Immunodominant Dengue Virus-Specific CD8⁺ T Cell Responses Are Associated with a Memory PD-1⁺ Phenotype

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ABSTRACT

Dengue disease is a large public health problem that mainly afflicts tropical and subtropical regions. Understanding of the correlates of protection against dengue virus (DENV) is poor and hinders the development of a successful human vaccine. The present study aims to define DENV-specific CD8⁺ T cell responses in general and those of HLA alleles associated with dominant responses in particular. In human blood donors in Nicaragua, we observed a striking dominance of HLA B-restricted responses in general and of the allele B*35:01 in particular. Comparing these patterns to those in the general population of Sri Lanka, we found a strong correlation between restriction of the HLA allele and the breadth and magnitude of CD8⁺ T cell responses, suggesting that HLA genes profoundly influence the nature of responses. The majority of gamma interferon (IFN- γ) responses were associated with effector memory phenotypes, which were also detected in non-B*35:01-expressing T cells. However, only the B*35:01 DENV-specific T cells were associated with marked expression of the programmed death 1 protein (PD-1). These cells did not coexpress other inhibitory receptors and were able to proliferate in response to DENV-specific stimulation. Thus, the expression of particular HLA class I alleles is a defining characteristic influencing the magnitude and breadth of CD8 responses, and a distinct, highly differentiated phenotype is specifically associated with dominant CD8⁺ T cells. These results are of relevance for both vaccine design and the identification of robust correlates of protection in natural immunity.

IMPORTANCE

Dengue is an increasingly significant public health problem as its mosquito vectors spread over greater areas; no vaccines against the virus have yet been approved. An important step toward vaccine development is defining protective immune responses; toward that end, we here characterize the phenotype of the immunodominant T cell responses. These DENV-reactive T cells express high levels of the receptor programmed death 1 protein (PD-1), while those from disease-susceptible alleles do not. Not only does this represent a possible correlate of immunodominance, but it raises the hypothesis that PD-1 might be a regulator that prevents excessive damage while preserving antiviral function. Further, as this study employs distinct populations (Nicaraguan and Sri Lankan donors), we also confirmed that this pattern holds despite geographic and ethnic differences. This finding indicates that HLA type is the major determinant in shaping T cell responses.

With over 390 million infections worldwide, dengue has become a pressing global public health problem (1). Globalization, international travel, and increasing temperatures have led to an expansion of the dengue mosquito vectors *Aedes aegypti* and *Aedes albopictus* in tropical and subtropical regions. Furthermore, the lack of an approved vaccine leaves one-third of the world's population at risk of infection (2).

Dengue fever and the more severe forms of the disease, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), are caused by infection with dengue virus (DENV), which exists as four related serotypes (DENV1 to -4). Primary infection with one serotype is thought to confer lifelong protection only against the serotype of infection (3, 4), while secondary infections with another serotype contribute to the risk of acquiring a more severe form of the disease (5–7). The higher frequency of severe disease during secondary DENV infections has been attributed to either weakly neutralizing antibodies leading to “antibody-dependent enhancement” (ADE) or an aberrant T cell response resulting in a “cytokine storm” (8–11).

Previous studies in our lab supported the notion that CD8⁺ T cells play a protective role against severe DENV infection and

disease (12). These studies defined the CD8⁺ T cell reactivity of the general population in the area of Sri Lanka where DENV is endemic and revealed that HLA class I alleles previously associated with increased disease susceptibility were also associated with responses of lower breadth and magnitude, while those associated with protection from severe disease were associated with responses of greater breadth and magnitude (12). A recently completed comprehensive screen of DENV-specific CD8⁺ T cell reactivity in Nicaragua resulted in the definition of a global epitope “mega-pool” allowing the study of DENV-specific responses (13).

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However, the exact phenotype of DENV-specific CD8⁺ T cells as a function of HLA restriction has not been detailed.

Toward this end, the characterization of these cells in the present study is informed by previously identified memory subsets that correlate with protection from other natural viral infections such as influenza virus (14) or after vaccination with yellow fever (YF) virus (15). The immune response to the yellow fever vaccine, YF-17D, involves terminally differentiated memory CD8⁺ T cells (CCR7⁺ CD45RA⁺), which are highly functional and associated with protective long-term memory and show upregulation of the programmed death 1 (PD-1) protein (15).

The inhibitory receptor PD-1 exerts a wide range of immunoregulatory roles in T cell activation and tolerance (16). It has been initially shown that PD-1 is highly expressed on exhausted CD8 T cells (17) and highly expressed on HIV-, hepatitis B virus (HBV)-, and hepatitis C virus (HCV)-specific cells (18–20) indicating that PD-1 may serve as a useful marker on virus-specific CD8⁺ T cells to determine the degree of T cell exhaustion. Other studies have suggested that PD-1 expression is increased as a result of T cell activation during a primary immune response (21).

Here, we found that immunodominant alleles such as HLA B*35:01 were associated with higher levels of PD-1 compared to disease-susceptible alleles, such as A*24:02. Following assessment of cytokine production, coexpression of other inhibitory receptors, and proliferation, we found that expression of PD-1 indicated activation rather than exhaustion and may be an indicator of highly functional CD8⁺ memory T cells.

MATERIALS AND METHODS

Ethics statement. All blood samples obtained were discarded buffy coats from routine blood donations and thus were exempt from human subject review. According to local standards, the University of Colombo Ethics Review Committee and the Institutional Review Board (IRB) of the La Jolla Institute for Allergy and Immunology reviewed and approved the project.

Human blood samples. Anonymous blood donations were obtained from the National Blood Center (NBC) of the Nicaraguan Red Cross in Managua, Nicaragua. Donors were of both sexes from the general population and were between 17 and 65 years old. All donors presented a broad neutralization profile, suggesting that the donors had experienced at least two DENV infections prior to blood donation, as previously described (13). Sri Lankan blood samples were collected in an identical fashion, as previously described (12).

PBMC isolation. Briefly, peripheral blood mononuclear cells (PBMCs) and serum were isolated by Ficoll density gradient centrifugation and frozen in fetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO) or Synth-a-freeze (Life Technologies, Carlsbad, CA) (12, 13). Seropositivity was determined by testing for total anti-DENV antibodies by inhibition enzyme-linked immunosorbent assay (ELISA) (22, 23) or the flow-based U937+DC-SIGN neutralization assay (conducted at the National Virology Laboratory of the Nicaraguan Ministry of Health and the University of North Carolina, Chapel Hill, respectively) as previously described (12, 24).

HLA typing. Genomic DNA for HLA typing was isolated from donor PBMCs using standard QIAmp DNA isolation kits (Qiagen, Valencia, CA). High-resolution Luminex-based typing of HLA class I genes was conducted as per the manufacturer's instructions (sequence-specific oligonucleotide typing [One Lambda, Canoga Park, CA]). Alternatively, high-resolution typing was performed by PCR-based methods (sequence-specific primer typing [One Lambda]) as described previously (12).

Peptide selection and synthesis. HLA-specific T cell reactivity was determined for both Nicaraguan and Sri Lanka samples as previously described (12, 13). The peptides were restricted by 1 or more of 27 com-

mon HLA MHC class I alleles, including 16 HLA A alleles (A*01:01, A*26:01, A*32:01, A*02:01, A*02:03, A*02:06, A*68:02, A*23:01, A*24:02, A*03:01, A*11:01, A*30:01, A*30:02, A*31:01, A*33:01, and A*68:01), and 11 HLA B alleles (B*40:01, B*44:02, B*44:03, B*57:01, B*58:01, B*15:01, B*07:02, B*35:01, B*51:01, B*53:01, and B*08:01). Together, the 27 class I alleles account for about 97% of the HLA A and HLA B allelic variation in the general worldwide population, as well as the Nicaraguan and Sri Lankan cohorts (25). Accordingly, we generated a "mega-pool" of 268 epitopes selected to account for 90% of the gamma interferon (IFN- γ) response in both sample sets, also as previously described (12, 13). Our analysis included predicted peptides with lengths of 9 or 10 amino acids. While the majority of MHC class I-restricted epitopes identified to date are 9 and 10 amino acids in length, we cannot exclude that we have missed nonconventional peptides as have been described in previously published studies (26). A pool of 196 Epstein-Barr virus (EBV)-specific epitopes derived from the Immune Epitope Database (IEDB [www.iedb.org]) was used in control experiments. Peptides for screening studies were purchased from A & A/Synthetic Biomolecules (San Diego) as crude material. Selected peptides were synthesized on a larger scale and purified (>95%) by reverse-phase high-performance liquid chromatography (HPLC).

Ex vivo IFN- γ ELISPOT assay. For the IFN- γ enzyme-linked immunosorbent spot (ELISPOT) assay, PBMCs were tested with sets of peptides predicted to bind the exact HLA alleles expressed in the particular donor (exact match) as previously described (13). Briefly, human PBMCs (2×10^5 cells/well) were incubated with 2 μ g/ml of HLA-matched peptides in plates coated with the anti-human IFN- γ antibody 1-D1K (5 μ g/ml [Mabtech]) at 37°C for 20 to 24 h. Plates were then developed as previously described (12). PBMCs were first screened with peptide pools containing 10 peptides per pool. Pools associated with positive IFN- γ responses were deconvoluted to identify the individual epitopes. Restriction has been inferred on the basis of the donor expressing the allele and the peptide predicted to bind the matching HLA. It is possible that the measure is not fully selective as some of the peptides might cross-react with other HLA alleles coexpressed in some of the donors.

ICS. For intracellular cytokine staining (ICS), PBMCs (2×10^6 cells/well) were incubated with the peptide pools (1 μ g/ml) for 2 h. Brefeldin A (1 μ g/ml [BD Bioscience, San Diego, CA]) was added to the mixture, and the mixture was then incubated for another 4 h. Cells were then washed, stained with extracellular markers for 30 min, and then washed, fixed with 4% paraformaldehyde, blocked with normal human sera (Gemini, West Sacramento, CA) and stained for intracellular IFN- γ . Samples were analyzed on an LSR-II flow cytometer (BD Immunocytometry Systems, San Diego, CA), and data were processed with FlowJo X software (Tree Star). The following antibodies (with conjugates in parentheses) used in this study were purchased from BD Pharmingen: CD3, UCHT1 (Alexa Fluor 100); CD8, RPA-T8 (V500); CD28, CD28.2 (phycoerythrin [PE]-Cy7). The following antibodies (with conjugates in parentheses) were purchased from eBioscience: CD4, RPA-T4 (allophycocyanin-eFluor780); CD45RA, HI100 (eFluor450); CD62L, DREG-56 (PE-Cy7); PD-1, MIH4 (fluorescein isothiocyanate [FITC]); CTLA4, 14D3 (PE-Cy7); Ki67, 20Raj1 (allophycocyanin); and IFN- γ , 4S.B3 (PE). The remaining antibodies (with conjugates in parentheses) were purchased from Biolegend: Tim3, F38-2E2 (BV605); CCR7, G043H7 (peridinin chlorophyll protein [PerCP]-Cy5.5); and KLRG1, 2F1/KLRG1 (PE-Cy7).

HLA B*35:01 and HLA A*24:02 tetramer staining. The same B*35:01 peptides that are contained in the peptide pool to stimulate IFN- γ production were used to produce tetramers. Tetrameric staining reagents incorporating eight HLA B*35:01-restricted DENV epitopes (i.e., HPGA GKTKRY, TPEGIPTLF, LPVWLAYKVA, TPEGIIPALF, TPEGIIPSMF, VATTFTVTPM, IANQATVLM, FTMRHKKATY) and eight HLA A*24:02-restricted DENV epitopes (i.e., TYGWNLVKL, CYSQVNPITL, NFLEVE DYGF, SWMIRILIGF, LWPKTHTLW, LYAVATTIL, YYMATLKNV, and VMLLVHYAI) were synthesized by the NIH Tetramer Core Facility at Emory University. Tetramers were pooled and used in staining experiments at a 1:50 dilution (i.e., 20 to 40 μ g/ml). PBMCs were stained with

tetramers for 90 min at room temperature. Sixty minutes postaddition of tetramers, the PBMCs were stained with the phenotypic markers described above for 30 min at room temperature. Tetramer-stained PBMCs were washed and analyzed on an LSR-II (BD).

CD8⁺ T cell proliferation assays. (i) **Ki-67.** PBMCs were cultured in RPMI 1640 (Ω Scientific, Tarzana, CA) supplemented with 5% human serum (Cellgro, Herndon, VA) at a density of 2×10^6 cells/ml in 24-well plates (BD Biosciences, San Jose, CA). Cells were then incubated with the DENV-specific peptide pool (2.5 μg/ml) at 37°C in 5% CO₂. Additional PBMCs were incubated with phytohemagglutinin (PHA [1 μg/ml]) or DMSO (1 μl/well) as positive and negative controls, respectively. On day 3 poststimulation, interleukin-2 (IL-2 [5 U/ml]) was added to all wells. After 6 days, cells were harvested, labeled with DENV B*35:01 tetramers, and stained for surface markers (CD3, CD4, CD8, CCR7, CD45RA, and PD-1). After fixation with 4% paraformaldehyde, cells were permeabilized and stained for the intracellular marker Ki-67. Data were collected using an LSR Fortessa (BD) and analyzed using FlowJo X (Tree Star).

(ii) **CFSE assay.** The carboxyfluorescein succinimidyl ester (CFSE) assay was conducted similarly to the proliferation assay with Ki-67, except that PBMCs were labeled with CFSE (Life Technologies, Eugene, OR) prior to being placed in culture for 6 days. Briefly, whole PBMCs were resuspended at 1×10^7 cells/ml in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Cell Trace CFSE (Molecular Probes) was added to the cells at a final concentration of 1 μM, and the cells were incubated for 10 min at 37°C. Following incubation, the cells were removed from the incubator and immediately diluted in 10× the volume of ice-cold RPMI. Cells were then washed and placed in *in vitro* culture for 6 days as described above.

In vitro cytolytic activity assay. For the cytolytic activity assay, cells were surface stained and sorted using a FACSAria III (BD Biosciences, San Diego, CA). Effector memory CD8⁺ T cells (CD3⁺ CD8⁺ CCR7[−]) and central memory CD8 T cells (CD3⁺ CD4⁺ CCR7⁺ CD45RA[−]) either positive or negative for the expression of PD-1 were sorted as the effector cells. Antigen-presenting cells (APCs) (CD3[−]) were sorted as targets. APCs were then stained with CFSE at 5 μM for 20 min at room temperature and pulsed with DENV-specific peptides (10 μg/ml) for 60 min at 37°C. The APCs and effector cells were cocultured overnight at 37°C. Cells were then harvested and analyzed by flow cytometry.

RESULTS

B*35:01-restricted responses dominate the CD8⁺ T cell response in Nicaragua. Our general approach relied on the prediction of potential epitopes binding to a panel of the most common HLA A and B allelic variants worldwide (25). Here, we first verified that the panel of alleles selected did indeed provide extensive population coverage in the Nicaraguan blood donor population studied. Out of four possible molecules expressed in each donor (two HLA A and two HLA B), three or more were covered in 86% of the donors. If two-digit or supertype matching was considered (27), the fraction of donors matched for three or more HLA alleles increased to 96%. Accordingly, we concluded that the panel of alleles selected provides extensive coverage of the study population considered. We next analyzed correspondence between the frequency of individuals positive for alleles expressed at frequencies high enough to allow meaningful analysis ($\geq 5\%$ HLA frequency in Nicaragua), and the frequency of responses to the exactly corresponding HLA-matched peptides (Fig. 1A). Despite high frequency in the Nicaragua population, HLA A*02:01 and A*24:02 displayed low response rates. Conversely, a general immunodominance of HLA B alleles, and particularly B*35:01, was apparent. Remarkably, B*35:01-restricted responses accounted for 39% of the donor responses, 27% of the epitopes, and 43% of the total response magnitude (Fig. 1B). The high level of immu-

nodominance of B*35:01 is relevant in the context of previous studies correlating B*35:01 expression with decreased risk of severe dengue disease (28, 29).

HLA restriction determines the magnitude and breadth of the DENV-specific T cell response. Since epitope mapping studies were previously performed following the exact same approach in another country where dengue is endemic, Sri Lanka (12), we compared the breadth and magnitude of CD8⁺ T cell responses in the Nicaragua and Sri Lanka populations. This unbiased comparison revealed a strong correlation in the magnitudes of the responses ($R^2 = 0.98$) (Fig. 1C) between the two countries. A similarly high correlation was found for the breadth (the number of responding epitopes per HLA) of responses per allele in these countries ($R^2 = 0.96$) (Fig. 1D). These results demonstrate that the restricting HLA type is a major determinant in shaping T cell responses, despite the profound genetic/ethnic distinctions between the two populations, the likely differences in infecting serotypes and strains, and the different burdens of disease.

Phenotypic analysis of HLA B*35:01-restricted DENV-specific CD8⁺ T cells. In both countries, responses restricted by the B*35:01 allele showed the strongest magnitude and breadth of responses (Fig. 1C and D). To examine the phenotype of DENV-specific CD8⁺ T cells restricted by B*35:01, we utilized ICS assays and an epitope pool (DENV mega-pool) encompassing 268 DENV epitopes, previously validated as a tool to detect and characterize DENV CD8⁺ responses in settings as diverse as Sri Lanka, Nicaragua, and Brazil (13).

The gating strategy applied to study DENV-specific T cell responses is shown in Fig. 2A. PBMCs from B*35:01-positive donors were stimulated with the peptide mega-pool and then stained with various phenotypic markers. Between 0.8 and 8.7% of CD8⁺ T cells in B*35:01 donors showed IFN-γ⁺ responses to this peptide pool (mean, 3.1%; $n = 6$) (Fig. 2B). Phenotypic analysis of the responding cells revealed that these IFN-γ⁺ cells were mostly contained in the T_{EM} (CD45RA[−] CCR7[−]) and T_{EMRA} (CD45RA⁺ CCR7[−]) memory T cell subsets (Fig. 2C).

In the same series of experiments, we tested a panel of different markers indicative of T cell differentiation, such as CD28, CD62L, and PD-1. For each marker, the expression levels in CD8⁺ IFN-γ-producing cells were compared with those in CD8⁺ IFN-γ-negative cells within the same donor. Compared to the total CD8⁺ population, DENV-specific IFN-γ-producing cells expressed similar levels of CD28 but downregulated CD62L (Fig. 2D). In the case of PD-1, we noted a significant upregulation in IFN-γ-producing cells compared to IFN-γ[−] cells (Fig. 2D). In parallel experiments, we also tested donors who did not express HLA B*35:01 or other alleles associated with strong responses. Accordingly, IFN-γ secretion was remarkably lower (in the 0.3 to 0.5% range) (Fig. 2B) in these donors. Despite the difference in levels of IFN-γ secretion, the phenotype of the antigen-specific cells was similar. IFN-γ-producing cells were also mostly T_{EM}/T_{EMRA} (Fig. 2C) and expressed low levels of CD62L and CD28 (Fig. 2D). However, PD-1 was not significantly upregulated in IFN-γ-producing cells (Fig. 2D) compared to the CD8⁺ IFN-γ-negative cells in the same donors.

Tetramer staining of B*35:01 DENV-specific T cells identifies a memory subset that does not produce IFN-γ *ex vivo*. The previous results demonstrated that DENV-specific B*35:01-restricted CD8⁺ T cells associated with IFN-γ secretion are predominantly memory cells of the T_{EM} and T_{EMRA} subsets. To investigate

FIG 1 HLA coverage, allele frequency, and DENV-specific CD8⁺ T cell reactivity in the Nicaraguan population. (A) The HLA frequency in the Nicaraguan population and percentage of responders for each HLA allele are shown. (B) The number of epitopes per allele and the total magnitude of exact HLA-matched IFN- γ responses (spot-forming cells [SFC]/10⁶ cells) are shown. The magnitude (C) and breadth (D) of responses were compared for HLA alleles that were expressed in both populations and for which responses were detected ($n = 11$ [A*02:01, A*02:06, A*03:01, A*24:02, A*33:01, A*68:01, B*07:02, B*15:01, B*35:01, B*40:01, and B*44:03]).

the potential presence of B*35:01-restricted CD8⁺ T cells that do not secrete cytokines and hence are not detectable in ICS assays, we utilized a pool of prelabeled tetramers incorporating eight of the most dominant HLA B*35:01-restricted DENV epitopes. Between 0.2 and 8.5% of CD8⁺ cells were stained by this tetramer pool (mean, 2.1%; $n = 8$), as shown in Fig. 2E.

Further characterization at the level of memory subsets revealed that a large fraction of tetramer-positive (Tet⁺) cells are T_{EM}/T_{EMRA}, but also revealed a sizeable subset of Tet⁺ central memory T cells (T_{CM}) (CD45RA[−] CCR7⁺ [Fig. 2F]). These cells were undetected in the IFN- γ assay (see Fig. 2C for comparison), suggesting that these cells are less functional in terms of IFN- γ production.

Next, the expression levels of CD62L, CD28, and PD-1 in Tet⁺ cells were compared with those of the same markers in tetramer-negative (Tet[−]) cells from the same donors. The DENV-specific Tet⁺ cells expressed lower levels of CD28 and CD62L (Fig. 2G). Similar to observations with DENV-specific IFN- γ -producing cells, expression of PD-1 in DENV-specific Tet⁺ T_{CM} and T_{EM/EMRA} cells was significantly upregulated compared to Tet[−] cells (Fig. 2G, right panel). In conclusion, the phenotype of T_{CM} tetramer-positive cells resembled that of T_{EM/EMRA} Tet⁺ cells.

DENV-specific CD8⁺ T cells restricted by HLA A*24:02 show no significant central memory or PD-1⁺ populations. The tetramer experiments with B*35:01 donors identified a significant DENV-specific T_{CM} population not detected by ICS for IFN- γ

FIG 2 Phenotypic analysis of DENV-specific CD8⁺ T cells (A) Gating strategy for evaluation of IFN- γ -producing and tetramer-binding CD8⁺ T cells. (B) DENV-specific IFN- γ -producing CD8⁺ T cells were measured in donors expressing the B*35:01 and non-B*35:01 HLA alleles. (C) The IFN- γ response of sorted central (CCR7⁺ CD45RA⁺) and effector memory (CCR7⁻ CD45RA⁺ and CCR7⁻ CD45RA⁻) T cells was measured by ICS after DENV-specific peptide stimulation. (D) IFN- γ -producing cells were stained for phenotypic markers, including CD28, CD62L, and PD-1. (E) Binding of B*35:01- and A*24:02-restricted tetramers was evaluated in donors seropositive for DENV. (F) Tetramer binding in central memory and the two effector memory T cell subsets (as defined in panel B). (G) B*35:01 or A*24:02 tetramer-positive CD8⁺ T cells were stained for phenotypic markers, including CD28, CD62L, and PD-1.

and upregulation of PD-1. To exclude that the lower IFN- γ responses seen in non-B*35:01 donors are due to an expansion of this nonresponsive central memory population, we next investigated whether this observation would hold true in DENV-specific CD8⁺ T cells restricted by A*24:02, an allele associated with subdominant responses and increased susceptibility to severe disease (30). Similarly, in both of our study populations this allele showed the lowest magnitude of response and also one of the lower breadths of responses (Fig. 1C and D).

Accordingly, a tetramer pool of the previously identified A*24:02-restricted epitopes was prepared and tested exactly as described above. The number of cells binding the A*24:02-restricted tetramer pool was significantly lower than for the B*35:01-restricted tetramer pool (Fig. 2E), confirming the results obtained by ICS. Interestingly, A*24:02 Tet⁺ cells contained a significantly lower proportion of T_{CM} than B*35:01 Tet⁺ cells (Fig. 2F). Furthermore, unlike B*35:01 Tet⁺ cells, A*24:02 Tet⁺ cells showed

no downregulation of CD62L and no upregulation of PD-1 expression (Fig. 2G). These experiments illustrate key phenotypic differences between CD8⁺ T cells restricted by the B*35:01 and A*24:02 alleles: whereas B*35:01-restricted responses are associated with PD-1 upregulation and a large T_{CM} population that does not produce IFN- γ , DENV-specific A*24:02-restricted T cells lack T_{CM} and do not upregulate PD-1.

PD-1 upregulation positively correlates with response magnitude. PD-1 has been described as a dual marker, reflective of cell exhaustion but also more recently of activation (21, 31). One cardinal feature of exhausted CD8⁺ T cells is the gradual loss of effector capabilities, such as production of IFN- γ (31). Thus, we plotted the number of PD-1-positive IFN- γ -producing cells for each donor against the proportion of CD8⁺ cells that produce IFN- γ (Fig. 3A and B) and found that the larger the proportion of IFN- γ -producing cells, the higher the proportion of cells expressing PD-1. Next, we plotted for each B*35:01 or A*24:02 subject the

FIG 3 Expression of PD-1 correlates with the magnitude of response. (A) Representative staining of PD-1⁺ and IFN- γ ⁺ B*35:01 CD8⁺ T cells. (B) DENV-specific IFN- γ -producing PD-1⁺ cells for each individual donor plotted against the total DENV-specific IFN- γ ⁺ CD8⁺ population. (C) Representative staining of PD-1⁺ and B*35:01 tetramer⁺ CD8⁺ T cells. (D) PD-1 expression on DENV-specific tetramer⁺ CD8⁺ T cells plotted against the total DENV-specific tetramer-positive CD8⁺ population.

fraction of tetramer-binding cells that were positive for PD-1 against the total CD8⁺ tetramer-binding population (Fig. 3C and D). Larger fractions of Tet⁺ cells correlated with higher proportions of cells expressing PD-1. Thus, PD-1 expression seems to correlate positively with the number of antigen-specific cells measured by either IFN- γ release or tetramer frequency.

PD-1 expression on DENV-specific T cells is not associated with expression of classical CD8⁺ T cell exhaustion receptors. The other cardinal feature of exhausted CD8⁺ T cells is the sustained high expression of multiple inhibitory receptors, including TIM3, CTLA4, 2B4, and KLRG1 (31). We thus aimed to determine the expression levels of these receptors on IFN- γ -producing PD-1-expressing cells (32, 33). As a control, we compared expression of the same markers on EBV-specific IFN- γ -producing T cells. We were able to detect IFN- γ ⁺ T cells for both DENV- and EBV-specific peptide pools (Fig. 4A). In DENV-specific PD-1⁺ CD8⁺ T cells, levels of expression of TIM3, CTLA4, and 2B4 were significantly lower than those found in EBV-specific PD-1⁺ CD8⁺ T cells (Fig. 4B to D). Interestingly, expression of TIM3 and 2B4 in DENV-specific PD-1⁺ cells did not show a significant difference, while expression of CTLA4 was lower than that in DENV-specific PD-1[−] cells (Fig. 4B to D). The levels of expression of KLRG1 did not differ between the two populations independent of whether cells were DENV or EBV specific (Fig. 4E). The low expression of inhibitory receptors on DENV-specific PD-1⁺ CD8⁺ T cells suggests that PD-1 expression is not an indicator of exhaustion in DENV-specific T cells.

DENV-specific CD8⁺ T cells proliferate in the presence of DENV peptides and are able to kill antigen-labeled target cells.

An important property of memory CD8⁺ T cells is their ability to undergo rapid proliferation upon reencountering antigen (15). To assess the proliferative capacity of DENV-specific CD8⁺ T cells, we used Ki-67 and CFSE staining and then costained with DENV-specific HLA B*35:01 tetramers. Following 6 days of *in vitro* stimulation with DENV-peptide epitopes, we observed a 3- to 4-fold increase in HLA B*35:01 Tet⁺ CD8⁺ T cells compared to unstimulated cells (Fig. 4F). Staining with Ki-67, a marker of cycling CD8 T cells (34), revealed that a significantly higher portion of the DENV-stimulated Tet⁺ cells were actively proliferating (Fig. 4G). Subsequent proliferation experiments with CFSE-labeled PBMCs indicated approximately two to three cell divisions in the Tet⁺ cells in response to DENV antigen stimulation

(Fig. 4H). Cells stimulated with PHA as a positive control showed four to five cell divisions, while cells that had not reencountered antigen did not divide more than once. Interestingly, when we analyzed the antigen-specific CD8⁺ T cells for expression of PD-1, we found PD-1⁺ cells were significantly enriched compared to CD8⁺ T cells that had not encountered antigen ($P = 0.008$) (Fig. 4I). In line with this, we found relatively high expression of CD127 on DENV-specific CD8⁺ T cells, indicative of the ability to respond to IL-7-mediated survival signals. Specifically in the case of the antigen-specific T_{EM}/T_{EMRA} Tet⁺ cells, CD127 expression was significantly higher than on the corresponding Tet[−] cells ($P = 0.04$) (Fig. 4J).

Next we were interested in whether DENV-specific memory T cells are able to fully exert their effector function by killing antigen-labeled target cells. We thus sorted PD-1⁺ and PD-1[−] central memory (CCR7⁺ CD45RA[−]) and effector memory (CCR7[−] CD45RA⁺) cells and incubated these subsets with antigen-presenting cells labeled with DENV-specific peptides (Fig. 4K). Interestingly, we saw no difference in the abilities to kill target cells between these subsets, even when incubated with increasing effector/target ratios (Fig. 4L), indicating that PD-1 does not limit cytotoxic function of these effector cells. In summary, these data indicate that PD-1⁺ DENV-specific CD8⁺ memory T cells are capable of rapid proliferation in response to antigen stimulation and are able to fully exert killing of antigen-labeled target cells.

DISCUSSION

The present study extends previous observations of Weiskopf et al. to the Nicaraguan population for whom DENV is endemic (13). The results are notable, as they highlight the crucial role of HLA class I genes in determining DENV responsiveness and define phenotypic features specifically associated with dominant T cell responses. Using PBMCs from individuals who have cleared DENV infection in Sri Lanka, a region of DENV infection hyperendemicity, we previously found that the magnitude and frequency of CD8⁺ T cell responses are higher in the context of alleles associated with decreased risk of severe DENV disease (12). These data suggest a role for CD8⁺ T cells in protection against DENV disease in humans, as found in animal models (35–37).

To examine the generality of these observations and to address which fraction of DENV responsiveness might be attributable to HLA genes, we undertook a similar analysis in a different popula-

FIG 4 PD-1-expressing cells express low levels of other coinhibitory molecules and retain proliferative capacity. (A) Peptide pools specific for DENV and EBV were used to stimulate donor PBMCs (at 1 μ g/ml per individual peptide), and corresponding IFN- γ ⁺ responses were characterized. IFN- γ ⁺⁺ PD-1⁺ and IFN- γ ⁺⁺ PD-1⁻ responses were analyzed for the coexpression of TIM3 (B), CTLA4 (C), KLRG1 (D), and 2B4 (E). The proliferative capacity of DENV-specific cells was assessed by expansion of tetramer-positive cells (F), expression of Ki-67 (G), and CFSE assays (H) after 6 days of stimulation with DENV-specific peptide pools. (I) PD-1 expression was measured on antigen-specific tetramer-positive CD8⁺ T cells in the presence or absence of antigen. (J) Expression of CD127 on tetramer-positive and -negative T_{CM} and T_{EM}/T_{EMRA} subsets. (K and L) PD-1⁺ and PD-1⁻ central memory (CCR7⁺ CD45RA⁻) and effector memory (CCR7⁻ CD45RA⁺) cells were sorted and incubated with antigen-presenting cells labeled with DENV-specific peptides. The difference in absolute numbers of APCs cocultured with either of these subsets compared to naive T cells was then expressed as the percentage of cytolytic activity.

tion, using the same approach and methodology (13). Nicaragua is also a country where DENV infection is highly endemic, but it is different from Sri Lanka in several respects, including ethnic composition, DENV variants, and epidemiology. Despite these differences, we found a strong correlation between HLA type and the breadth and magnitude of CD8⁺ T cell responses, suggesting that HLA genes profoundly influence the nature of these responses.

We further examined the phenotype of T cells restricted by the HLA B*35:01 allele, which is associated with dominant T cell responses and accounts for a large fraction of CD8⁺ T cell responses in Nicaragua, and compared it to the phenotype of A*24:02-restricted T cells, since the allele A*24:02 is subdominant and is associated with increased susceptibility to severe disease (30). For HLA B*35:01 DENV-specific T cells, we detected sizeable central memory and effector memory subsets that highly expressed PD-1. DENV-specific PD-1-expressing T cells were found to proliferate readily and exert effector functions. It is not clear why there is a difference in the phenotype of B*35:01-restricted IFN- γ and tetramer-positive (Tet⁺) CD8 T cells. A large fraction of Tet⁺ cells detected were T_{EM}/T_{EMRA}, but analysis also revealed a sizeable subset of Tet⁺ central memory T cells. These cells were undetected in the IFN- γ assay, suggesting that these cells are less functional in terms of IFN- γ production. It is possible that this T cell subset while unresponsive in terms of IFN- γ production might be associated with production of alternative cytokines. It is also possible that PD-1 expression on Tet⁺ CD8 cells makes them dysfunctional, or alternatively, it is possible that this specific T_{CM} subset needs a prolonged time (>6 h) to get activated and release cytokines.

The magnitude of HLA B*35:01-restricted responses further correlated with the expression of PD-1, which was not detected in HLA A*24:02 DENV-specific T cells. In several systems, PD-1 is highly expressed on activated T cells during acute virus infection, and its expression is transient (38). In contrast, PD-1 is persistently expressed on T cells during chronic/persistent virus infections (31). Since PD-1 expression varies with distinct stages of CD8 T cell differentiation, it would be interesting to know if PD-1 was consistently expressed on B*35:01-restricted CD8 T cells during different stages of differentiation. Collection of longitudinal samples will allow following the response and PD-1 expression over time.

Recent work on the function of PD-1 allows us to speculate on its biological significance in DENV-specific T cells. Studies in mice have shown that the absence of PD-1 results in early overactivation, excessive proliferation, and diminished survival of virus-specific CD8⁺ T cells during both acutely resolved and chronic viral infections (39). We therefore hypothesize that PD-1 might act in the context of acute DENV infection as a “blunter”—a regulator to prevent excessive damage while preserving dominant antiviral effects. This unique phenotype might be a correlate of protection specifically associated with clearance of DENV infection due to an immunodominant allele.

Protection from dengue virus is most likely multifactorial. Future studies will thus analyze correlations between CD4 and CD8 T cell responses and antibody titers. Definition of the transcriptional and proteomic signatures associated with this potentially unique protective antiviral cellular program will reveal possible targets for modulation of, and intervention with, viral infection, as well as providing molecular correlates of vaccine-induced protection.

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