Title

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Phenotypic and Functional Signatures of Herpes Simplex Virus-Specific Effector Memory CD73^{+}CD45RA^{high}CCR7^{low}CD8^{+} T_{EMRA} and CD73^{+}CD45RA^{low}CCR7^{low}CD8^{+} T_{EM} Cells are Associated with Asymptomatic Ocular Herpes

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Abstract

Herpes Simplex Virus type 1 (HSV-1) -specific CD8^{+} T cells protect from herpes infection and disease. However, the nature of protective CD8^{+} T cells in HSV-1 seropositive healthy asymptomatic individuals (with no history of clinical herpes disease) remains to be determined. In this study, we compared the phenotype and function of HSV-specific CD8^{+} T cells from HLA-A*02:01 positive asymptomatic (ASYMP) and symptomatic (SYMP) individuals (with a documented history of numerous episodes of recurrent ocular herpetic disease). We report that, while SYMP and ASYMP individuals have similar frequencies of HSV-specific CD8^{+} T cells, the "naturally" protected ASYMP individuals have a significantly higher proportion of multi-functional HSV-specific effector memory CD8^{+} T cells (CD73^{+}CD45RA^{high}CCR7^{low}CD8^{+} T_{EMRA} and CD73^{+}CD45RA^{low}CCR7^{low}CD8^{+} T_{EM}) as compared to SYMP individuals. Similar to humans, HSV-1 infected ASYMP B6 mice had frequent multi-functional HSV-specific CD73^{+}CD8^{+} T cells in the cornea, as compared to SYMP mice. Moreover, in contrast to wild-type (WT) B6, CD73 $^{-/-}$ deficient mice infected ocularly with HSV-1 developed more recurrent corneal herpetic infection and disease. This was associated with less functional CD8^{+} T cells in the cornea and trigeminal ganglia, the sites of acute and latent infection. The phenotypic and functional characteristics of HSV-specific circulating and *in situ* CD73^{+}CD8^{+} T cells, demonstrated in both ASYMP humans and mice, suggest a positive role for effector memory CD8^{+} T cells expressing the CD73 costimulatory molecule in the protection against ocular herpes infection and disease. These findings are important for the development of safe and effective T cell-based herpes immunotherapy.

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INTRODUCTION

Herpes Simplex Virus type 1 (HSV-1) infection is widespread in human populations (1–5). A staggering 3.72 billion individuals worldwide currently carry the virus that causes a wide range of mild to life-threatening diseases (1–7). Complications range from mild, such as cold sores and genital lesions, to more serious complications including permanent brain damage from encephalitis in adults and neonates and blinding corneal inflammation (5, 8). HSV infections are prevalent and permanent, as the virus establishes latency in the neurons of sensory ganglia after a primary infection (9–12). Although the virus reactivates from latency and is shed multiple times each year in body fluids (i.e. tears, saliva, nasal and vaginal secretions), most reactivations are subclinical due to an efficient immune-mediated containment of the infection and disease (2, 3, 13, 14). Thus, most infected individuals are asymptomatic (ASYMP) and do not present any apparent recurrent herpetic disease (e.g. cold sores, genital or ocular herpetic disease). However, a small proportion of individuals experience endless recurrences of herpetic disease, usually multiple times a year, often necessitating continuous antiviral therapy (i.e. Acyclovir and derivatives) (15, 16). In those symptomatic (SYMP) individuals, HSV-1 frequently reactivates from latency, re-infects the eyes and may trigger recurrent and severe corneal herpetic disease, a leading cause of infectious corneal blindness in the industrialized world (17–19). In the United States, up to 450,000 individuals have a history of recurrent herpetic stromal keratitis (rHSK), a T cell mediated immune-pathological lesion of the cornea (17–19). Ergo, a better understanding of the immune mechanisms that protect from HSV-1 is highly desirable for the development of more efficacious vaccines and immunotherapies to reduce herpes infection and disease.

Despite recent progress, a clear understanding of the molecular and cellular basis of memory T cells in herpes simplex infection is still lacking. In animal models of herpes infection and disease, HSV-specific memory $CD8^+$ T cells play a critical role in aborting attempts of virus reactivation from latency and in reduction of herpetic disease (1, 7, 13, 20–22). However, herpetic corneal disease is also associated with HSV-specific $CD8^+$ T cell responses (23, 24). While HSV glycoprotein B (gB) and glycoprotein D (gD) are major targets of $CD8^+$ T cells in seropositive ASYMP individuals (14, 25), they only produced a transient protective immunity in vaccine clinical trials (19, 26, 27). In B6 mice, an immunodominant $CD8^+$ T cell epitope, $gB_{498–505}$, achieved at least partial protection against herpes infection and disease (15, 19, 28, 29). Considering the wealth of data addressing the phenotype and function of HSV-1 $gB_{498–505}$ epitope-specific $CD8^+$ T cells in B6 mice (2, 3, 8, 13, 30), it is surprising how only a few reports characterizing the phenotype and function of “protective” $CD8^+$ T cells, specific to human epitopes (instead of mouse epitopes) that are developed from HSV-seropositive healthy ASYMP individuals who appear to have acquired a “natural” protection from recurrent herpetic disease (1, 31), actually exist. This information is necessary for the successful design of effective T cell-based immunotherapeutic strategies.

While memory $CD8^+$ T cell sub-populations are heterogeneous in terms of phenotype, function, and anatomical distribution, they can generally be divided into two major subsets: effector memory $CD8^+$ T cells (T_{EM}) and central memory $CD8^+$ T cells (T_{CM}) (31, 32). We recently reported two distinct phenotypic and functional patterns of protective and non-protective HSV-1 gB-specific $CD8^+$ T cells that are associated with ASYMP versus SYMP

ocular herpes, respectively. While a significantly higher proportion of HSV-1 gB-specific $CD8^+$ T_{EM} cells were detected in ASYMP individuals, a significantly higher proportion of HSV-1 gB-specific $CD8^+$ T_{CM} cells were detected in SYMP patients. The mechanisms by which HSV-specific $CD8^+$ T_{CM} and T_{EM} cells play different roles in herpes infection and disease remain to be fully determined.

The CD73 receptor is both a coactivator molecule of T cells and an immunosuppressive ecto-enzyme through adenosine production (33, 34). However, the precise role of the CD73 co-stimulatory molecule and its involvement in $CD8^+$ T-cell function during HSV infection has not been reported. In this report, we hypothesized that: (*i*) CD73 may have a role in the modulation of T-cell responses to herpes infection and disease; and (*ii*) that ASYMP individuals develop more protective HSV-specific $CD73^+CD8^+$ T_{EM} cells, while SYMP patients develop more non-protective (or possibly pathogenic) HSV-specific $CD73^+CD8^+$ T_{CM} cells, compared to SYMP individuals (35, 36). Herein, we report for the first time, that "naturally" protected ASYMP individuals have a significantly higher proportion of multi-functional HSV-1 $VP11/12_{220–228}$ epitope-specific effector memory $CD8^+$ T cells ($CD73^+CD45RA^{high}CCR7^{low}CD8^+$ T_{EMRA} and $CD73^+CD45RA^{low}CCR7^{low}CD8^+$ T_{EM} cells) when compared to SYMP individuals who present with repetitive recurrent herpetic disease. Moreover, ASYMP wild type (WT) B6 mice, that are ocularly infected with HSV-1 but did not develop corneal disease, have more HSV-specific $CD73^+CD8^+$ T cells in the cornea and TG, compared to SYMP mice that did develop corneal disease. Similar to ASYMP humans, the HSV-specific effector $CD73^+CD8^+$ T cells from ASYMP mice are multi-functional. In contrast to WT B6 mice, $CD73^{-/-}$ deficient mice develop significantly more herpes infection and disease. These findings draw attention to a role of circulating and *in situ* effector memory $CD73^+CD8^+$ T cells in protection against herpes infection and disease and this should unequivocally be considered in the development of a safe and effective T cell-based herpes immunotherapy.

MATERIALS AND METHODS

Human study population:

All clinical investigations in this study were conducted according to the Declaration of Helsinki. All subjects were enrolled at the University of California, Irvine under approved Institutional Review Board-approved protocols (IRB#2003–3111 and IRB#2009–6963). Written informed consent was received from all participants prior to inclusion in the study.

During the last fifteen years (i.e. January 2003 to January 2018), we have screened 875 individuals for HSV-1 and HSV-2 seropositivity. Five hundred seventy-four were White, 301 were non-White (African, Asian, Hispanic and other), 446 were female, and 429 were male. Among this sample, a cohort of 306 immuno-competent individuals, ranging from 21–67 years old (median 39), were seropositive for HSV-1 and seronegative for HSV-2. All patients were negative for HIV and HBV, with no history of immunodeficiency. 792 patients were HSV-1, HSV-2 or HSV-1/HSV-2 seropositive, among them 698 patients were healthy and defined as asymptomatic (ASYMP). These patients have never had any herpes disease (ocular, genital or dermal) based on self-reporting and clinical examination. Even a single episode of any herpetic disease would exclude the individual from this group. The remaining

94 patients were defined as HSV-seropositive symptomatic (SYMP) individuals who suffered from frequent and severe recurrent genital, ocular and/or oro-facial lesions. Signs of recurrent disease in SYMP patients were defined as herpetic lid lesions, herpetic conjunctivitis, dendritic or geographic keratitis, stromal keratitis, and iritis consistent with HSK, with one or more episodes per year for the past two years. However, at the time of blood collection, SYMP patients had no recurrent disease (other than corneal scarring) and had no recurrences during the past 30 days. They had no ocular disease other than HSK, no history of recurrent genital herpes, and were HSV-1 seropositive and HSV-2 seronegative. Because the spectrum of recurrent ocular herpetic disease is wide, our emphasis was mainly focused on the number of recurrent episodes and not on the severity of the recurrent disease. No attempt was made to assign specific T cell epitopes to the severity of recurrent lesions. Patients were also excluded if they: (1) had an active ocular (or elsewhere) herpetic lesion, or had one within the past 30 days, (2) were seropositive for HSV-2, (3) pregnant or breastfeeding, or (4) were on Acyclovir and other related anti-viral drugs or any other immunosuppressive drugs at the time of blood draw. Among this large cohort of SYMP and ASYMP individuals, 29 patients were enrolled in this study (**Table I**). SYMP and ASYMP groups were matched for age, gender, serological status and race. We also collected and tested blood samples from ten healthy control individuals who were seronegative for both HSV-1 and HSV-2 and had no history of ocular herpes, genital lesions or oro-facial herpes disease.

HSV specific serotyping through Enzyme-Linked Immunosorbent Assay:

The sera collected from random donors were tested for anti-HSV antibodies. ELISA was performed on sterile 96-well flat-bottom microplates coated with the HSV-1 antigen in coating buffer overnight at 4°C. The next day, plates were washed with PBS-1% Tween 20 (PBST) five times. Nonspecific binding was blocked by incubating them with a 5% solution of skimmed milk in PBS (200 μl/well) at 4°C for 1 hour at room temperature (RT). The microplates were washed three times with PBS–Tween and incubated with various sera at 37°C for two hours. Following five washes, biotinylated rabbit anti-human IgG, diluted 1: 20,000 with PBST, was used as the secondary antibody and incubated at 37°C for two hours. After five washes, streptavidin was added at a 1: 5,000 dilution and incubated for 30 minutes at RT. After five additional washes, the color was developed by adding 100 μl of TMB substrate. The mixture was incubated for 5–15 minutes at RT in the absence of light. The reaction was terminated by adding 1 M H_2SO_4. The absorbance was measured at 450 nm.

HLA-A2 typing:

The HLA-A2 status was confirmed by PBMC staining with 2 μl of anti-HLA-A2 mAb, (clone BB7.2) (BD Pharmingen, Franklin Lakes, NJ), at 4°C for 30 minutes. The cells were washed and analyzed by flow cytometry using a LSRII (Becton Dickinson, Franklin Lakes, NJ). The acquired data were analyzed with FlowJo software (BD Biosciences, San Jose, CA).

Tetramer/VP11/12 peptide staining:

Fresh PBMCs were analyzed for the frequency of CD8+ T cells recognizing the VP11/12 peptide/tetramer complexes, as we previously described (9–12). The cells were incubated with VP11/12 peptide/tetramer complex for 30–45 min at 37°C. The cell preparations were then washed with FACS buffer and stained with FITC-conjugated anti-human CD8 mAb (BD Pharmingen). The cells were washed and fixed with 1% paraformaldehyde in PBS and subsequently acquired on a BD LSRII. Data were analyzed using FlowJo version 9.5.6 (Tree Star).

CD107 cytotoxicity assay:

To detect VP11/12-specific cytolytic $CD8^+$ T cells in PBMC, spleen, TG and cornea cells, intracellular CD107a/b cytotoxicity assay was performed as described by Betts *et al.* (2003) with a few modifications (37, 38). Briefly, 1×10^6 PBMCs from patients, in addition to spleen cells, DLN cells and TG cells from HSV-infected mice infected, were transferred into 96-well V-bottomed FACS plates (BD) in R10 medium and stimulated with 10 different VP11/12 peptides (10 μg/mL) in the presence of anti-CD107a-FITC and CD107b-FITC (BD Pharmingen) and BD-Golgi stop (10μg /ml) for 5–6 hours at 37°C. PHA (10 μg/mL) (Sigma) and no peptide were used as positive and negative controls, respectively. At the end of the incubation period, the cells were harvested into separate tubes and washed once with FACS buffer and then stained with PE-conjugated anti-human CD8 antibody for 30 minutes. Cells were then fixed, permeabilized and stained with additional antibodies against IFN-γ and TNF- α using FIX/PERM and PERM/Wash solution (BD).

Human peripheral blood mononuclear cells (PBMC) isolation:

Individuals (negative for HIV, HBV, and with or without any HSV infection history) were recruited at the UC Irvine Institute for Clinical and Translational Science (ICTS). Between 40 and 100 mL of blood was drawn into yellow-top Vacutainer® Tubes (Becton Dickinson). The serum was isolated and stored at −80°C for the detection of anti-HSV-1 and HSV-2 antibodies, as we have previously described (16). PBMCs were isolated by gradient centrifugation using leukocyte separation medium (Life Sciences, Tewksbury, MA). The cells were then washed in PBS and re-suspended in complete culture medium consisting of RPMI1640, 10% FBS (Bio-Products, Woodland, CA) supplemented with 1x penicillin/streptomycin/L-glutamine, 1x sodium pyruvate, 1x non-essential amino acids, and 50 μM of 2-mercaptoethanol (Life Technologies, Rockville, MD). Freshly isolated PBMCs were also cryo-preserved in 90% FCS and 10% DMSO in liquid nitrogen for future testing.

Human T cells flow cytometry assays:

The following anti-human antibodies were used for the flow cytometry assays: CD3 (clone SK7) PE-Cy7, CD8 (clone SK1) APC-Cy7, CD73 (AD2) PE-Cy7, PD-1 (clone EH12.1) A647, CD45RA (L48) FITC, CCR7 (clone 3D12) PE-Cy7, IFN-γ (clone B27) Alexa Fluor 647, TNF-α (MAb11) (BD Biosciences), $CD107^b$ (clone H4B4) FITC (BioLegend). For surface stain, mAbs against cell markers were added to a total of 1×10^6 cells in 1X PBS containing 1% FBS and 0.1% sodium azide (FACS buffer) for 45 minutes at 4°C. After washing with FACS buffer, cells were then permeabilized for 20 minutes on ice using the

Cytofix/Cytoperm Kit (BD Biosciences) and then washed twice with Perm/Wash Buffer (BD Bioscience). Intracellular cytokine mAbs were then added to the cells and incubated for 45 minutes on ice in the dark. Cells were washed again with Perm/Wash and FACS Buffer and fixed in PBS containing 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). For each sample, 100,000 total events were acquired on the BD LSRII. Ab capture beads (BD Biosciences) were used as individual compensation tubes for each fluorophore in the experiment. To define positive and negative populations, we employed fluorescence minus controls for each fluorophore used in this study when initially developing staining protocols. In addition, we further optimized gating by examining known negative cell populations for background expression levels. The gating strategy was similar to that used in our previous work (2). Briefly, we gated single cells, dump cells, viable cells (Aqua Blue), lymphocytes, $CD3^+$ cells, and $CD8^+$ cells before finally gating human epitope-specific $CD8^+$ T cells using HSV-specific tetramers. Data analysis was performed using FlowJo version 9.9.4 (TreeStar, Ashland, OR). Statistical analyses were done using GraphPad Prism version 5 (La Jolla, CA).

The intracellular assay to detect IFN-γ, TNF-α and $CD107^{a/b}$ in response to *in vitro* peptide stimulations were performed as described (39–41) with a few modifications. On the day of the assay, $1X10^6$ PBMCs were stimulated *in vitro* with peptide pools (10 μg/ml/peptide) at 37°C for an additional six hours in a 96-well plate with BD Golgi Stop (BD Biosciences), 10 μl of $CD107^a$ FITC and $CD107^b$ FITC. PHA (5 μg/mL) (Sigma) and no peptides were used as positive and negative controls, respectively. At the end of the incubation period, the cells were transferred to 96-well round-bottomed plate and washed twice with FACS buffer then stained with PE-conjugated anti-human CD8 for 45 minutes at 4°C. Intracellular staining for the detection of IFN-γ and $CD107^{a/b}$ was performed as outlined above. The cells were washed again and fixed, then 100,000 total events were acquired on the BD LSRII and data analysis was performed using FlowJo version 9.9.4 (TreeStar).

Peptide synthesis:

HLA-A*0201 binding peptide $VP11/12_{220–228}$ and $HLA\text{-}2^b$ binding peptide $gB_{498–505}$ were synthesized by Magenex (San Diego, CA) on a 9050 Pep Synthesizer instrument using solid-phase peptide synthesis and standard 9-fluorenylmethoxy carbonyl technology (PE Applied Biosystems, Foster City, CA). Stock solutions were made at 1 mg/ml in PBS. $gB_{498–505}$ and $VP11/12_{220–228}$ peptides were aliquoted and were stored at −20°C until assayed.

Virus production:

HSV-1 (strain McKrae) was grown and titrated on RS (rabbit skin) cells, as we have previously described (3).

Mice:

Colonies of wild-type (WT) B6 and CD73 knockout (CD73 KO) mice (42) were bred and maintained at University of California Irvine, Laboratory Animal Resources, under specific pathogen free (SPF) conditions. All studies were conducted in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care and according to

Institutional Animal Care and Use Committee-approved animal protocols (IACUC # 2002–2372).

Ocular infection of mice with HSV-1:

Two groups of age-matched female CD73$^{-/-}$ deficient and WT B6 mice were infected with 2 × 10^5 PFU of strain McKrae as eye drops, without corneal scarification. Control mice were inoculated using mock samples of the virus. Following ocular infection, mice were monitored for ocular herpes infection and disease.

Monitoring of ocular herpes infection and disease:

Animals were examined for signs of ocular disease via slit lamps. Clinical assessments were made immediately before inoculation and on days 1, 3, 5, 7, 10, 14 and 21 thereafter. The examination was performed by investigators blinded strain the mice and scored according to a standard 0–4 scale: 0, no disease; 1, 25%; 2, 50%; 3, 75%; and 4, 100% disease, as previously described (43, 44). While some mice remained asymptomatic (exhibited no symptoms or disease), others developed symptoms and were considered symptomatic. At a given and same time point post-infection, mice were segregated into ASYMP mice that are infected but never developed any corneal herpetic disease (score of 0) and SYMP mice that are infected and developed corneal herpetic disease with corneal neovascularization and opacity development of a score of 1 to 4. To quantify replication and clearance of HSV-1 from the eyes, mice were swabbed daily with moist, type 1 calcium alginate swabs. Swabs were placed in 1.0 ml titration media (Media 199, 2% penicillin/streptomycin, 2% newborn calf serum) and frozen at −80°C until titrated on RS cell monolayers, as described previously (43, 44).

Statistical analyses:

Data for each assay were compared by analysis of variance (ANOVA) and Student's *t* test using GraphPad Prism version 5.03. Differences between the groups were identified by ANOVA and multiple comparison procedures, as we previously described (45). Data are expressed as the mean ± SD. Results were considered statistically significant at $p < 0.05$.

RESULTS

1. HSV-seropositive asymptomatic individuals have frequent HSV-1 VP11/12$_{220–228}$ epitope-specific CD73$^+$CD8$^+$ T cells compared to symptomatic individuals:

The characteristics of the symptomatic (SYMP) and asymptomatic (ASYMP) study population used in this present study, with respect to gender, age, HLA-A*02:01 frequency distribution, HSV-1/HSV-2 seropositivity and status of ocular herpetic disease are presented in Table I and detailed in the *Materials and Methods section*. Since HSV-1 is the main cause of ocular herpes, only individuals who are HSV-1 seropositive and HSV-2 seronegative were enrolled in the present study. HSV-1 seropositive individuals were divided into two groups: (*i*) ten HLA-A*02:01 positive, HSV-1-infected ASYMP individuals who have never had any clinically detectable herpes disease; and (*ii*) ten HLA-A*02:01 positive HSV-1-infected SYMP individuals with a history of numerous episodes of well-documented recurrent clinical herpes diseases, such as herpetic lid lesions, herpetic conjunctivitis, dendritic or

geographic keratitis, stromal keratitis, and iritis consistent with rHSK, with one or more episodes per year for the past five years. One patient had over two severe recurrent episodes during the last ten years that necessitated multiple corneal transplantations. Only SYMP patients who were not on Acyclovir or other anti-viral or anti-inflammatory drug treatments at the time of blood sample collections were enrolled.

We first compared the frequency of HSV-specific $CD73^+CD8^+$ T cells, using HLA-A*02:01 specific immunodominant $VP_{220–228}$ epitope tetramer/anti-CD73/CD8 mAbs, in the peripheral blood of nine HLA-A*02:01 positive, HSV-1 seropositive ASYMP, nine HLA-A*02:01 positive, HSV-1 seropositive SYMP individuals, and ten seronegative healthy donors (controls). The low frequencies of PBMC-derived HSV-specific $CD8^+$ T cells complicate a direct *ex vivo* detection with tetramers using a typical number of PBMC ($\sim 10^6$ cells), and a prior expansion of $CD8^+$ T cells by HSV-1 or peptide stimulation in an *in vitro* culture will hamper a reliable determination of the frequency, phenotype and function of epitope-specific $CD8^+$ T cells. We, therefore, opted to measure the frequencies of $VP_{220–228}$ epitope-specific $CD8^+$ T cells *ex vivo* using a large number of PBMCs ($\sim 10 \times 10^6$) per tetramer/CD8 mAbs panel.

The representative dot plots shown in **Fig. 1A** indicate although similar frequencies of $VP_{220–228}$ peptide $VP_{220–228}$ epitope tetramer $^{(+)}$ $CD8^+$ T cells were detected in ASYMP versus SYMP individuals, higher frequencies of $CD73^+CD8^+$ T cells specific to the HSV-1 immunodominant $VP_{220–228}$ epitope were detected in one ASYMP individual (55.8%, *left two panels*) compared to one SYMP individual (33.2%, *right two panels*). **Fig. 1B** shows median frequencies of $CD73^+CD8^+$ T cells detected in eight SYMP and nine ASYMP individuals. The highest and most significant frequencies of $VP_{220–228}$ specific $CD73^+CD8^+$ T cells were consistently detected in ASYMP individuals (49.5% ± 10.5%). The frequencies of $VP_{220–228}$ specific $CD73^+CD8^+$ T cells were significantly lower in SYMP individuals compared to ASYMP individuals (49.5% ± 5.5% versus 30.5% ± 10.5%, respectively; $P = 0.01$). Despite repeated attempts, with and without *in vitro* expansions, $VP_{220–228}$ specific $CD73^+CD8^+$ T cells were consistently undetectable in seronegative healthy donors (*data not shown*).

We next determined whether there were any differences in the level of expression of CD73 on $VP_{220–228}$ specific $CD73^+CD8^+$ T cells from the peripheral blood of eight HLA-A*02:01 positive, HSV-1 seropositive ASYMP, six HLA-A*02:01 positive, HSV-1 seropositive SYMP individuals. The representative histograms shown in **Fig. 1C** indicated higher levels of the CD73 molecules detected on $CD8^+$ T cells, specific to the HSV-1 immunodominant $VP_{220–228}$ epitope, from one ASYMP individual (MFI of 3614, *left panel*) compared to one SYMP individual (MFI of 1371, *right panel*). **Fig. 1D** shows the median expression of CD73 on $CD8^+$ T cells (expressed as MFI) detected in eight ASYMP and six SYMP individuals. The highest and most significant levels of CD73 on $VP_{220–228}$ specific $CD73^+CD8^+$ T cells were consistently detected in ASYMP individuals (MFI of 3550± 355). The level of CD73 on $VP_{220–228}$ specific $CD73^+CD8^+$ T cells were significantly lower in SYMP individuals compared to ASYMP individuals (MFI of 1250 ± 165 vs. MFI of 3550 ± 355, respectively; $P = 0.03$).

The frequency of A2AR$^+$CD8$^+$ T cells specific to VP$_{220–228}$ epitopes complex was analyzed among ASYMP and SYMP individuals. **Fig. 1E**, shows representative FACS data of the high frequency of A2AR$^+$CD8$^+$ T cells detected in one ASYMP (27.1% ± 3.5%, *left panel*) as compared to one SYMP individual (12.7% ± 1.5%, *right panel*). **Fig. 1F** shows median frequencies of A2AR$^+$CD8$^+$ T cells detected in eight ASYMP and six SYMP individuals. The highest and most significant frequencies of VP$_{220–228}$ specific A2AR$^+$CD8$^+$ T cells were consistently detected in ASYMP individuals (25.5% ± 5.5% versus 11.5% ± 1.5%, respectively; $P < 0.05$).

Altogether, these results: (*i*) indicate that while HSV-seropositive SYMP and ASYMP individuals have similar frequencies of HSV-1 VP11/12$_{220–228}$ epitope-specific CD8$^+$ T cells, ASYMP individuals develop frequent HSV-specific CD73$^+$CD8$^+$ T cells compared to SYMP individuals; (*ii*) suggest that herpetic disease is not a consequence of a clonal deletion of specific repertoires of CD8$^+$ T cells in SYMP individuals; and (*iii*) suggest a positive role for the CD73 co-stimulatory molecule in the protection against ocular herpes.

2. Asymptomatic individuals have frequent HSV-1 VP11/12$_{220–228}$ epitope-specific effector memory CD73$^+$CD8$^+$ T$_{EM}$ and CD73$^+$CD8$^+$ T$_{EMRA}$ cells:

Next, we studied the expression levels of CD73 on the memory CD8$^+$ T cell sub-populations at various stages of differentiation: Naïve T cells (CD45RA^{high}CCR7^{high}CD8$^+$ T$_{NAIVE}$ cells); central memory T cells, (CD45RA^{low}CCR7^{high}CD8$^+$ T$_{CM}$ cells); effector memory T cells, (CD45RA^{high}CCR7^{low}CD8$^+$ T$_{EMRA}$ cells and CD45RA^{low}CCR7^{low}CD8$^+$ T$_{EM}$ cells). In the peripheral blood of 10 HLA-A*02:01 positive, HSV-1 seropositive ASYMP and 10 HLA-A*02:01 positive, HSV-1 seropositive SYMP individuals, we compared the CD73 expression in CD8$^+$ T cells specific to VP11/12$_{220–228}$ epitopes and divided them into T$_{NAIVE}$, T$_{CM}$, T$_{EM}$ and T$_{EMRA}$ phenotypes (**Figs. 2A to 2H**). Similar percentages of CD73$^+$CD45RA^{high}CCR7^{high}CD8$^+$ T$_{NAIVE}$ cells were detected in ASYMP and SYMP individuals (**Figs. 2A and 2B**). There was an increase in the CD73$^+$CD45RA^{low}CCR7^{high}CD8$^+$ T$_{CM}$ cells detected in ASYMP and SYMP individuals (**Figs. 2C and 2D**). Significantly higher percentages of CD73$^+$CD45RA^{high}CCR7^{low}CD8$^+$ T$_{EMRA}$ cells (**Figs. 2E and 2F**) and CD73$^+$CD45RA^{low}CCR7^{low}CD8$^+$ T$_{EM}$ cells (**Figs. 2G and 2H**) were detected in ASYMP individuals as compared to SYMP individuals ($P = 0.001$).

Altogether, the phenotypic properties of HSV-1 VP11/12$_{220–228}$ epitope-specific memory CD8$^+$ T cells revealed a clear dichotomy in memory CD8$^+$ T cell sub-populations in SYMP versus ASYMP individuals. ASYMP individuals appeared to develop frequent HSV-specific effector memory CD73$^+$CD8$^+$ T$_{EMRA}$ and CD73$^+$CD8$^+$ T$_{EM}$ cells, as compared to SYMP individuals. By maintaining high frequencies of the “experienced” HSV-specific CD73$^+$CD8$^+$ T$_{EMRA}$ cells and CD73$^+$CD8$^+$ T$_{EM}$ cells, the ASYMP individuals should be better protected against infection and/or disease. The higher expression of CD73 seen on CD8$^+$ T cells in ASYMP individuals may favor effector to memory CD8$^+$ T cell transition (E>>>M) and the formation of more HSV-specific CD73$^+$CD8$^+$ T$_{EMRA}$ cells and CD73$^+$CD8$^+$ T$_{EM}$ cells (46). Thus, these results suggest that during a second pathogen encounter (e.g. following HSV-1 reactivation from latency), ASYMP individuals, but not

SYMP individuals, would mount a much faster and stronger protective antiviral $CD73^+CD8^+$ T_{EMRA} and $CD73^+CD8^+$ T_{EM} cell responses, allowing for better clearance of infection and disease.

3. HSV-specific $CD73^{(+)}CD8^+$ T cells from asymptomatic individuals are multi-functional compared to HSV-specific $CD73^{(-)}CD8^+$ T cells:

We next compared the effector functions of HSV-1 $VP11/12_{220–228}$ epitope-specific $CD73^{(+)}CD8^+$ T cells versus $CD73^{(-)}CD8^+$ T cells from ASYMP and SYMP individuals. $VP11/12_{220–228}$ epitope-primed $CD8^+$ T cells from ASYMP individuals were divided into $CD73^+CD8^+$ T cells and $CD73^-CD8^+$ T cells (**Fig. 3A**) and their functions analyzed.

We first compared the expression levels of the $CD107^{a/b}$ cytotoxic degranulation molecules on gated $VP11/12_{220–228}$ epitope-specific $CD73^+CD8^+$ T cells and $CD73^-CD8^+$ T cells (**Fig. 3B**). We found high levels of CD107 expressed on $VP11/12_{220–228}$ epitope-specific $CD73^+CD8^+$ T cells from ASYMP individuals as compared to $VP11/12_{220–228}$ epitope-specific $CD73^-CD8^+$ T cells, suggesting a positive correlation of strong HSV-specific $CD73^+CD8^+$ T cell cytotoxic responses with protection from ASYMP ocular herpetic disease. A significant increase in the expression level of CD107, as determined by mean fluorescent intensity (**Fig. 3B**, $P \leq 0.05$), and higher percentages of $VP11/12_{220–228}$ epitope-specific $CD107^{high}CD73^+CD8^+$ T cells were consistently detected in ASYMP individuals as compared to SYMP individuals (**Figs. 3C and 3D**, $P = 0.01$). Cross-linking of the CD73 and CD3 molecules did not lead to increased levels of CD107 molecules on $CD8^+$ T cells from ASYMP individuals (**Fig. 3E**), suggesting that the expression level of CD107 had already reached its maximum.

We next determined the ability of HSV-specific $CD73^+CD8^+$ T cells versus $CD73^-CD8^+$ T cells to produce IFN-γ. Freshly isolated $CD8^+$ T cells from ASYMP individuals were stimulated *in vitro* for six hours with the immunodominant $VP11/12_{220–228}$ epitope peptide, as described in the *Materials and Methods* section. The percentages and numbers of IFN-γ^+CD8^+ T cells were compared among gated $VP11/12_{220–228}$ epitope-specific $CD73^+CD8^+$ T cells and $CD73^-CD8^+$ T cells by intracellular FACS staining. Significantly higher percentages of $VP11/12_{220–228}$ epitope-specific $CD73^+CD8^+$ T cells producing IFN-γ were detected in ASYMP individuals, as compared to lower percentages of $VP11/12_{220–228}$ epitope-specific IFN-$\gamma^+CD73^-CD8^+$ T cells (**Figs. 3F and 3G**, $P = 0.03$). Cross-linking of the CD73 and CD3 molecules significantly increased the level of IFN-γ by $CD8^+$ T cells produced from ASYMP individuals following $VP11/12_{220–228}$ peptide stimulation (**Fig. 3H**).

To confirm the polyfunctional nature of $CD73^+CD8^+$ T cells, we further studied the production of TNF-α on $VP11/12_{220–228}$ epitope-specific $CD8^+$ T cells. Freshly isolated $CD8^+$ T cells from ASYMP individuals were stimulated *in vitro* for six hours with the immunodominant $VP11/12_{220–228}$ epitope peptide, as described in *Materials and Methods section*. Percentages and numbers of TNF-α^+CD8^+ T cells were compared among gated $VP11/12_{220–228}$ epitope-specific $CD73^+CD8^+$ T cells and $CD73^-CD8^+$ T cells by intracellular FACS staining. Significantly higher percentages of $VP11/12_{220–228}$ epitope-specific $CD73^+CD8^+$ T cells producing TNF-α were detected in ASYMP individuals, as

compared to lower percentages of VP11/12$_{220–228}$ epitope-specific TNF-α^{+}CD73^{-}CD8^{+} T cells (**Figs. 3I and 3J**, $P = 0.01$). Following VP11/12$_{220–228}$ peptide stimulation and cross-linking of CD73 and CD3 molecules, there was significant increase in the levels of both IFN-γ and TNF-α produced by CD8^{+} T cells from ASYMP individuals (**Figs. 3H and 3K**).

In ASYMP individuals, we detected a positive correlation between the frequency of VP11/12$_{220–228}$ epitope-specific CD73^{+}CD8^{+} T cells and the expression of CD107$^{a/b}$ ($P = 0.3$, **Fig. 4A**) and the production of IFN-γ ($P = 0.01$, **Fig. 4B**). However, no correlation was found between the frequency of VP11/12$_{220–228}$ epitope-specific CD73^{+}CD8^{+} T cells and the production of TNF-α (**Fig. 4C**) ($P > 0.5$). Moreover, no correlation was found between the frequency of VP11/12$_{220–228}$ epitope-specific CD73^{-}CD8^{+} T cells and any of T cell functions studies (**Figs. 4D–4F**) ($P > 0.5$).

Altogether, these results suggest that in contrast to HSV-specific effector CD73^{-}CD8^{+} T cells, the HSV-specific effector CD73^{+}CD8^{+} T cells from ASYMP individuals are multi-functional. Overall, ASYMP individuals had higher proportions of multi-functional CD73^{+}CD8^{+} T cells with expression of three functions (IFN-γ, TNF-α and/or CD107$^{a/b}$).

4. HSV-specific CD73$^{(-)}$CD8^{+} T cells are exhausted compared to HSV-specific CD73$^{(+)}$CD8^{+} T cells:

VP11/12$_{220–228}$ epitope-specific CD8^{+} T cells from ASYMP and SYMP individuals were divided into CD73^{+}CD8^{+} T cells and CD73^{-}CD8^{+} T cells and FACS was used to determine the expression level of PD-1, a marker of T cell exhaustion, on tetramer gated CD8^{+} T cells specific to VP11/12$_{220–228}$ epitope, in CD73^{+}CD8^{+} and CD73-CD8^{+} T cell populations (**Figs. 5A and 5B**). The VP11/12$_{220–228}$ epitope-specific CD73^{+}CD8^{-} T cells expressed high level of PD-1 marker of exhaustion compared to CD73^{+}CD8^{+} T cells (**Figs. 5A and 5B**). The numbers on the top of each histogram represent the mean fluorescent intensity (MFI) depicting the expression level of PD-1 molecule on CD73^{+}CD8^{+} T and CD73^{-}CD8^{+} T cells **(Figs. 5A**). The percentages of PD-1^{high}CD73^{+}CD8^{+} T cells and PD-1^{high}CD73^{-}CD8^{+} T cells were also compared between the two T cell sub-populations (**Fig. 5C**). Significantly higher frequency of VP11/12$_{220–228}$ epitope-specific PD-1^{high}CD73^{-}CD8^{+} T cells were detected in SYMP individuals, as compared to lower frequency in ASYMP individuals (**Fig. 5C**). In both SYMP and ASYMP individuals, an inverse correlation was detected between the frequency of VP11/12$_{220–228}$ epitope-specific CD73^{+}CD8^{+} T cells and the level expression of PD-1 (**Fig. 5D**). Moreover, we compared the frequency of PD-1^{high}TIM-3^{high}CD73^{+}CD8^{+} T cells and PD-1^{high}TIM-3^{high}CD73^{-}CD8^{+} T cells side-by-side in SYMP and ASYMP individuals (**Fig. S1**). Significantly higher percentages of VP11/12$_{220–228}$ epitope-specific PD-1^{high}TIM3^{high}CD73^{-}CD8^{+} T cells were detected in SYMP individuals compared to ASYMP individuals (**Fig. S1C-S1F**).

These results confirm that: (*i*) more HSV-specific effector CD73^{-}CD8^{+} T cells from SYMP individuals are dysfunctional (exhausted). In contrast, HSV-specific effector CD73^{+}CD8^{+} T cells from ASYMP individuals are mostly multifunctional; (*ii*) the increase in the frequency of HSV-specific effector CD73^{-}CD8^{+} T cells from SYMP individuals expressing PD-1 and TIM-3 markers of T cell exhaustion is consistent with continuous antigenic stimulation (47);

(*iii*) a higher expression of PD-1 on HSV-specific effector $CD8^+$ T cells from SYMP individuals also suggests more exposure to Ag compared to ASYMP individuals, leading to partial dysfunction.

Because of the obvious ethical and practical considerations of obtaining tissue-resident $CD8^+$ T cells (i.e. from the cornea or from TG, the site of acute and latent infections, respectively), our investigations in humans were limited to human PBMC-derived $CD8^+$ T cells. To gain more insight into the nature of protective versus non-protective cornea and TG-resident HSV-specific $CD8^+$ T cell sub-populations in SYMP and ASYMP herpes, the remainder of the study utilized a mouse model of ocular herpes.

5. Frequent functional HSV-1 $gB_{495–505}$ epitope-specific $CD73^+CD8^+$ T cells are present in the cornea of asymptomatic mice compared to the cornea of symptomatic mice:

We first compared, in a kinetic study, the frequency of HSV-specific $CD8^+$ T cells expressing CD73 molecule from the cornea and trigeminal ganglia (TG) of HSV-1-infected B6 mice. Representative FACS data of the percentages of $CD73^+CD8^+$ T cells (**Fig. 6A**) and average percentages of $CD73^+CD8^+$ T cells detected on 0, 8, 14, 23 and 41 days post infection (**Fig. 6B**) showed a peak frequency for $gB_{498–505}$ epitope-specific $CD73^+CD8^+$ T cells, which was reached in the cornea of HSV-1 infected mice around day 23 post-infection (PI). However, this peak was reached in infected TG as early as day 14 PI. The numbers outlined in the top left corner of each dot plot in **Fig. 6A** indicate the highest percentages of $gB_{495–505}$ epitope-specific $CD73^+CD8^+$ T cells were at 44% in cornea on day 23 PI and 62% in TG on day 14 PI. Kinetics of the average percentages of $CD73^+CD8^+$ T cells showed a decline of $gB_{495–505}$ epitope-specific $CD73^+CD8^+$ T cells in the cornea starting around day 23 PI and around day 14 PI in the TG (**Fig. 6B**).

We next compared the frequency of the HSV-specific $CD73^+CD8^+$ T cells in the cornea and TG of HSV-1 infected SYMP versus ASYMP B6 mice PI (**Fig. 6C**). The low frequencies of HSV-specific $CD8^+$ T cells in the TG and cornea of ocularly infected SYMP and ASYMP B6 mice complicate a direct *ex vivo* detection of $gB_{498–505}$ epitope-specific $CD73^+CD8^+$ T cells from a typical number of $\sim 10^6$ cells using $gB_{498–505}$/tetramers. Moreover, a prior expansion of $CD8^+$ T cells by HSV-1 or $gB_{498–505}$ peptide stimulation in an *in vitro* culture would hamper determination of a reliable frequency of $gB_{495–505}$ epitope-specific $CD8^+$ T cells. We therefore opted to measure the frequencies of $gB_{498–505}$ epitope-specific $CD8^+$ T cells *ex vivo* by pooling ten corneas and ten TG from SYMP or from ASYMP mice and then using a large number of cells ($\sim 10 \times 10^6$) per tetramer/CD8 mAbs panel.

The representative dot plots shown in **Fig. 6C** indicate that a significantly higher frequency of $CD73^+CD8^+$ T cells specific to the HSV-1 $gB_{495–505}$ epitope was present in the cornea of ASYMP mice (61.9%, *top left panel*) as compared to SYMP mice (43.7%, $P = 0.04$, *top right panel*). However, similar frequencies of $CD73^+CD8^+$ T cells were detected in TG of SYMP and ASYMP mice (49.2% and 56.4%, $P > 0.05$, respectively, *two bottom panels*). Despite repeated attempts, with and without *in vitro* expansions, $gB_{495–505}$ specific $CD73^+CD8^+$ T cells were consistently undetectable in mock-infected B6 mice (*data not shown*).

We then compared the effector functions of the HSV-1 $gB_{495–505}$ epitope epitope-specific $CD73^{(+)}CD8^+$ T cells from cornea and TG ASYMP versus SYMP mice **(Fig. 6D**). We found a significantly higher frequency of $gB_{495–505}$ epitope-specific IFN-$\gamma^+CD73^+CD8^+$ T cells in the corneas of ASYMP mice as compared to corneas of SYMP mice (10.9% and 5.6%, $P = 0.03$, respectively, *top panels*). However, similar frequencies of IFN-$\gamma^+CD73^+CD8^+$ T cells were detected in the TG of SYMP and ASYMP mice (12.4% and 9.8%, $P > 0.05$, respectively, *bottom panels*). Moreover, significantly higher percentages of $gB_{495–505}$ epitope-specific $CD107^+CD73^+CD8^+$ T cells were detected in both the corneas and TGs of ASYMP mice, as compared to lower percentages of $gB_{498–505}$ epitope-specific $CD107^+CD73^+CD8^+$ T cells in the corneas and TGs of SYMP mice (**Fig. 6E**, $P = 0.03$ and $P = 0.04$, respectively).

Altogether, these results indicate that, similar to ASYMP humans: (*i*) HSV-1 infected ASYMP mice developed frequent HSV-specific $CD73^+CD8^+$ T cells compared to SYMP mice; (*ii*) the HSV-specific effector $CD73^+CD8^+$ T cells from ASYMP mice produce IFN-γ and have cytotoxic activity; (*iii*) the phenotypic and functional studies in both humans and mice suggest a positive role for effector memory $CD8^+$ T cells expressing the CD73 co-stimulatory molecule in protection against ocular herpes infection and disease.

6. $CD73^{-/-}$ deficient mice develop significantly less memory $CD8^+$ cells in the cornea and trigeminal ganglia and more recurrent herpetic infection and disease:

Next, we determined whether the lack of CD73 would directly affect HSV-specific $CD8^+$ T cells mobilization and function in the cornea and TG and whether this would affect recurrent herpetic infection and disease. $CD73^{-/-}$ deficient and WT mice were infected ocularly with 2×10^5 pfu of HSV-1 (strain McKrae) without corneal scarification, as described in *Materials and Methods*. On day 35 (i.e. during latent phase), the eyes of 15 infected animals that survive acute infection were exposed to UV-B irradiation in order to induce reactivation of HSV-1 from latently infected TG. UV-B irradiation led to virus shedding in tears and recurrent corneal herpetic disease, as we recently described (43, 44). The timeline of HSV-1 infection, UV-B irradiation and subsequent immunological and virological assays is illustrated in **Fig. 7A**.

Virus replication in the cornea (the site of HSV acute replication) and corneal herpetic disease were compared in $CD73^{-/-}$ deficient mice and wild type (WT) B6 mice (**Figs. 7B to 7D**). The frequency of HSV-specific $CD73^+CD8^+$ T cells and the function of $CD8^+$ T cells were compared in the cornea (**Figs. 7E to 7H**) and TG (**Figs. 7I to 7L**) of $CD73^{-/-}$ deficient mice and WT mice.

Viral titers measured in corneal swabs taken on day 8 after UV-B exposure demonstrated significantly less control of virus replication ($P < 0.05$, **Fig. 7B**) leading to significantly more recurrent ocular herpetic disease ($P = 0.04$, **Figs. 7C and 7D**) in $CD73^{-/-}$ deficient mice compared to WT mice. $CD73^{-/-}$ mice developed severe cornea lesions (average score of 3 on a scale of 0 to 4) compared to WT mice (average score of 1.5). This was associated with a significant decrease in $CD103^+CD8^+$ T cells infiltrating the cornea ($P < 0.05$, **Figs. 7E and 7F**) and TG ($P < 0.05$, **Figs. 7I and 7J**) of $CD73^{-/-}$ mice compared to WT mice. However, similar frequencies of HSV-1 $gB_{498–505}$-specific IFN-γ^+CD8^+ T cells were

detected in the cornea (**Fig. 7G**) and TG (**Fig. 7K**) of $CD73^{-/-}$ and WT mice. Moreover, we detected significant decreases in HSV-1 $gB_{498–505}$-specific $CD107^{+}$ $CD8^{+}$ T cytotoxic T cells infiltrating the cornea (**Fig. 7H**) and TG (**Fig. 7L**) of $CD73^{-/-}$ mice as compared to WT mice.

These results indicated that the lack of CD73 led to a decreased function of HSV-specific $CD8^{+}$ T cells in the cornea and TG. In addition, this was associated with severe recurrent herpes infection and disease in both humans and mice confirming a positive role for effector memory $CD8^{+}$ T cells expressing the CD73 costimulatory molecule in the protection against ocular herpes infection and disease. The association of HSV-specific $CD73^{+}CD8^{+}$ T cell response with ASYMP herpes makes this memory sub-population desired in T-cell based immunotherapies against ocular herpes, as it might be involved, with a yet-to-be determined mechanism, in the protection against recurrent herpes infection and disease.

DISCUSSION

Characterizing the phenotype and function of HSV-specific effector and memory $CD8^{+}$ T cells associated with ASYMP herpes infection is critical for the design of T cell-based herpes immuno-therapies. In this study, we report that HSV-specific effector memory $CD73^{+}CD45RA^{high}CCR7^{low}CD8^{+}$ T_{EMRA} and $CD73^{+}CD45RA^{low}CCR7^{low}CD8^{+}$ T_{EM} cells are present in significantly higher proportions in HSV-1 infected ASYMP individuals. In contrast, SYMP individuals had significantly lower frequencies of HSV-specific $CD73^{-}CD8^{+}$ T_{CM} cells; (*ii*) similar to ASYMP individuals, ASYMP B6 mice developed polyfunctional HSV-specific $CD73^{+}CD8^{+}$ T cells in the cornea and TG following ocular infection with HSV-1, suggesting a protective role of CD73 pathway in herpes infection and disease. The functional results of $CD73^{+}CD8^{+}$ T cells from ASYMP individuals suggest that, compared to SYMP individuals, ASYMP individuals are better prepared to mount protective cytotoxic and IFN-γ-producing cytotoxic $CD8^{+}$ T cell responses, which are the two effector arms of immunity that protect against herpes; (*iii*) The role of CD73 in herpes protection was further clarified following ocular infection of wild-type (WT) and CD73 $^{-/-}$ deficient mice with HSV-1. In contrast to WT mice, HSV-1 infected CD73 $^{-/-}$ mice were highly susceptible to immune-mediated pathology. The $CD8^{+}$ T cell phenotypic and functional studies, in both humans and mice, suggest a positive role for memory $CD8^{+}$ T cells expressing the CD73 costimulatory molecule in protection against ocular herpes infection and disease. The association of HSV-specific $CD73^{-}CD8^{+}$ T cell response with SYMP herpes makes memory $CD73^{-}CD8^{+}$ T cell sub-population undesired in immunotherapeutic strategies against herpes, as $CD73^{-}CD8^{+}$ T cells might be involved, with a yet-to-be determined mechanism, in the pathogenesis of herpetic disease. Our findings should hopefully guide a successful design of effective T cell-based vaccines and immunotherapeutic strategies.

Memory $CD8^{+}$ T cells play a major role in host defense by enabling the immune system to respond more rapidly and vigorously to previously encountered infectious pathogens by rapid recognition and lysis of virus-infected cells. The importance of both T_{CM} and T_{EM} subsets for the control of infectious diseases and the effectiveness of vaccines has been shown in several human and murine studies (48–51). Our understanding of the population

size, the phenotype and the function of protective versus non-protective HSV-specific $CD8^+$ T cell sub-populations mainly comes from studies of B6 mouse model of herpes infection (18, 29, 36, 52–54). To gain insight into the nature of human protective versus non-protective HSV-specific $CD8^+$ T cell sub-populations, we used several human focused immunological assays to compare the protective versus non-protective HSV-1 $VP11/12_{220–228}$ epitope-specific $CD8^+$ T cells in the peripheral blood of ASYMP and SYMP individuals at both effector and memory levels (1). Our findings from this study indicate that ASYMP and SYMP individuals have similar frequencies of HSV-1 $VP11/12_{220–228}$ epitope-specific $CD8^+$ T cells, thereby suggesting that herpetic disease is not a consequence of a clonal deletion of specific repertoires of $CD8^+$ T cells in SYMP individuals. This result is in agreement with a recent study which exemplifies that once the clonal repertoire of HSV-specific memory $CD8^+$ T cells is established, it is kept constant for several years (55). Nevertheless, there are phenotypic and functional differences in T cells between ASYMP and SYMP individuals. Within the overall memory $CD8^+$ T cell population, two distinct major sub-populations have been described and can be recognized by the differential expression of chemokine receptor CCR7 and L-selectin (CD62L). Central memory T_{CM} cells express CD62L and CCR7 and secrete IL-2, but not IFN-γ or IL-4. Effector memory T_{EM} cells do not express CD62L or CCR7, rather, the cells produce effector cytokines like IFN-γ and IL-4, consistent inasmuch as our findings. A lack of CCR7 expression, which is required for T cell egress and recirculation, impairs the capacity of T_{EM} cells for homing to lymphoid tissues (56). We previously reported on phenotypic and functional differences of HSV-specific effector and memory $CD8^+$ T cells from ocular herpes ASYMP and SYMP individuals. ASYMP individuals had a significantly higher proportion of differentiated poly-functional HSV--specific $CD8^+$ T_{EM} cells. Conversely, SYMP patients had significantly higher frequencies of less-differentiated mono-functional HSV-specific $CD8^+$ T_{CM} cells (39, 41, 57). The present study extends those findings by demonstrating that the “naturally” protected ASYMP individuals have significantly higher proportions of multi-functional HSV-specific effector memory $CD8^+$ T cells ($CD73^+CD45RA^{high}CCR7^{low}CD8^+$ T_{EMRA} and $CD73^+CD45RA^{low}CCR7^{low}CD8^+$ T_{EM} cells compared to mono-functional HSV-specific central memory $CD8^+$ T cells ($CD73^+CD45RA^{low}CCR7^{high}CD8^+$ T_{CM} cells in SYMP individuals; and *ii*) In like manner, T_{EM} and T_{EMRA} appeared predominantly in most ASYMP individuals and were most effector-like, with a high expression of $CD107^{a/b}$ and high production of IFN-γ. With less effector like conditions, T_{EM} and T_{EMRA} in SYMP individuals, comparatively, possessed a low expression of $CD107^{a/b}$ and less production of IFN-γ. We are aware that the information gained from peripheral blood T cells may not be completely reflective of tissue-resident T cells. However, due to the obvious ethical and practical considerations of obtaining tissue-resident $CD8^+$ T cells from the cornea or from the TG, the sites of acute and latent infections, our investigations in humans were limited to peripheral blood-derived $CD8^+$ T cells. Nevertheless, the results obtained from the B6 mouse model showed there was a significant increase in the frequency of $CD73^+CD8^+$ T cells in the corneas (but not TG) of ASYMP mice compared to the corneas of SYMP mice. The reason why we found high frequency of $CD73^+CD8^+$ T cells in peripheral blood of ASYMP individuals (which is away from the sites of latency) but not in TG of ASYMP mice remains to be determined. In spite of this, our results indicate that maintaining high frequencies of the “experienced”

HSV-specific $CD73^+CD45RA^{high}CCR7^{low}CD8^+$ T_{EMRA} and $CD73^+CD45RA^{low}CCR7^{low}CD8^+$ T_{EM} cells can better arm ASYMP individuals to prevent and/or efficiently clear new infections and reactivations. At a second pathogen encounter (e.g., following HSV-1 reactivation from latency), ASYMP individuals, but not SYMP individuals, can mount markedly stronger and faster protective HSV-specific $CD73^+CD45RA^{high}CCR7^{low}CD8^+$ T_{EMRA} and $CD73^+CD45RA^{low}CCR7^{low}CD8^+$ T_{EM} cell responses allowing for better clearance of herpes infection and disease.

Naïve T cells are commonly characterized by the surface expression of CD62L, CCR7, CD45RA and the absence of memory CD45RO isoform (reviewed in (58)). While naïve T cells are regularly regarded as a quiescent cell population, there is increasing evidence that naïve T cells are actually heterogeneous in phenotype, function, dynamics and differentiation status, resulting in a whole spectrum of naïve cells with different properties (58). For instance, some non- naïve T cells express surface markers similar to naïve T cells (T_{SCM}, stem cell memory T cells (59). T_{MP}, memory T cells, carry a naïve phenotype (60), while some antigen-specific naïve T cells may have lost their naïve phenotype (reviewed in (61)). In this study, we found antigen-specific tetramer-binding T cells expressing a high level of CD45RA and CCR7 falling into the phenotype of naïve cells (Fig. 2). Nonetheless, these naïve T cells could be heterogeneous and therefore must be further characterized in ASYMP and ASYMP individuals to better appreciate potential differences in the phenotype, including expression of CD73, function, dynamics and differentiation status.

Furthermore, previous studies in mice have shown that, following HSV-1 infection, $CD8^+$ T cells persist in the cornea and TG for prolonged periods of time following clearance of corneal herpes lesions (13, 62–68). The phenotype and function of these persistent $CD8^+$ T cells have not been fully elucidated. In this report, we investigated the association between HSV-1 disease severity and the quantity, quality and diversity of HSV-specific memory $CD8^+$ T cell sub-populations from blood (in human) and cornea (in mice). We found that: (*i*) circulating and tissue resident HSV-1 $VP11/12_{220–228}$ epitope-specific memory $CD8^+$ T cells differ among individuals with mild to nonexistent symptoms (ASYMP) versus individuals with severe symptoms (SYMP) of ocular herpetic diseases; and (*ii*) specific phenotype and function of HSV-1 $VP11/12_{220–228}$ epitope-specific, tissue resident memory $CD8^+$ T cells are associated with ocular herpes disease in mice. Whether the phenotype and function of $CD8^+$ T cells found in B6 mice can be extrapolated to human corneal tissue resident memory, as reported in other herpes systems (69, 70), is yet to be determined. Investigating the phenotype and function of $CD8^+$ T cells in human cornea and TG samples from ASYMP and SYMP cohorts is warranted to determine the quantity, quality and diversity of cornea- and TG-resident HSV-specific memory $CD8^+$ T cell sub-populations, as well as their association with corneal disease severity. This information would help guide our ongoing efforts in developing a T-cell based immunotherapeutic strategy to cure ocular herpes.

HSV-specific memory $CD8^+$ T cells elicited during a primary infection persist for years after clearance of the virus and mediate recall responses that halt future attempts of virus reactivation from latency. We previously reported that circulating $CD8^+$ T cells from HSV-1-seropositive ASYMP individuals had a greater frequency of polyfunctional HSV-specific

$CD8^+$ T_{EM} cells compared to more $CD8^+$ T_{CM} cells from SYMP patients (57). The present study extends those findings by reporting that polyfunctional HSV-specific $CD8^+$ T_{EM} cells that are predominant in ASYMP individuals expressed higher levels of CD73 costimulatory molecules. The HSV-1 $VP11/12_{220–228}$ epitope-specific $CD73^+CD8^+$ T cells, but not the HSV-1 $VP11/12_{220–228}$ epitope-specific $CD73^-CD8^+$ T cells, in ASYMP individuals displayed high levels of CD107 and produced elevated IFN-γ, implying a protective role for the $CD73^+CD8^+$ T cells in ocular herpes. The present results confirm our previous reports that up to 95.5% of ASYMP individuals had $CD8^+$ T cells that were able to produce 3 to 5 functions concurrently compared to only 32% of SYMP individuals (1, 57). Additionally, it suggests that polyfunctional $CD73^+CD8^+$ T cells, but not the $CD73^-CD8^+$ T cells from ASYMP individuals may provide a better and faster immune surveillance to protect against recurrent herpetic disease. Based on these findings, we propose a mechanism built on a novel "symptomatic/asymptomatic concept," in which the immunopathology associated with corneal lesions are the result from the balance between immune-pathological $CD73^-CD8^+$ T cell responses specific to SYMP HSV-1 epitopes and immuno-protective $CD73^+CD8^+$ T cell responses specific to ASYMP HSV-1 epitopes. These mechanisms might involve $CD73^+CD8^+$ T_{EM} and $CD73^+CD8^+$ T_{EMRA} cell-mediated protection in ASYMP individuals. Accordingly, the polyfunctionality of HSV-specific ASYMP $CD73^+CD8^+$ T_{EM} and $CD73^+CD8^+$ T_{EMRA} cells is likely to be one factor, among others, that accounts for the T cell control of herpes infection and disease. Mono-functional HSV-specific SYMP $CD73^-CD8^+$ T cells, on the other hand, may be involved in the immuno-pathological recurrent corneal disease. However, this does not mitigate or exclude the possibility of other hosts or viral factors either acting alone or in concert with non-protective $CD73^-CD8^+$ T cell responses in determining the course of herpetic disease. Regardless the mechanism, if ASYMP individuals develop more of protective $CD73^+CD8^+$ T_{EMRA} and $CD73^+CD8^+$ T_{EM} cells while SYMP patients do not, it would be logical to exclude epitopes that do not induce protective $CD73^+CD8^+$ T_{EMRA} and $CD73^+CD8^+$ T_{EM} cells from future herpes vaccines.

We recently demonstrated the requirement of $CD4^+$ T cell help for efficient priming and maintenance of HSV-specific $CD8^+$ T cells (18, 45). Others have shown that $CD4^+$ T cells are required to pave the way for efficient migration of memory $CD8^+$ T cells into restricted tissues, such as the cornea and TG (71–74). We do not exclude that $CD73^+CD4^+$ T cells act directly as effector cells or as helper cells in the mobilization of effector and memory $CD73^+CD8^+$ T_{EM} cells into HSV infected tissues of ASYMP individuals. Future studies will determine whether: (*i*) HSV-specific effector $CD73^+CD4^+$ T cells are frequent in ASYMP compared to SYMP individuals; and (*ii*) helper $CD73^+CD4^+$ T cells would contribute in the mobilization of HSV-specific effector and memory $CD8^+$ T_{EM} cells into HSV-1 infected cornea and TG, and the results will be the subject of future reports.

T cell exhaustion contributes to the failure to control persistent infections, and this likely includes latent/chronic HSV-1 and HSV-2 infections. High expression of inhibitory receptors, including PD-1, and the inability to sustain functional T cell responses contribute to T cell exhaustion. We detected a higher expression of PD-1 exhaustion receptor on HSV-specific $CD73^-CD8^+$ T cells compared to HSV-specific $CD73^+CD8^+$ T cells. This suggests that functional exhaustion of PD-$1^{high}CD73^-CD8^+$ T cells, predominantly in SYMP patients, may contribute to symptomatic herpes disease. These findings do not exclude that

infection with some highly pathological clinical isolates of HSV-1 in SYMP individuals leads, by a yet-to-be-determined mechanism, to functionally compromised HSV-specific PD-$1^{high}CD73^{-}CD8^{+}$ T cells. The latter is supported by a recent report of differences in the protective and pathological properties of HSV-2 clinical isolates from the United States and South Africa (75). The mechanism that leads to phenotypic and functional exhaustion of PD-$1^{high}CD73^{-}CD8^{+}$ T cells in SYMP individuals remains to be determined. Although continuous production of antigens does not occur during latent HSV-1 infections, antigenic stimulation during sporadic reactivations of HSV-1 from latency may lead to T cell exhaustion. We are currently testing whether blockade of the negative T cell co-stimulatory pathway PD-1/PDL-1 using specific mAb therapy in combination with vaccination will restore the function of exhausted HSV-specific $CD8^{+}$ T cells in latently infected mice following UV-B induced virus reactivation. This might enhance the epitope-specific T_{EM} cell responses in otherwise SYMP mice and protect against herpetic disease.

The association of HSV-specific SYMP $CD73^{-}CD8^{+}$ T cell response with herpetic disease makes this sub-population undesired in T-cell based immunotherapies against ocular herpes, as it may be involved with a yet-to-be determined mechanism in the pathogenesis of ocular herpes. In contrast, the HSV-specific ASYMP $CD73^{+}CD8^{+}$ T cells might be involved, with a yet-to-be determined mechanism, in protection against ocular herpes. In this context, it is worth recapping that CD73 is an ecto-5'-nucleotidase, an enzyme that is critically involved in the conversion of ATP into extracellular adenosine (76–81). It is therefore likely that increased expression of CD73 on T_{EM} cells and the high frequency of $CD73^{+}CD8^{+}$ T_{EM} cells detected ASYMP individuals may contribute to an increase in the amount of extracellular adenosine which in turn is associated to express type A2 adenosine receptors, A2AR, expressed on other types of immune cells, including $CD8^{+}$ T cells (82). Moreover, our results show that a higher proportion of $CD8^{+}$ T cells in ASYMP individuals expressed A2AR, likely allows them to bind and clear the extracellular adenosine (Fig. 1E). Our future studies will exploit available knockout mice lacking A2ARs and determine whether the protective effect of $CD73^{+}CD8^{+}$ T cells is limited to their ability to bind adenosine and whether selective A2AR agonists/antagonists can be used to effectively improve $CD73^{+}CD8^{+}$ T cell responses against herpes infection and disease. Such studies will elucidate the cellular and molecular mechanisms by which CD73 and A2AR, both appear to be expressed on ASYMP HSV-specific $CD8^{+}$ T_{EM} cells and the results from those studies will be the subject of future reports.

In this study, we show that the HSV-1-specific $CD73^{+}CD8^{+}$ T cells play crucial role in protection against ocular herpes infection and disease. CD73, an enzyme that convert extracellular AMP into adenosine, is expressed on various types of immune cells, including $CD4^{+}$ T cells, and binds to the A2A adenosine receptor (A2AR) (76–78). CD73 also plays an important role in regulating T cells migration into infected tissues (76–78, 81). Moreover, compared to SYMP individuals, in ASYMP individuals we found significantly higher frequency of HSV-specific $CD8^{+}$ T cells expressing the A2A adenosine receptor (i.e. $A2AR^{+}CD8^{+}$ T cells) (Fig. 1C). The A2AR receptor allows expansion of T cells lacking effector functions in extracellular adenosine-rich microenvironment (83–85). T cells are targets of adenosine-mediated immune-regulation because they predominantly express A2AR (83, 86). Whether A2AR promote expansion of HSV-2-specific $CD73^{+}CD8^{+}$ T_{EM} cells and/or

mobilize effector and memory HSV-2 specific $CD73^{+}CD8^{+}$ T cells into the infected vaginal mucosa and dorsal ganglia (the sites of acute and latent HSV-2 infections). Whether this would contribute to the natural immunity seen in the genital herpes ASYMP individuals is being currently investigated, and the results will be the subjects of future reports.

In summary, our investigation provides new insights about the phenotype and the function of protective HSV-specific $CD8^{+}$ T cell sub-populations that are associated with the immunologic control of herpes infection and disease in ASYMP individuals. It suggests an import role of polyfunctional HSV-specific $CD73^{+}CD8^{+}$ T_{EMRA} cells and $CD73^{+}CD8^{+}$ T_{EM} cells in anti-viral defense against ocular HSV-1 infection and disease. The association of HSV-specific $CD73^{-}CD8^{+}$ T cell response with SYMP herpes makes memory $CD73^{-}CD8^{+}$ T cell sub-populations undesired in immunotherapeutic strategies against herpes, as $CD73^{-}CD8^{+}$ T cells might be involved, with a yet-to-be determined mechanism, in the pathogenesis of herpetic disease. Thus, the findings demonstrate quantitative and qualitative features of effective HSV-specific $CD8^{+}$ T cell responses that should be taken into consideration in designing effective T cell-based ocular herpes immunotherapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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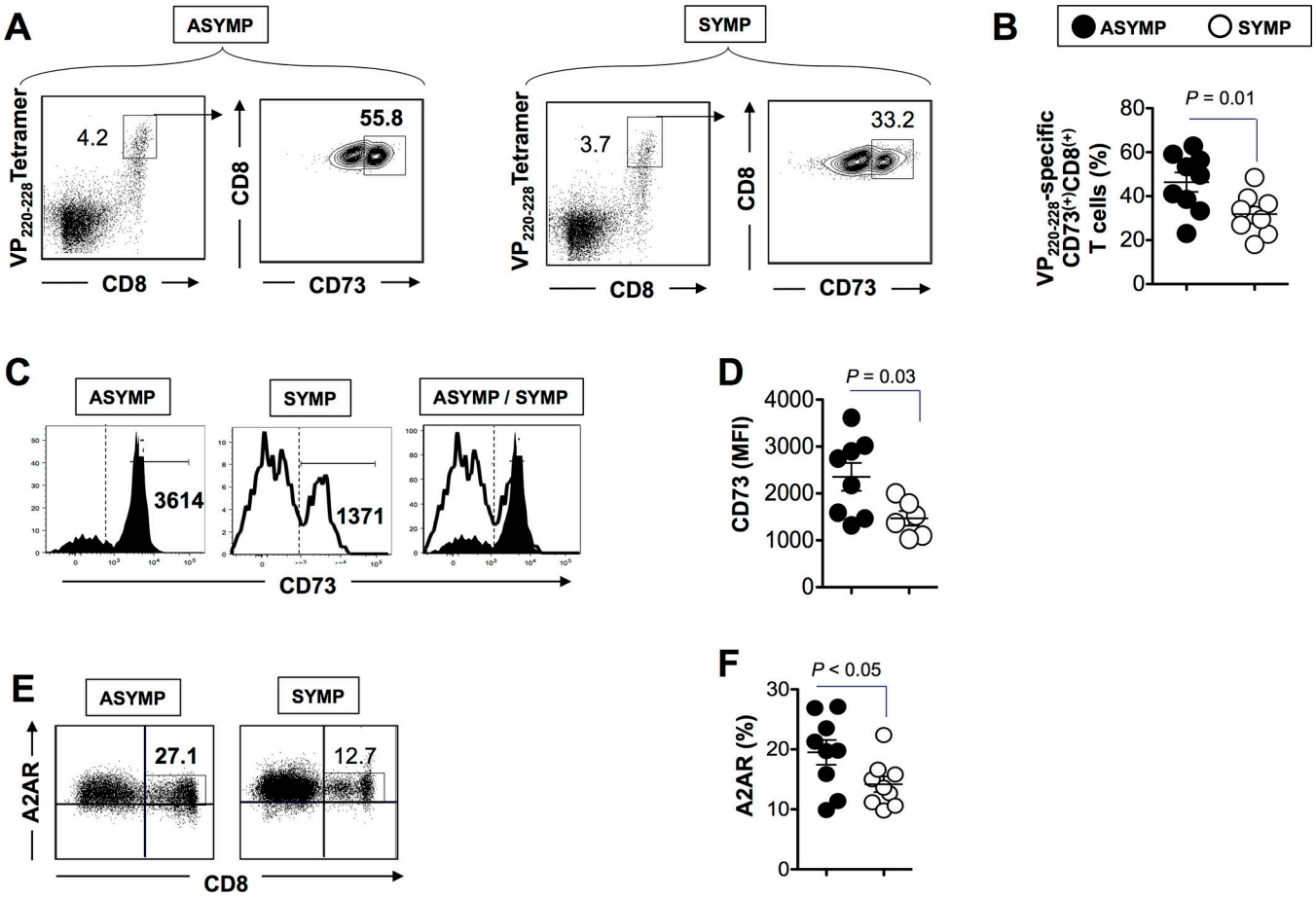

Figure 1: Frequent HSV-1 VP11/12$_{220–228}$ epitope-specific CD73^{+}CD8^{+} memory T cells detected in ASYMP individuals compared to SYMP individuals:

The frequency of CD73^{+}CD8^{+} T cells specific to the VP$_{220–228}$ peptide/tetramer complex was analyzed in HLA-A*02:01 positive HSV-1 seropositive ASYMP and SYMP individuals. (**A**) Representative FACS data of the frequencies of CD73^{+}CD8^{+} T cells, specific to VP11/12$_{220–228}$ epitope, detected in PBMCs from one HLA-A*02:01 positive HSV-1 seropositive ASYMP individual (*left two panels*) and one HLA-A*02:01 positive HSV-1 seropositive SYMP individual (*right two panels*). (**B**) Average frequencies of PBMC-derived CD8^{+} T cells, specific to VP11/12$_{220–228}$ epitope, detected from nine HLA-A*02:01 positive HSV-1 seropositive ASYMP individuals compared to nine HLA-A*02:01 positive HSV-seropositive SYMP individuals. The CD73 molecule expression level on CD8^{+} T cells specific to VP$_{220–228}$ epitope was analyzed in HLA-A*02:01 positive HSV-1 seropositive ASYMP and SYMP individuals. (**C**) Representative FACS data of the CD73 expression level on CD8^{+} T cells specific to VP$_{220–228}$ epitope detected from one HLA-A*02:01 positive HSV-1 seropositive ASYMP individual (*left panels*) and one HLA-A*02:01 positive HSV-1 seropositive SYMP individual (*right panels*). (**D**) Average CD73 expression level on CD8^{+} T cells, specific to VP11/12$_{220–228}$ epitope, detected from eight HLA-A*02:01 positive HSV-1 seropositive ASYMP individuals compared to six HLA-A*02:01 positive HSV-seropositive SYMP individuals. The frequency of A2AR^{+}CD8^{+} T cells specific to VP$_{220–228}$ epitopes complex was analyzed in ASYMP and SYMP individuals. (**E**)

Representative FACS data of the frequencies of $A2AR^+CD8^+$ T cells detected from one ASYMP (*left panel*) and one SYMP individual (*right panel*). (**F**) Average frequencies of $A2AR^+CD8^+$ T cells from nine ASYMP compared to nine SYMP individuals. The results are representative of two independent experiments in each individual. The indicated *P* values, calculated using unpaired t test, show statistical significance between SYMP and ASYMP individuals.

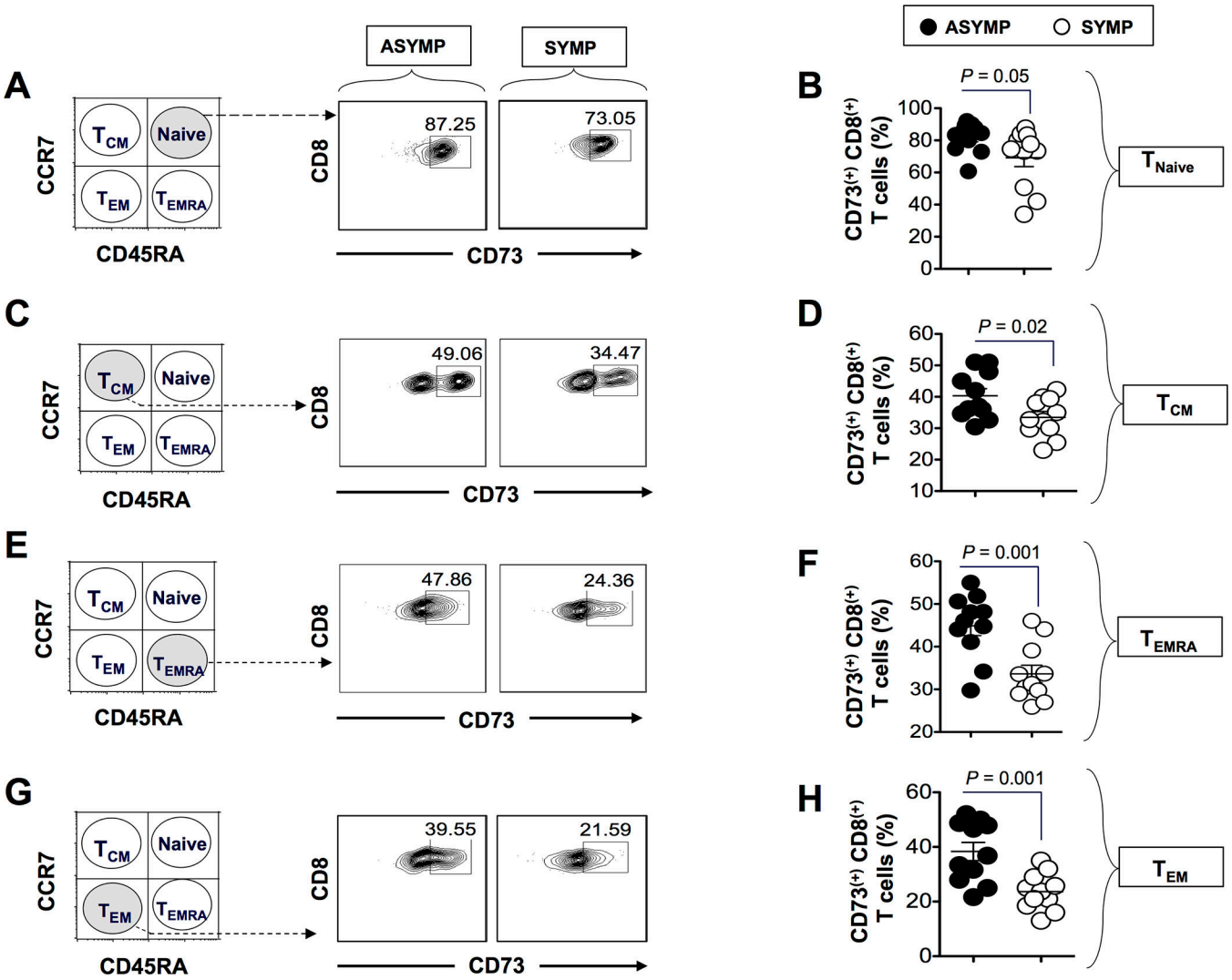

Figure 2. Frequent HSV-1 VP11/12$_{220–228}$ epitope-specific CD8$^+$ T cells with effector memory phenotype (CD73$^+$CD8$^+$ T$_{EMRA}$ and CD73$^+$CD8$^+$ T$_{EM}$ cells) detected in ASYMP individuals compared to SYMP individuals:

The CD8$^+$ T cells specific to VP11/12$_{220–228}$ peptide/tetramer representing shown in Fig. 1 were analyzed in terms of T_{NAIVE}, T_{CM}, T_{EMRA} and T_{EM} phenotypes. Representative FACS data of the frequencies of (**A**) CD45RA^{high}CCR7^{high}CD8$^+$ T_{NAIVE} cells, (**C**) CD45RA^{low}CCR7^{high}CD8$^+$ T_{CM} cells, (**E**) CD45RA^{high}CCR7^{low}CD8$^+$ T_{EMRA} cells, and (**G**) and CD45RA^{low}CCR7^{low}CD8$^+$ T_{EM} cells detected in one ASYMP individual and one SYMP individual. Representative FACS data of the frequencies of (**B**) CD45RA^{high}CCR7^{high}CD8$^+$ T_{NAIVE} cells, (**D**) CD45RA^{low}CCR7^{high}CD8$^+$ T_{CM} cells, (**F**) CD45RA^{high}CCR7^{low}CD8$^+$ T_{EMRA} cells and (**H**) CD45RA^{low}CCR7^{low}CD8$^+$ T_{EM} cells detected from 10 ASYMP and 10 SYMP individuals. The results are representative of two independent experiments in each individual. The indicated *P* values, calculated using unpaired t test, show statistical significance between SYMP and ASYMP individuals.

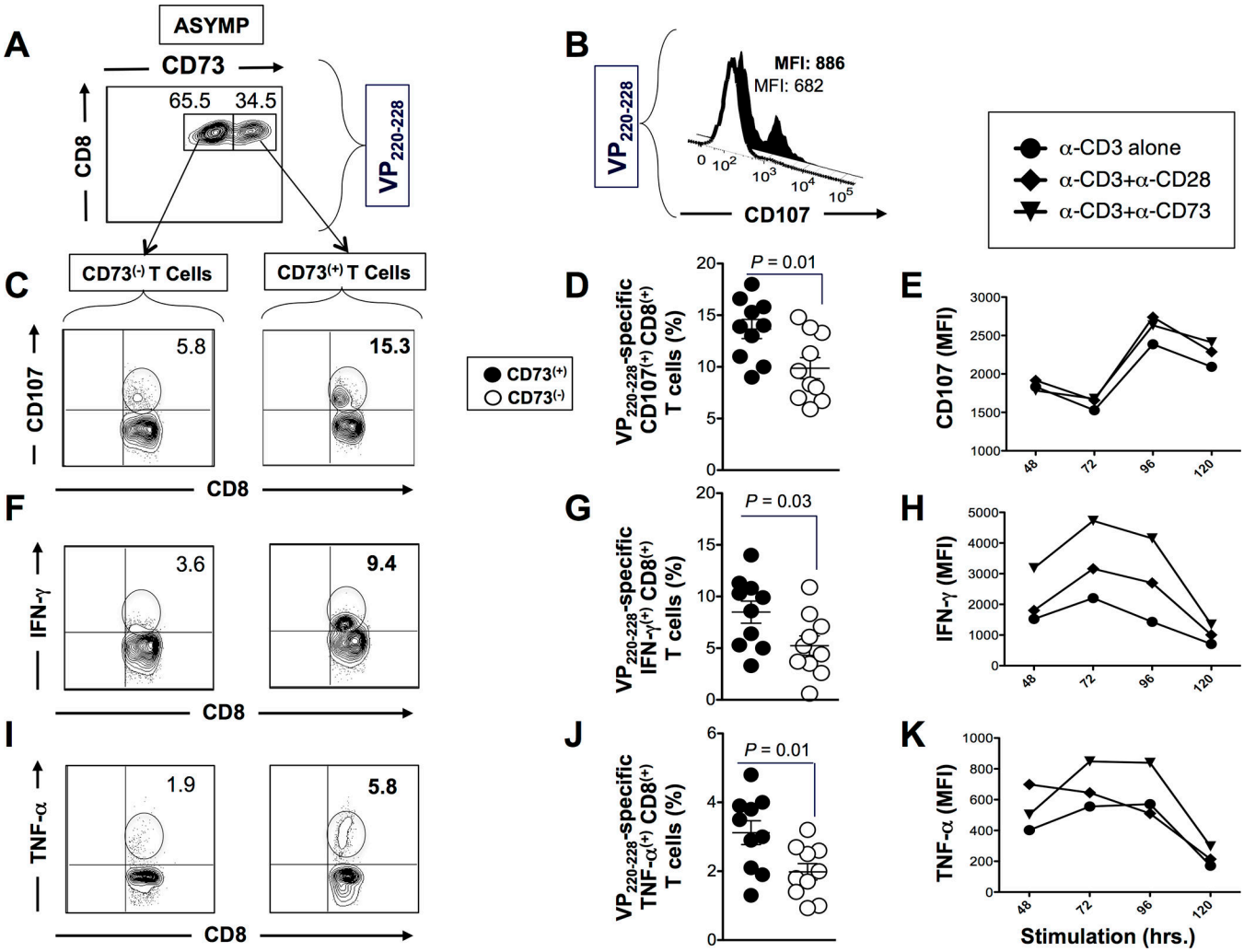

Figure 3. Frequent HSV-1 VP11/12$_{220–228}$ epitope-specific CD73^{+}CD8^{+} polyfunctional T cells detected in symptomatic individuals:

(**A**) VP11/12$_{220–228}$ epitope-primed CD8^{+} T cells from ASYMP individuals were divided into CD73^{+}CD8^{+} T cells and CD73^{-}CD8^{+} T cells and their functions compared. (**B**) FACS was used to determine the expression level of CD107$^{a/b}$ on tetramer gated CD8^{+} T cells specific to VP11/12$_{220–228}$ epitope, as described in the *Materials and Methods section*. VP11/12$_{220–228}$ epitope-specific CD73^{+}CD8^{+} T cells express high levels of CD107$^{a/b}$ cytotoxic degranulation compared to CD73^{-}CD8^{+} T cells. The numbers on the top of each histogram represent the mean fluorescent intensity (MFI) depicting the expression level of CD107$^{a/b}$ molecules. Numbers in bold represent mean fluorescent intensity (MFI) on CD73^{+}CD8^{+} T cells from ASYMP individual. Representative FACS data (**C**) and average percentage (**D**) of VP11/12$_{220–228}$ epitope-specific CD107a/b^{+}CD73^{+}CD8^{+} T cells versus CD107a/b^{+}CD73^{-}CD8^{+} T cells. (**E**) Level of CD107$^{a/b}$ molecules expression on VP11/12$_{220–228}$ epitope-specific CD8^{+} T cells following co-stimulation with (*i*) mAbs anti-CD3 alone, (*ii*) mAbs anti-CD3 + mAbs anti-CD28 (*iii*) anti-CD3 + mAbs anti-CD73. Representative FACS data (**F**) and average percentage (**G**) of VP11/12$_{220–228}$ epitope-specific IFN-γ^{+}CD73^{+}CD8^{+} T cells versus IFN-γ^{+}CD73^{-}CD8^{+} T cells are shown. (**H**) IFN-

γ expression level on VP11/12$_{220–228}$ epitope-specific CD8$^+$ T cells following co-stimulation on with (*i*) mAbs anti-CD3 alone, (*ii*) mAbs anti-CD3 + mAbs anti-CD28 (*iii*) anti-CD3 + mAbs anti-CD73. Representative FACS data (**I**) and average percentages (**J**) of VP11/12$_{220–228}$ epitope-specific TNF-α$^+$CD73$^+$CD8$^+$ T cells versus TNF-α$^+$CD73$^-$CD8$^+$ T cells are shown. (**K**) TNF-α expression levels on VP11/12$_{220–228}$ epitope-specific CD8$^+$ T cells following co-stimulation on with (*i*) mAbs anti-CD3 alone, (*ii*) mAbs anti-CD3 + mAbs anti-CD28 (*iii*) anti-CD3 + mAbs anti-CD73. The average frequencies of CD8$^+$ T cells from 10 HLA-A*02:01-positive ASYMP and 10 SYMP individuals in response to stimulation with the VP11/12$_{220–228}$ peptide are shown. The results are representative of two independent experiments in each individual. The indicated *P* values, calculated using one-way ANOVA Test, show statistical significance between SYMP and ASYMP individuals.

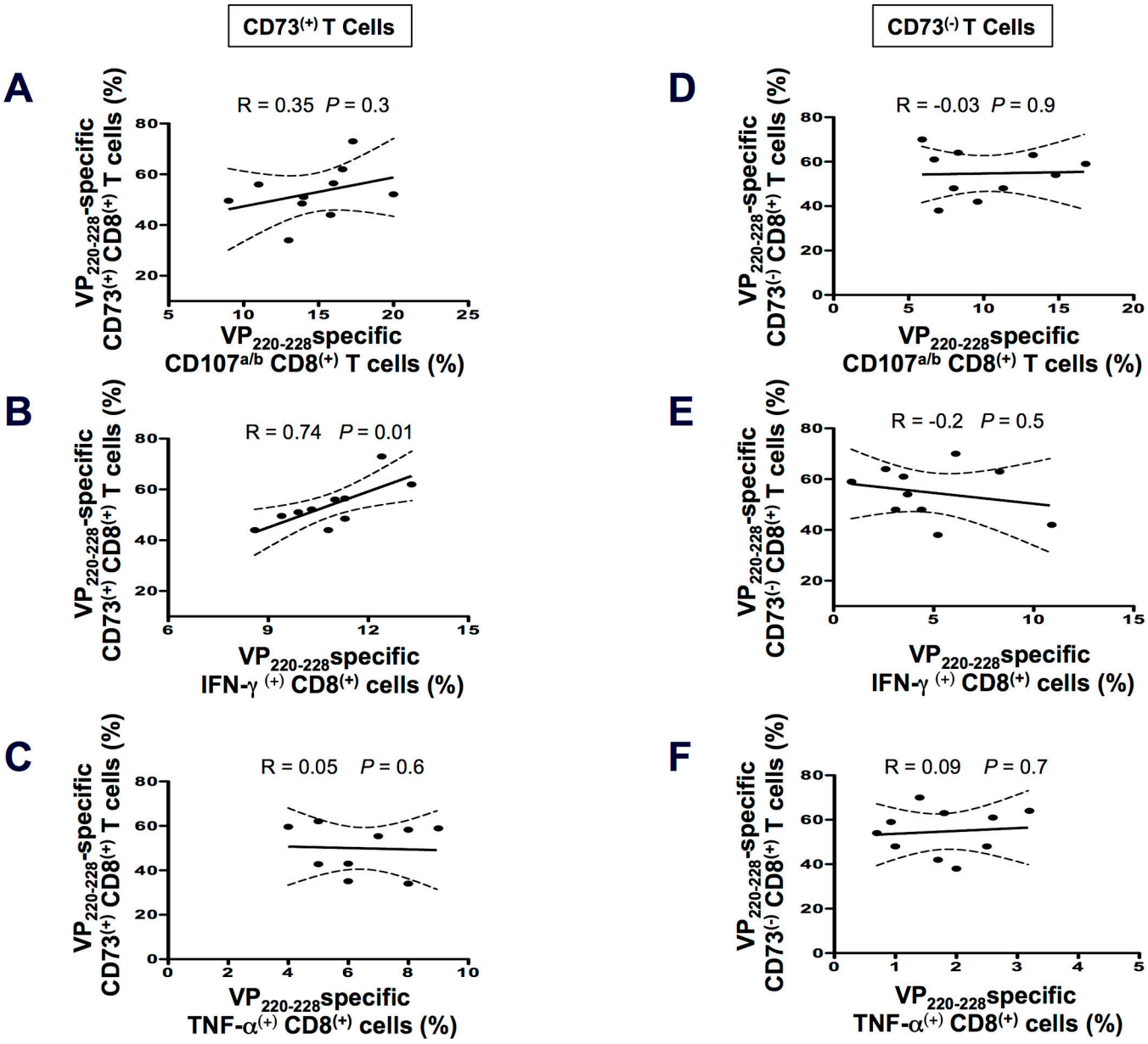

Figure 4. HSV-1 VP11/12$_{220–228}$ epitope-specific CD73^{+}CD8^{+} T cells are more functional:
Correlation of VP11/12$_{220–228}$ epitope-specific CD73^{+}CD8^{+} T cells percentage in PBMC with (**A**) expression of CD107$^{a/b}$ cytotoxic degranulation molecules, and production of (**B**) IFN-γ and (**C**) TNF-α. Correlation of VP11/12$_{220–228}$ epitope-specific CD73^{-}CD8^{+} T cells percentage in PBMC with (**D**) expression of CD107$^{a/b}$ cytotoxic degranulation molecules, and production of (**E**) IFN-γ and (**F**) TNF-α. The nominal *P* values show statistical significance between the percentage of VP11/12$_{220–228}$ epitope-specific CD8^{+} T cells and production of IFN-γ function.

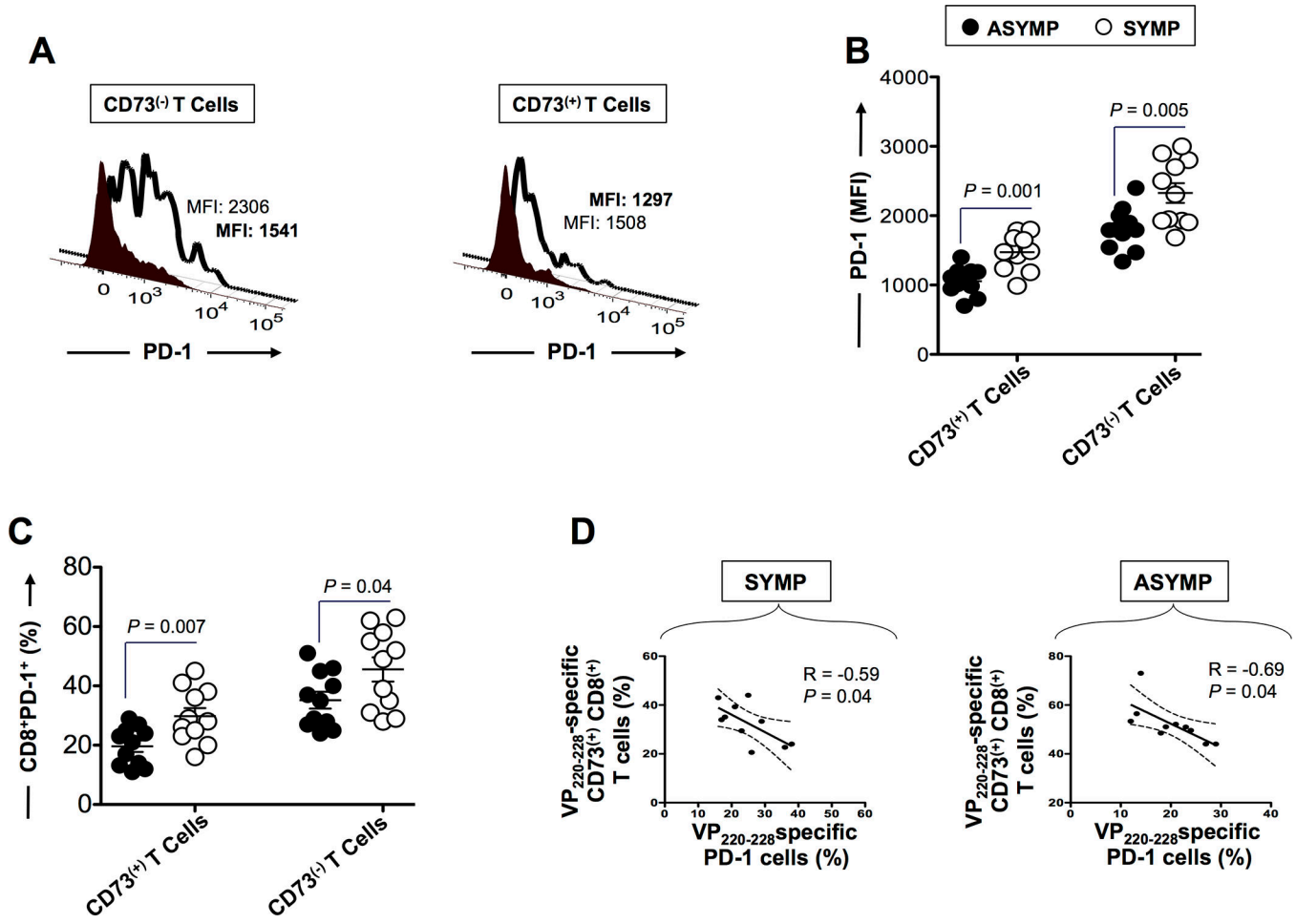

Figure 5. HSV-1 VP11/12$_{220–228}$ epitope-specific CD73^{-}CD8^{+} T cells are dysfunctional (exhausted):

VP11/12$_{220–228}$ epitope-specific CD8^{+} T cells from ASYMP and SYMP individuals were divided into CD73^{+}CD8^{+} T cells and CD73^{-}CD8^{+} T cells and their function compared. VP11/12$_{220–228}$ epitope-specific CD73^{-}CD8^{+} T cells express high level of PD-1 marker of exhaustion compared to CD73^{+}CD8^{+} T cells. FACS was used to determine the expression level of PD-1 on tetramer gated CD8^{+} T cells specific to VP11/12$_{220–228}$ epitope, as described in the *Materials & Methods* section. The numbers on the top of each histogram represent mean fluorescent intensity (MFI) depicting the expression level of PD-1 molecule. (**A**) Representative FACS data (**B**) average MFI (**C**) and average percentage of VP11/12$_{220–228}$ epitope-specific PD-1^{high}CD73^{+}CD8^{+} T cells versus PD-1^{high}CD73^{-}CD8^{+} T cells are shown. (**D**) An inverse correlation of VP11/12$_{220–228}$ epitope-specific CD73^{+}CD8^{+} T cells percentage in PBMC with PD-1 expression in SYMP and ASYMP individuals. The results are representative of two independent experiments in each individual. The indicated *P* values, calculated using unpaired t test, show statistical significance between SYMP and ASYMP individuals.

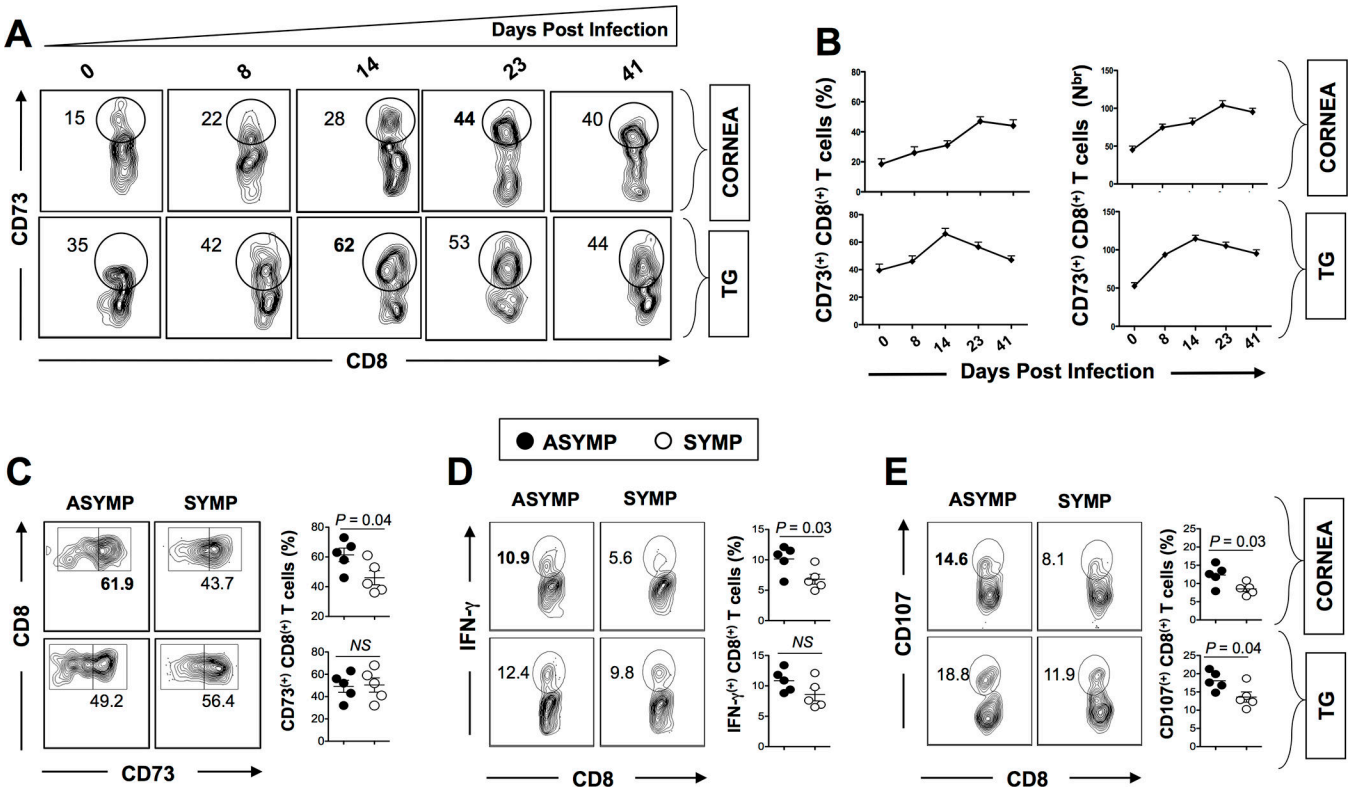

Figure 6. Frequent functional $gB_{498–505}$ epitope-specific $CD73^+CD8^+$ T cells detected in the cornea and trigeminal ganglia of HSV-1 infected ASYMP B6 mice:

(**A** and **B**) Kinetics of CD73 molecule expression in cornea- and trigeminal ganglia-derived $CD8^+$ T cells. Flow cytometry of the frequency of $gB_{498–505}$ epitope specific $CD73^+CD8^+$ T cells in the cornea and trigeminal ganglia (TG) of B6 mice (n =10) following ocular infection with HSV-1 (McKrae, 2×10^5 pfu/eye). (**C**) Representative FACS data (*left panel*) and average frequency (*right panel*) of the $gB_{498–505}$ epitope-specific $CD73^+CD8^+$ T cells in the cornea and trigeminal ganglia (TG) of ASYMP (n = 5) versus SYMP (n = 5) mice. (**D**) Representative FACS data (*left panel*) and average frequency (*right panel*) of the $gB_{498–505}$ epitope-specific IFN-γ^+CD8^+ T cells (**E**) and $CD107^+CD8^+$ T cells in the cornea and trigeminal ganglia (TG) of ASYMP (n = 5) versus SYMP (n = 5) mice. Data are representative of two independent experiments. The indicated P values, calculated using unpaired t test, show statistical significance between SYMP and ASYMP individuals.

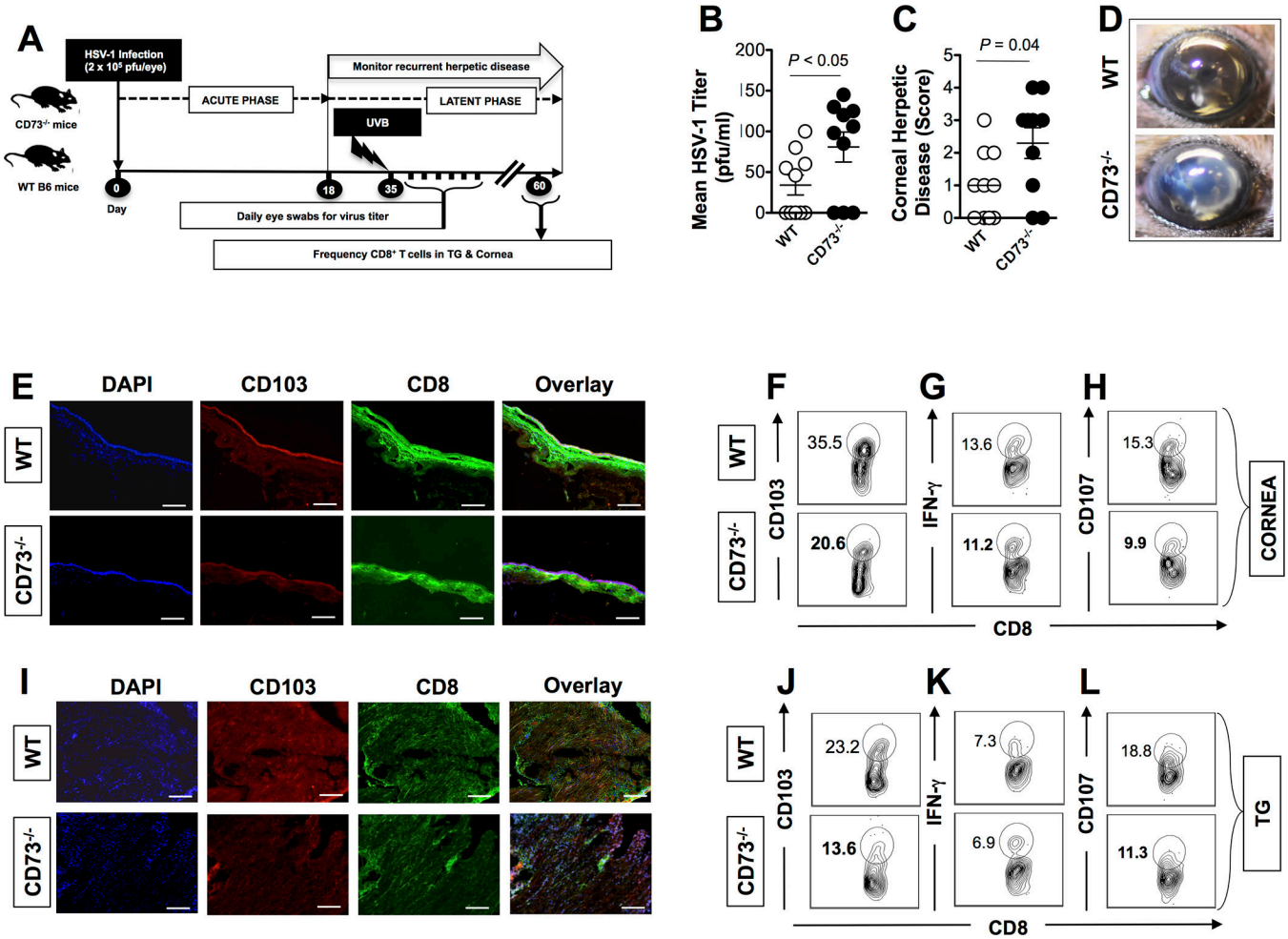

Figure 7. CD73$^{-/-}$ deficient mice developed more corneal infection and severe herpetic disease associated with less functional HSV-specific CD8^{+} T cells in cornea and trigeminal ganglia compared to WT B6 mice:

(**A**) Schematic representation of the timeline of HSV-1 infection and UV-B induced recurrent disease in WT B6 mice and CD73$^{-/-}$ deficient mice. A group of CD73$^{-/-}$ deficient mice and WT mice (6–8 weeks old) were ocularly infected on day 0 with 2 × 10^{5} pfu of HSV-1 (strain McKrae) following scarification. After establishment of latency (35 days post-infection), reactivation of latent virus was induced following irradiation with UV-B. Tears were collected daily for six days post-UV-B and recurrent corneal disease was observed daily for eye disease for 25 days post UV-B exposure. (**B**) Presence of infectious virus in the tears of WT and CD73$^{-/-}$ mice post UV-B treatment. Viral titer estimation detected in tear samples six days post UV-B irradiation expressed as mean of virus load. The data are expressed as mean of virus load (plaque forming units (pfu)/ml). (**C**) Recurrent corneal herpetic disease detected for up to 25 days following UV-B irradiation and scored on a scale of 0 to 4. (**D**) Representative slit lamp images of WT and CD73$^{-/-}$ mice corneas. Mice were euthanized on day 60 post UV-B exposure and single cell suspensions from cornea and TG was obtained after collagenase treatment and stained for markers of CD8^{+} T cells, IFN-γ and CD107 and analyzed by immunostaining and FACS. Sections of the cornea (**E**) and TG (**I**) from WT and CD73$^{-/-}$ mice were co-stained using Blue - DAPI (DNA stain), mAb

specific to CD103-Red - Green - CD8 Infiltration. Single cell suspension from the cornea was obtained after collagenase treatment at 37^0C for an hour and stained for markers of $CD8^+$ T_{RM} cells, IFN-γ and $CD107^{a/b}$ and analyzed by FACS. Representative FACS plot of the frequency of HSV-specific $CD103^+CD8^+$ T cells (**F** and **J**), IFN-γ^+CD8^+ T cells (**G** and **K**) and $CD107^{a/b+}CD8^+$ T cells (**H** and **L**) detected in cornea and TG of WT and $CD73^{-/-}$ deficient mice. The results are representative of two independent experiments. The indicated *P* values, calculated using an unpaired t test, show statistical significance between WT and $CD73^{-/-}$ deficient mice.

Table I:

Cohorts of HLA-A*02:01 positive, HSV seropositive Symptomatic and Asymptomatic individuals enrolled in the study.

Subject-level characteristic	All subjects (*n* = 39)
Gender [no. (%)]:	
Female	15 (51%)
Male	14 (49%)
Race [no. (%)]	
Caucasian	19 (66%)
Non-Caucasian	10 (34%)
Age [median (range) yr.]	39 (21–67 yr.)
HSV status [no. (%)]	
HSV-1 seropositive	29 (100%)
HSV-2 seropositive	0 (0%)
HSV-1 and −2 seropositive	0 (0%)
HSV seronegative	10 (100%)
HLA [no. (%)]	
HLA-A*02:01 positive	24 (83%)
HLA-A*02:01 negative	5 (17%)
Herpes disease status [no. (%)]	
Asymptomatic (ASYMP)	19 (66%)
Symptomatic (SYMP)	10 (34%)

Definition of symptomatic and asymptomatic individuals are detailed in Materials and Methods.