher in magnitude. The single epitope recognized in the IFN-α/β⁻/⁺ strain elicited an IFN-γ response of 50 SFC/10^6 CD8+ T cells compared with an average of 857 SFC/10^6 CD8+ T cells in the IFN-α/β⁻/⁻/+ mouse.

**Dengue-specific T cell responses in an MHC class II-transgenic mouse model**

To determine if the observations made in the case of MHC class I-transgenic mice were also applicable to MHC class II-restricted responses, we determined the antigenicity of HLA DRB1*0101 predicted binding peptides in HLA DRB1*0101, IFN-α/β⁻/⁻, and IFN-α/β⁻/⁺ mice, respectively. Using the same experimental conditions described above for the MHC class I-transgenic mice, we infected HLA DRB1*0101, IFN-α/β⁻/⁻, and IFN-α/β⁻/⁺ mice with DENV2 and isolated CD4+ T cells 7 d postinfection. A panel of 12 predicted S221-specific peptides predicted to bind DRB1*0101 was then tested for the ability to elicit IFN-γ production by ELISPOT. Five epitopes that could be identified in the DRB1*0101 IFN-α/β⁻/⁻/+ mice in two independent experiments (Fig. 2, black bars) were identified. Similarly to the observation in MHC class I-transgenic mice described above, only a single peptide could be identified in the corresponding DRB1*0101 IFN-α/β⁻/⁺ mice (NS2A_{1999–1235}; Fig. 2, white bars). This epitope in the IFN-α/β⁻/⁺ did not represent a novel epitope, as it was also...
observed in the corresponding IFN-αβR−/− mice. Similar to the MHC class I-transgenic mice, all observed responses were stronger in the IFN-αβR−/− mice than their wild-type counterparts.

In summary we identified a total of 55 epitopes in the HLA-transgenic IFN-αβR−/− mice, whereas the same screen in HLA-transgenic IFN-αβR+/+ mice only revealed 8 epitopes. Importantly, these eight epitopes were also detected in the HLA-transgenic IFN-αβR−/− mice. The broader repertoire seen in IFN-αβR−/− mice, as well as the stronger and more robust IFN-γ responses, suggest that HLA-transgenic mice, backcrossed with IFN-αβR−/− mice, represent a more suitable model to study T cell responses to DENV infection than HLA-transgenic wild-type mice, presumably because the latter do not establish a productive infection.

Mapping optimal epitope in respect to peptide length

Within the 50 MHC class I-restricted epitopes identified, 9 pairs of nested epitopes were identified in which the 10-mer as well as a nested 9-mer peptide was able to elicit an immune response (Fig. 1). To determine which peptide in each nested epitope pair was the optimal epitope, we employed peptide titration assays as shown in Fig. 3A. For one epitope (NS4A 2205–2213), both the 9- and the 10-mer displayed similar kinetics upon peptide titration (Fig. 3A). Because the 9-mer was able to elicit slightly higher responses in all conditions tested, we considered the 9-mer version of this epitope for further experiments. In all other cases, an optimal epitope length peptide could be unequivocally identified.

Similarly, for two of the B*0702-restricted epitopes (NS4B 2296–2305 and NS5 2646–2655), 8- and 9-mer peptides carrying alternative dominant B7 motifs were synthesized and tested for T cell recognition. In one case, the corresponding 8-mer (NS4B 2296–2304) showed dominant IFN-γ responses compared with the 9-mer. In the other case, the 10-mer originally identified (NS5 2646–2655) was able to elicit higher responses than the newly synthesized 8- and 9-mer. In both cases, the optimal epitope length peptide could be unequivocally identified.
Seven days postinfection, CD4+ T cells were purified and tested against a panel of DENV2 predicted peptides. The data are expressed as mean number of IFN-γ ELISPOT responses per 10^6 CD4+ T cells of two independent experiments. Error bars represent SEM. Responses against peptides were considered positive if the stimulation index exceeded double the mean negative control wells (effector cells plus APCs without peptide), and net spots were above the threshold of 20 SFC/10^6 CD4+ T cells in two individual experiments. Asterisks indicate peptides, which were able to elicit a significant IFN-γ response, according to the criteria described above.

Further characterization of the identified epitopes

Of the five HLA-transgenic mouse strains tested, only the A*0201 and the A*1101-transgenic strains coexpressed murine MHC molecules together with the respective HLA molecule. Thus, it was necessary to assure that the observed responses were restricted by the human HLA class I molecule and not by murine class I. We therefore tested purified T cells for their capacity to recognize the specific epitopes when pulsed on APC expressing only human class I. For this purpose, we used the tumor cell line 721.221, which is negative for expression of any human or murine class I molecule, and transfected it with either HLA A*0201 or HLA*A1101. As shown in Fig. 4A, all 10 HLA*A0201-restricted epitopes were recognized when presented by APC exclusively expressing HLA*A0201 molecules. Nine out of 13 of the HLA*A1101-restricted epitopes identified stimulated a CD8+ T cell response when presented exclusively on HLA*A1101 molecules (Fig. 4B). When the four remaining epitopes were tested in non-HLA-transgenic IFN-α/βR−/− mice as described above, all elicited a significant T cell response (data not shown). Furthermore, one of the epitopes has already been described to be recognized by T cells from DENV2-infected BALB/c mice [E633-642 (41)]. We therefore consider these four epitopes (M111-120, E274-282, E633-642, and NS4B2280-2289) as solely mouse MHC restricted and excluded them from our further studies. Among those epitopes were also the two epitopes that were able to elicit a stronger response in the HLA A*1101 IFN-α/βR−/− mice compared with the IFN-α/βR−/− strain (M111-120 and NS4B2287-2296).

To further confirm the MHC restriction of the identified epitopes inferred on the basis of the bioinformatic predictions, we measured their MHC-binding capacity to their predicted allelic molecule using purified HLA molecules in an in vitro binding assay. The results of these assays are also shown in Table I. Thirty-two of the 42 tested peptides (76%) bound the corresponding predicted allele with high affinity as indicated by an IC50 < 50 nM. Sixteen out of these even showed an IC50 < 10 nM and can therefore be considered as very strong binders. Of the remaining peptides, 7 (17%) were able to bind the predicted allele with intermediate affinities, as indicated by IC50 < 150 nM. Only three of the identified epitopes (7%) bound with low affinity, showing an IC50 value between 500 and 1500 nM. A summary of all 42 epitopes identified, after conclusion of these experiments and elimination of redundancies is shown in Table I.

Validation of the identified epitopes in human DENV-seropositive donors

To validate the epitopes identified in the HLA-transgenic IFN-α/βR−/− mice, we tested the capacity of these epitopes to stimulate PBMC from human donors previously exposed to DENV as determined by ELISA (IgG OD450 mean = 1.5; range 0.8–1.9). The limited sample size did not allow us to detect a significant correlation between the level of seropositivity and the robustness of T cell response. Because the IFN-γ response to these peptides was not detectable directly ex vivo (data not shown), we re-stimulated HLA-matched PBMC from the four respective peptides and IL-2. As a control, we restimulated PBMC from donors that expressed neither the exact HLA molecule nor one from the same supertype, as well as PBMC from DENV-seronegative donors. The average IFN-γ response from these control donors, plus three times the SD, was set as a threshold of positivity. Fig. 5A–D (HLA A*0101, A*0201, A*1101, and B*0702) show the capacity of the identified epitopes to stimulate PBMC from the various donor categories. Each of the A*0101 and A*0201 epitopes was antigenic in at least one HLA-matched donor, although the magnitude as well as the frequency of responses was higher for the A*0201-restricted epitopes (Fig. 5A, 5B, Table I). Out of the nine A*1101-restricted epitopes, three have been detected once in HLA-matched donors. These three epitopes, though, have been able to stimulate a robust IFN-γ response, as indicated by net SFC > 800 (Fig. 5C). In the case of the B*0702-restricted epitopes, 10 out of the 12 were antigenic in one or more HLA-matched donors, as shown in Fig. 5D and Table I. Interestingly, the two epitopes, not detected in human donors, were also demonstrated to require high concentrations in the HLA B*0702 IFN-α/βR−/− mice (NS4B2280-2289, Fig. 3A, and NS52546-2655, Fig. 3B), which suggests that these epitopes might be more weakly stimulatory and maybe less relevant. No significant response could be detected in non-HLA–matched donors tested. In contrast, all five restricted DRB1*0101 epitopes have been detected in three out of four HLA-matched donors tested and were able to elicit significant IFN-γ responses in non-HLA–matched donors. This is in accordance with recent reports demonstrating a high degree of repertoire sharing across MHC class II molecules (42). Overall, we could detect responses to 34 of the 42 epitopes in at least one donor, which corresponds to an overlap of 81% between the murine and human system. In addition to the
we also performed an Immune Epitope Database query (43) with the epitopes identified in the mouse model. By this analysis, we found that 13 of our 42 epitopes were previously described to elicit an IFN-γ response in DENV-seropositive individuals, as indicated in Table I. Thirty percent overlap with known epitopes contributes to the validation of our mouse model and shows, in contrast, that 70% of the epitopes identified are novel, contributing to an extended knowledge of T cell-mediated responses to DENV.

**Experimental approach**

A especially notable observation in this study was that out of all HLA-transgenic mouse strains tested, the strongest CD8+ T cell responses were detected in the B*0702-transgenic IFN-α/βR−/− mice infected with 1 × 10^{10} GE of DENV2, and spleens were harvested 7 d postinfection. CD8+ T cells were purified and incubated for 24 h with ascending concentrations of nested peptides. A shows pairs of nested epitopes in which the 10-mer as well as a nested 9-mer peptide were able to elicit an immune response. B shows two B*0702-restricted epitopes in which 8- and 9-mer peptides carrying alternative dominant B7 motifs were synthesized and tested for T cell recognition. The peptides, which were able to elicit stronger IFN-γ responses at various concentrations, were then considered the optimal epitope.

**Figure 3.** Determination of the optimal epitope. To determine the optimal epitope, HLA-transgenic IFN-α/βR−/− mice were infected with 1 × 10^{10} GE of DENV2, and spleens were harvested 7 d postinfection. CD8+ T cells were purified and incubated for 24 h with ascending concentrations of nested peptides. A shows pairs of nested epitopes in which the 10-mer as well as a nested 9-mer peptide were able to elicit an immune response. B shows two B*0702-restricted epitopes in which 8- and 9-mer peptides carrying alternative dominant B7 motifs were synthesized and tested for T cell recognition. The peptides, which were able to elicit stronger IFN-γ responses at various concentrations, were then considered the optimal epitope.
mice. Four B*0702-restricted epitopes were able to elicit an IFN-γ response of 1000 SFC/10^6 CD8+ T cells (Table I, column 4). On average, B*0702 epitopes were able to elicit an IFN-γ response of 877 SFC/10^6 CD8+ T cells compared with an average of 350, 365, and 476 SFC/10^6 CD8+ T cells for the HLA A*0101-, A*0201-, and A*1101-restricted epitopes, respectively (Fig. 5F, black bars).

Most interestingly, the exact same response pattern could be observed testing PBMC from HLA-matched donors previously exposed to DENV (Fig. 5F, white bars). As seen in mice, out of all epitopes tested, B*0702-restricted epitopes were able to elicit the strongest IFN-γ responses when tested in HLA-matched human donors, reaching an average of 688 SFC/10^6 PBMC, followed by an average of 530, 423, and 119 SFC/10^6 PBMC for HLA*1101-, A*0202-, and A*0101-restricted epitopes, respectively (Table I, column 5). Considering that the human PBMCs were stimulated for 7 d in the presence of IL-2, compared with 1 d for murine cells, the magnitude of responses observed with the two approaches cannot be compared. However, it is interesting that the hierarchy of responses observed in both systems is the same. The highest responses are seen in B*0702, followed by A*1101, DRB1*0101, and A*0201, and the weakest responses in both systems are observed for A*0101. The fact that the mouse model described in this study reflects response patterns observed in humans makes it an even more valuable model to study epitopes of human relevance during infection with DENV.

Protein location of identified epitopes

As shown in Fig. 6, the identified epitopes are derived from 9 out of the 10 DENV proteins, with the membrane protein being the only protein for which no epitope could be detected. The majority of epitopes, however, were derived from the seven nonstructural proteins. Thirty-nine out of 42 of the identified epitopes (93%) originate from the 7 NS proteins, accounting for 97% of the total IFN-γ response observed. Within the NS proteins, NS3 and NS5 alone account for 67% of the total response, representing a total of 23 epitopes detected from these two proteins. Furthermore, NS5 was the only protein in which at least one epitope was identified in all five HLA-transgenic mouse strains. These results support the previously reported high immunogenicity of NS3 and also suggest NS5 as a major target of T cell responses.

Conservancy of identified epitopes within the DENV2 serotype

Cross-reactivity of T cells is a well-described phenomenon in the context of DENV infection (5). To circumvent this issue, we have exclusively tested T cell reactivity to S221-derived peptides, which was also used as infectious agent in this study. However, to
assess the relevance for infections with other DENV2 strains, we investigated the conservancy of the S221-derived epitopes within other strains of the DENV2 serotype. We retrieved 171 full-length DENV2 polyprotein sequences from the NCBI Protein database and used them for conservancy analysis. Of the 42 epitopes identified, 30 were conserved in \(90\%\) of all DENV2 strains, with 8 being conserved in all 171 strains analyzed. Of the remaining 12 epitopes, 6 were conserved in \(75\%\) of all strains analyzed, and 6 were found in the 30–65% range. This accounts for an average conservancy of 92% for the epitopes identified, which is significantly higher than the average conservancy of non-epitopes (73%; \(p < 0.001\)). We then asked if the epitopes identified were also conserved in serotypes other than DENV2. Accordingly, we retrieved 162 DENV1, 169 DENV3, and 53 DENV4 sequences from the NCBI protein database and investigated the conservancy of the identified epitopes within the respective serotypes. In contrast to the high degree of conservancy within the DENV2 serotype, 35 out of the 42 epitopes did not occur in any of the 384 DENV1, -3, and -4 sequences tested, and only 7 epitopes had sequence homologs in one or more of the other serotypes. Interestingly, most of the epitopes that showed conservancy across serotypes have been identified in the B*0702-transgenic mice. Four of the identified B*0702-restricted epitopes (NS3 1682–1690, NS3 1700–1709, NS3 1753–1761, and NS4B 2280–2292) were additionally conserved in 89–100% of sequences derived from serotypes other than DENV2. The same was observed for two DRB1*0101-restricted epitopes, which were conserved across serotypes (NS3 1742–1756, NS5 2966–2980). In this study, the epitopes were conserved in \(99\%\) of polyprotein sequences of two serotypes other than DENV2. Finally, one of the A*0101-restricted epitopes (NS1 1090–1099) was also conserved in 100% of DENV3 sequences. The results from this analysis are shown in Table I.

### Table I. Identified DENV2-derived epitopes in HLA-transgenic IFN-α/βR2/2 mice

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Sequence</th>
<th>Restriction</th>
<th>T Cell Responses (SFC)</th>
<th>Frequency in Humans (%)</th>
<th>HLA Binding (IC50)</th>
<th>Conservancy within Serotypes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E451–459</td>
<td>ITEAELTGY</td>
<td>A*0101</td>
<td>327 67</td>
<td>20 (1/5)</td>
<td>25</td>
<td>85 0 0 0</td>
</tr>
<tr>
<td>NS1 1090–1099</td>
<td>RSCTPLPERY</td>
<td>A*0101</td>
<td>228 104</td>
<td>20 (1/5)</td>
<td>5.9</td>
<td>100 0 100 0</td>
</tr>
<tr>
<td>NS2A 1192–1200</td>
<td>MTDGDGMGV</td>
<td>B*0702</td>
<td>430 163</td>
<td>20 (1/5)</td>
<td>19</td>
<td>84 0 0 0</td>
</tr>
<tr>
<td>NS2A 1251–1259</td>
<td>LTDALALGM</td>
<td>B*0702</td>
<td>465 143</td>
<td>40 (2/5)</td>
<td>129</td>
<td>91 0 0 0</td>
</tr>
<tr>
<td>NS4B 2399–2407</td>
<td>VIDLPPY</td>
<td>B*0702</td>
<td>153 92</td>
<td>20 (1/5)</td>
<td>17</td>
<td>53 0 0 0</td>
</tr>
<tr>
<td>NS5 3375–3383</td>
<td>YTDYMSMK</td>
<td>B*0702</td>
<td>495 143</td>
<td>20 (1/5)</td>
<td>37</td>
<td>98 0 0 0</td>
</tr>
</tbody>
</table>

| Mean | 350 119 |

| E631–639 | RLITVNPIV | A*0201 | 265 393 | 43 (3/7) | 2.8 | 98 0 0 0 |
| NS2A 1212–1221 | RPTFAAGLLL | B*0702 | 400 335 | 20 (1/5) | 4.8 | 92 0 0 0 |
| NS3 1509–1517 | SQIQAGVYK | A*1101 | 436 0 | 0 (0/5) | 33 | 98 0 0 0 |
| NS5 2608–2616 | STGWNLVR | B*0702 | 1030 0 | 0 (0/5) | 22 | 100 0 0 0 |

| Mean | 476 530 |

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| NS3 1509–1517 | SQIQAGVYK | A*1101 | 436 0 | 0 (0/5) | 33 | 98 0 0 0 |

| Mean | 476 530 |

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Discussion

There have been several attempts to develop mouse models that allow studying of T cell responses in DENV disease in humans. Wild-type mice are resistant to DENV-induced disease. As such, development of mouse models for DENV infection to date has been challenging and has had to rely on infection of immunocompromised mice, nonphysiologic routes of infection, and mouse-human chimeras (reviewed in Ref. 44). Due to the importance of the IFN system in the host antiviral response, mice lacking IFN-$\alpha$/$\beta$R support a productive infection. A mouse-passaged DENV2 strain, S221, is highly immunogenic and also replicates to high levels in IFN-$\alpha$/$\beta$R$^{-/-}$ mice. A possible concern is that the lack of type I IFN may alter the T cell responses observed in IFN-$\alpha$/$\beta$R$^{-/-}$ mice. It has been previously shown that the specificity of T cell responses postinfection with DENV is similar in wild-type and IFN-$\alpha$/$\beta$R$^{-/-}$ mice, thus allowing the study of CD4$^+$ and CD8$^+$ T cell responses in DENV infection. It was further demonstrated in this murine model that vaccination with T cell epitopes prior to DENV2 infection provided significant protection (8, 9). However, a small animal model in which epitope specificity can be linked with elements of the human immune system to study T cell responses to DENV Ags would add greatly to the relevance of the model. Therefore, we backcrossed IFN-$\alpha$/$\beta$R$^{-/-}$ mice with mice transgenic for HLA A*0101, A*0201, A*1101, B*0702, and DRB1*0101. Although significant differences exist between human and murine TCR repertoires and processing pathways, HLA-transgenic mice are fairly accurate models of human immune responses, especially when peptide immunizations are used. Numerous studies to date show that these mice develop T cell responses that mirror the HLA-restricted responses observed in humans in the context of various pathogens (45–53).

The findings presented in this study demonstrate that HLA-transgenic IFN-$\alpha$/$\beta$R$^{-/-}$ mice represent a valuable model to systematically map the epitopes recognized in humans. In fact, we could not only identify a number of HLA-restricted T cell responses, but our genomewide screen also provided further insight into the proteins targeted by T cells during DENV infection. Whereas we identified T cell epitopes from 9 out of 10 DENV...
proteins, the majority of responses (97%) were derived from the NS proteins. More than half of the epitopes identified originated from the NS3 and NS5 protein. Thus, we were able to confirm the suggested highly immunogenic role of the conserved NS3 and NS5 proteins as a major target of T cell responses (54, 55). Interestingly, the proteins previously described as Ab targets (pre-membrane, E, and NS1) (56) accounted for <5% of all T cell responses, with only three epitopes identified from these proteins. The observation that T and B cell epitopes after primary DENV infection are not derived from the same proteins may factor in future vaccine design strategies, because immunizing with Ags containing NS3 and NS5 T cell epitopes would induce a robust T cell response without the risk of Ab-dependent enhancement.

Another unique challenge in vaccine development is the high degree of sequence variation in a pathogen, characteristically associated with RNA viruses. This is of particular relevance in the case of DENV infections, in which it is well documented that prior exposure to a different serotype may lead to more severe disease and immunopathology (16). The fact that there is also significant genetic variation within each serotype adds to the complexity of this topic (57, 58). It is hypothesized that in certain cases, peptide variants derived from the original Ag in the primary infection, with substitutions at particular residues, can induce a response that is qualitatively different from the response induced by the original Ag (for example, inducing a different pattern of lymphokine production; partial agonism) or even actively suppress the response (TCR antagonism). Variants associated with this phenotype are often collectively referred to as altered peptide ligands [APLs (59)]. During secondary infections, the T cell response directed at the APL may lead to altered or aberrant patterns of lymphokine production and TCR antagonist-mediated inhibition of T cell responses (60). Therefore, a balanced immunity to all four serotypes is required for a suitable vaccine candidate, and it is necessary to explore DENV vaccine concepts that might overcome these potential problems. It is generally recognized that conserved protein sequences represent important functional domains (61) and that mutations at these important protein sites could be detrimental to the survival of the virus. T cells that target highly conserved regions of a protein are therefore likely to target the majority of genetic variants of a pathogen (62). Most interestingly, in this context, we could show that epitopes that are highly conserved within the DENV2 serotype are the major target for T cells. These data suggest that immunization with peptides from a given serotype would protect from the majority of genotypes within this serotype. In contrast to the conservancy of epitopes within a serotype, our analyses showed that most DENV2-derived epitopes identified were not conserved in other serotypes. These findings point to a tetravalent immunization strategy with a collection of multiple non–cross-reactive epitopes derived from each of the four DENV serotypes. The induction of separate non–cross-reactive responses would avoid issues arising from incomplete cross-reactivity and APL/TCR antagonism effects.

In addition to sequence variation, HLA polymorphism adds to the complexity of studying T cell responses to DENV. Human MHC molecules are extremely polymorphic, with several hundred different variants known at most loci (63, 64). Therefore, selecting multiple peptides matching different MHC-binding specificities will increase coverage of the patient population for basic investigations and diagnostic or vaccine applications alike. However, different MHC types are expressed at dramatically different frequencies in different ethnicities. To address this issue, we backcrossed IFN-α/βR−/− mice with mice transgenic for HLA A*0101, A*0201, A*1101, B*0702, and DRB1*0101. These four MHC class I alleles were chosen as representatives of four supertypes (A1, A2, A3, and B7, respectively), allowing a combined coverage of ∼90% of the worldwide population (65) and with >50% expressing at least one of the specific alleles selected. HLA supertypes are not limited to class I molecules. Several studies have demonstrated the existence of HLA class II supertypes (66–68) and functional classification has revealed a surprising degree of repertoire sharing across class II supertypes (42). This is in accordance with our data because we could identify the DRB1*0101-restricted epitopes in almost every donor, regardless if the donor expressed the DRB1*0101 allele (Fig. 5E). The fact that some of the DRB1*0101-restricted epitopes were able to elicit an even higher response in HLA-nonmatched donors suggests that these epitopes might additionally be restricted by MHC class II molecule(s) other than DRB1*0101. In the case of the MHC class I-restricted epitope, all of the A*0101- and A*0201-restricted, and 10 out of 12 B*0702-restricted, epitopes have been able to elicit an IFN-γ response when tested in human PBMC (Fig. 5A, 5B, 5D). In the case of the A*1101-restricted epitopes, only three out of nine were detected in both experimental systems (Fig. 5C). Because of the fact that responses to the two B*0702 peptides not detected in humans required higher amounts of peptide in the HLA B*0702 IFN-α/βR−/− mice, we hypothesized that a similar phenomenon might explain the low overlap in A*1101 epitopes. To test this hypothesis, we have performed titrations for all of the A*1101-restricted epitopes not recognized in the corresponding HLA-matched donors (data not shown). However, we did not detect any correlation between the epitope dose response in HLA-transgenic IFN-α/βR−/− mice and recognition in humans. Another possible explanation for this low overlap could be the substrate specificity of the murine TAP transporter. Murine TAP molecules prefer peptides with hydrophobic C termini (69, 70), whereas the HLA A*1101 preference is for positively charged residues (71). Accordingly, it is possible that the optimal A*1101 peptides recognized in humans have not been presented and identified in the HLA A*1101-transgenic mice. Overall, we could demonstrate that the mouse model significantly reflects the response pattern observed in humans and that HLA B-restricted responses seem to be dominant in transgenic mice as well as in human donors (Fig. 5F). The dominance of HLA B responses has been also shown in the context of several other viruses, such as HIV, EBV, CMV, and influenza (72–75). These studies suggest that this observation is not limited to RNA viruses. In fact, it has even been described for an intracellular bacterial pathogen, Mycobacterium tuberculosis (76, 77). Furthermore, HLA B-restricted T cell responses have been described to be of higher magnitude (73) and to influence infectious disease course and outcome. In the case of DENV, it has been shown that one particular B*07 epitope was able to elicit higher responses in patients with DHF compared with patients suffering from DF only and could therefore be associated with disease outcome (78). Other studies suggest a role for HLA B44, B62, B76, and B77 alleles in protection against developing clinical disease after secondary DENV infection, whereas other alleles were associated with contribution to pathology (79, 80). This suggests that HLA alleles are associated with clinical outcome of exposure to DENV, in previously exposed and immunologically primed individuals. All of this is in agreement with the observations we made in this study. Similarly, the fact that the stronger B*07 response occurs in our human samples as well as in our mouse model of DENV infection underscores the value of this mouse model because it seems to mimic patterns of immunodominance observed in humans.

In conclusion, the exact role of DENV sequence variation in protection and immunopathology has not been fully elucidated, and addressing this issue is key to the development of vaccines against...
DENV infection and DHF/DSS. In this study, we provide a novel model to study the mechanisms of T cell-mediated mechanisms of protection and pathogenesis relevant for vaccine design against DENV. In addition, our studies have led to the identification of 24 novel human epitopes, which contributes to an extended knowledge of T cell-mediated responses against DENV infection. Future experiments will continue the evaluation of these novel epitopes to provide a more detailed examination of the epitope-specific responses, such as analysis of multicytokine production, CTL activity, and the persistence of these epitope-specific responses into memory. Further studies will also test the efficacy of vaccination strategies using the epitopes identified in this study.

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Disclosures

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References


