Intraocular varicella-zoster virus (VZV) and HSV type 1 (HSV-1) infections cause sight-threatening uveitis. The disease is characterized by an intraocular inflammatory response involving herpesvirus-specific T cells. T cell reactivity to the noncausal human alphaherpesvirus (αHHV) is commonly detected in the affected eyes of herpetic uveitis patients, suggesting the role of cross-reactive T cells in the disease. This study aimed to identify and functionally characterize intraocular human alphaherpesvirus cross-reactive T cells. VZV protein immediate early 62 (IE62), which shares extensive homology with HSV ICP4, is a previously identified T cell target in VZV uveitis. Two VZV-specific CD4 T cell clones (TCC), recovered from the eye of a VZV uveitis patient, recognized the same IE6218-927 peptide using different TCR and HLA-DR alleles. The IE621918-927 peptide bound with high affinity to multiple HLA-DR alleles and was recognized by blood-derived T cells of 5 of 17 HSV-1/VZV-seropositive healthy adults but not in cord blood donors (n = 5). Despite complete conservation of the IE62 epitope in the orthologous protein ICP4 of HSV-1 and HSV-2, the TCC recognized VZV and HSV-1– but not HSV-2–infected B cells. This was not attributed to proximal epitope-flanking amino acid polymorphisms in HSV-2 ICP4. Notably, VZV/HSV-1 cross-reactive CD4 T cells controlled VZV but not HSV-1 infection of human primary retinal pigment epithelium (RPE) cells. In conclusion, we report on the first VZV/HSV-1 cross-reactive CD4 T cell epitope, which is HLA-DR promiscuous and immunoprevalent in coinfected individuals. Moreover, ocular-derived peptide-specific CD4 TCC controlled VZV but not HSV-1 infection of RPE cells, suggesting that HSV-1 actively inhibits CD4 T cell activation by infected RPE cells.

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was to identify and functionally characterize intraocular eHHV cross-reactive T cells in relation to the pathology of eHHV uveitis.

Materials and Methods

Cell lines and T cell clones

Human melanoma MeWo cells (ATCC ID number HTB-65) and African green monkey kidney epithelial Vero cells (ATCC ID number CCL-81) were grown in DMEM (Invitrogen) containing 10% heat-inactivated FBS (Sigma-Aldrich) and antibiotics. The human RPE cell line ARPE-19 (ATCC ID number number ACM62283) and HSV-2 ICp4 residues 972–1082 (GenBank accession number NP_044530) were amplified by PCR from DNA isolated from VZV-infected wild-type VHS-1 (KOS strain) and HSV-2 (strain MS) DNA using the following primers: HSV-1_Fw (5′-gaagatccagtcatGGCggcctgcggggcgcg-3′), HSV-1_Rv (5′-ggaattcgCTCGAGCAGCGTTCCTG-3′), HSV-2_Fw (5′-gaagatcctcatgtGCGccggcgggggcccg-3′), and HSV-2_Rv (5′-gaagatccCTCGAGCAGCGTTCCTG-3′). Amplification was performed using Phi29 DNA polymerase (New England Biolabs) and the following conditions (denaturation for 5 min at 95 °C, 40 cycles (1 min at 95 °C, 1 min at 65 °C, and 1 min at 72 °C), and final extension for 15 min at 72 °C. The resulting amplifications were purified using the MinElute Gel Extraction kit (Qiagen) and cloned into pEGFP-N1 plasmid (BD Biosciences) in frame with the ORF of EGFP, using restriction enzymes BglII and EcoRI. Plasmid DNA was isolated using the Qiagen Plasmid Midi kit to confirm appropriate in-frame insertions of the target sequences in the vector by sequencing analysis using the BidDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems) and the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Synthetic peptides and HLA-DR allele binding affinity

The N-terminal FITC-conjugated and nonmodified peptides, varying in length from 9 to 12 aa, were synthesized by Sigma-Aldrich and GeneCust, respectively, to >70% purity. The HLA-DR binding potential was predicted using the following HLA-DR binding epitope prediction software (http://www.ims.uni-bielefeld.de/Software/HCA/Propred/) and NetMHCIIpan (37), and SVRMHC (38). Peptides were considered to bind to a specific HLA-DR allele if more than or equal to four of six programs predicted high-affinity peptide binding. The HLA-DR allele–specific peptide binding affinity was determined as previously described (39), using affinity-purified HLA-DR molecules and FITC-conjugated peptides known to bind with high affinity to the respective HLA-DR alleles, combined with an interfering concentration of the virus-derived peptide of interest. Binding affinities were considered to be high (<1 μM), intermediate (1–10 μM), weak (10–100 μM), or absent (>100 μM) (39).

Plasmids

Sequences coding for HSV-1 ICp4 residues 945–1054 (GenBank accession number ACNM26283) and HSV-2 ICp4 residues 972–1082 (GenBank accession number NP_044530) were amplified by PCR from DNA isolated from VZV- and HSV-infected wild-type VHS-1 (KOS strain) and HSV-2 (strain MS) DNA using the following primers: HSV-1_Fw (5′-gaagatccagtcatGGCggcctgcggggcgcg-3′), HSV-1_Rv (5′-ggaattcgCTCGAGCAGCGTTCCTG-3′), HSV-2_Fw (5′-gaagatcctcatgtGCGccggcgggggcccg-3′), and HSV-2_Rv (5′-gaagatccCTCGAGCAGCGTTCCTG-3′). Amplification was performed using Phi29 DNA polymerase (New England Biolabs) and the following conditions (denaturation for 5 min at 95 °C, 40 cycles (1 min at 95 °C, 1 min at 65 °C, and 30 s at 72 °C), and final extension for 10 min at 72 °C. The resulting amplifications were purified using the MinElute Gel Extraction kit (Qiagen) and cloned into pEGFP-N1 plasmid (BD Biosciences) in frame with the ORF of EGFP, using restriction enzymes BglII and EcoRI. Plasmid DNA was isolated using the Qiagen Plasmid Midi kit to confirm appropriate in-frame insertions of the target sequences in the vector by sequencing analysis using the BidDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems) and the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

IFN-γ ELISPOT assay

Autologous BLCL were used as APC for IFN-γ ELISPOT assays in three different ways. First, BLCL were infected overnight with HSV at a multiplicity of infection (MOI) of 10 or pulsed overnight with predefined optimal concentrations of protein lysates obtained from VZV-, HSV-, and mock-infected cells. Second, BLCL were nucleofected with pEGFP-N1 control plasmid with the indicated synthetic peptides in RPMI 1640 medium (2 μM or as indicated) (20). Third, BLCL were nucelofected with pEGFP-N1 control plasmid and plasmids expressing HSV-1 ICp4 aa 945–1054 (pEGFP-N1/HHSV-1: ICp4945-1054) or HSV-2 ICp4 aa 972–1082 (pEGFP-N1/HHSV-2 ICp4972-1082) using Amaxa Cell Line Nucleofector kit V (Lonza). IFN-γ ELISPOT assays were performed in triplicate, according to the manufacturer’s conditions (Mabtech Biolog). Briefly, T cells (5 × 10⁶ cells/well) were precultured with BLCL (2 × 10⁶ cells/well) for 1.5 h in U-bottom 96-well plates (Greiner Bio-One) at 37 °C, followed by an additional 6 h incubation at 37 °C in anti–IFN-γ precoated Multiscreen HTS IP ELISPOT plates (Millipore) (20). T cells were washed away, and the secondary anti–IFN-γ mAb, followed by the streptavidin-alkaline-phosphatase mAb were added to the wells. Spots were visualized by adding NBT-5-bromo-4-chloro-3-indolylphosphate substrate (Lucron Elitech). Resulting blue IFN-γ spots were counted with an automated ELISPOT reader (Saanquin Reagents). Each experiment was repeated at least twice.

Detection of VZV peptide IE62915–929–reactive T cell responses in blood samples

PBMC were isolated from heparinized peripheral blood samples of 17 HLA-typed VHS-1– and VZV-seropositive healthy adult individuals and 5 cord blood donors. Both the VZV uveitis patients and (cord) blood donors were recruited from the same geographic area of The Netherlands (South-Holland province), which is composed of an ethnically heterogeneous population for more densely populated areas in The Netherlands. The donors’ HLA-DR genotype were determined as described previously (17). The PBMC and cord blood mononuclear cells (CBMC) fractions (10⁶ each) were incubated with the VZV peptide IE62915–929 (0.1 μM) in TCIM with IL-2 (50 U/ml; Erotech) added after 3 d and supplemented after an additional 5 d of culture. After 1.5–2 wk of culture, the resulting short-term peripheral blood– and cord blood–derived T cell cultures were assayed for reactivity to VZV IE62915–929 peptide, and medium as negative control, by IFN-γ ELISPOT as described above.
Primary human RPE cell lines were grown to confluence in 48-well plates (~5 × 10^4 cells/well) and the expression of HLA-II and costimulatory molecules was induced by incubation with recombinant human IFN-γ (500 U/ml; PeproTech) for 3 d in S10F as described previously (17). The HLA-II phenotype of the uveitis patients and RPE cell lines, and the HLA-II restriction of TCC were previously determined (17, 18, 20). The RPE 171 cell line expresses HLA-DRB1*0301,0701 compatible with the HLA-DRB1*0703 restriction element of TCC 53, whereas RPE 172 cells express HLA-DRB1*1201,1501 compatible with the HLA-DRB1*1501 restriction element of TCC 44 (17, 18, 20). Human RPE cells were infected with HSV-1-GFP-VP16 (5 × 10^5 PFU/well), resulting in a MOI of 0.5–1, or infected with VZV-GFP-66-infected MeWo cells (ratio of approximately one infected cell to four uninfected cells) for 4 h at 37°C. Subsequently, the RPE cells were washed three times, and 7.5–15 × 10^4 to 1.5 × 10^5 T cells in 200 µl TCM were added to each well. At the indicated times, cell-free conditioned medium was collected, RPE cells were harvested by trypsinization, and the frequency of viable EGFP-expressing RPE cells was determined by flow cytometry. Supernatants were clarified by centrifugation for 5 min (1000 × g) and stored at −20°C for subsequent assay by ELISA for IFN-γ levels (eBioscience). Experiments were performed in triplicate and repeated at least three times. Alternatively, RPE cells were incubated with predefined optimal concentrations of viral Ag overnight at 37°C. Ag-pulsed RPE cells were washed, and 7.5–15 × 10^4 T cells were added to each well for 24 h. Cell-free conditioned TCM was assayed for the amount of secreted IFN-γ by ELISA (eBioscience). Experiments were performed in triplicate and repeated at least twice.

**Effect of soluble T cell factors on VZV replication in RPE cells**

Primary human RPE 172 cells were grown to confluence in 24-well plates, stimulated with IFN-γ (500 U/ml) for 3 d in S10F, and infected with VZV, EGFP-ORF66 (ratio of approximately one infected cell to four uninfected cells) for 4 h at 37°C. RPE cells were washed three times, 600 µl TCM was added to the wells, and Transwell Permeable Supports (polyester membrane, 0.4-µm pore size; Costar) were placed in the wells containing VZV-infected RPE cells. Subsequently, 7.5 × 10^4 autologous medium- or VZV-EGFP-ORF66-pulsed BLCL and 7.5 × 10^4 T cells in 100 µl TCM (TCC 53; HLA-DR mismatch with RPE 172) were added to the Transwell inserts. Alternatively, RPE 172 cells were grown in 48-well plates and infected with VZV-EGFP-ORF66 as described above. RPE cells were washed three times, and 200 µl S10F containing the indicated concentration of recombinant human IFN-γ (500 U/ml) for 3 d in S10F, and infected with VZV-EGFP-ORF66 (ratio of approximately one infected cell to four uninfected cells) for 4 h at 37°C. Virus-infected RPE cells were fixed using 4% paraformaldehyde (w/v) dissolved in PBS for 15 min at room temperature, permeabilized with 0.1% (v/v) Triton X-100 dissolved in PBS for 10 min, and incubated with mAb anti-HSV-1 IC0 (Santa Cruz Biotechnology) or polyclonal rabbit (w/v) dissolved in PBS for 15 min at room temperature, permeabilized with 4% paraformaldehyde (w/v) dissolved in PBS for 15 min at room temperature, permeabilized with 0.1% (v/v) Triton X-100 dissolved in PBS for 10 min, and incubated with mAb anti-HSV-1 IC0 (Santa Cruz Biotechnology) or polyclonal rabbit serum. The cytoskeleton was stained with phalloidin-labeled Alexa Fluor 546 (1:500; Invitrogen). Double labeling with mAbs and phalloidin-labeled Alexa Fluor 546 was performed essentially as described (35). Alexa Fluor 546-labeled HLA class II molecules were detected using a confocal microscope (Zeiss;510 Meta) and examined using Zeiss LSM 710 Meta software. Images were obtained using ×2 frame averaging and the pinhole adjusted to 1 airy unit. ZEN 2010 software (Zeiss) was used to adjust brightness and contrast.

**Immunofluorescent staining and confocal microscopy**

Primary human RPE cell lines were grown to confluence on glass coverslips in 24-well plates, stimulated with IFN-γ (500 U/ml) for 3 d in S10F, and infected with HSV-1-EGFP-VP16 (MOI = 0.5–1) and VZV-EGFP-ORF66 (ratio of approximately one infected cell to four uninfected cells) for 4 h at 37°C. Virus-infected RPE cells were fixed using 4% paraformaldehyde (w/v) dissolved in PBS for 15 min at room temperature, permeabilized with 0.1% (v/v) Triton X-100 dissolved in PBS for 10 min, and incubated with mAb anti-HSV-1 IC0 (Santa Cruz Biotechnology) or polyclonal rabbit serum. The cytoskeleton was stained with phalloidin-labeled Alexa Fluor 546 (1:500; Invitrogen). Double labeling with mAbs and phalloidin-labeled Alexa Fluor 546 was performed essentially as described (35). Alexa Fluor 546-labeled HLA class II molecules were detected using a confocal microscope (Zeiss;510 Meta) and examined using Zeiss LSM 710 Meta software. Images were obtained using ×2 frame averaging and the pinhole adjusted to 1 airy unit. ZEN 2010 software (Zeiss) was used to adjust brightness and contrast.

**Results**

Two ocular-derived CD4 TCC recognize VZV peptide IE62_918–927 using discordant HLA-DR alleles and TCR

Two protein domains of VZV IE62 and the orthologous HSV-1 protein ICP4 are highly conserved among alphaherpesvirinae: a domain that contains sites for DNA binding and homodimerization (IE62_636–636 and ICP4_3126–3126) and the large C-terminal region (IE62_910–918 and ICP4_814–814) involved in transcription of virus genes (28, 40). Previously, we reported that IE62 is a common target Ag of the intraocular VZV-specific T cell response in VZV uveitis patients (18). Two IE62-specific CD4 TCC (TCC 7 and TCC 53), which were recovered from intraocular fluid from the same affected eye of a VZN ARN patient, reacted to the same VZV IE62 region (IE62_910–918) using HLA-DR*1404 and -DR*0703 as HLA restriction element, respectively (18). In this study, we determined the minimal T cell epitope of both TCC using partially overlapping 9- to 12-meric peptides that cover the IE62_910–918 region (Fig. 1A; data not shown). Both TCC recognized IE62_918–926 and IE62_919–927 peptides, indicating that the TCC are directed to the same T cell epitope (IE62_918–927: LLLSTRDLAF). Because both TCC recognized the same IE62 peptide by disparate HLA-DR alleles, we determined their TCRα and TCRβ genotype. First, the TCC’s TCRβ gene (TRBV) usage was determined by flow cytometry using a panel of commercial single or dual FITC- and PE-conjugated human TRBV-specific mAbs. TCC 7 expressed TRBV5-1 and TCC 53 reacted with a mAb recognizing TRBV6-5, TRBV6-6, and TRBV6-9 (Fig. 1B). Finally, the TCC’s TCR V, J, and D gene usage, and the corresponding CDR3 region, of the TCC’s TCRα and TCRβ-chain were defined by multiplex RT-PCR and sequencing. The data showed that both TCC expressed not only different TCR V, J, and D gene segments but also had distinct TCR CDR3 regions (Table I). Collectively, the data demonstrate that two ocular-derived VZV-specific CD4 TCC recognize the same IE62_918–927 T cell epitope, located within the highly conserved C-terminal region of VZV IE62, using discordant HLA-DR alleles and TCR.

The VZV IE62_918–927 CD4 T cell epitope is highly conserved among alphaherpesvirinae

We aligned the amino acid sequences of published orthologous alphaherpesvirus proteins to determine whether the VZV IE62_918–927 T cell epitope is conserved among alphaherpesvirinae by diverse animal species. Notably, VZV IE62_918–927 is completely conserved among human and monkey simplexviruses and varicelloviruses, whereas orthologous proteins of more distant varicelloviruses varied only one to two residues (Table II). At high concentrations of peptides corresponding to the IE62_918–927 variants to determine cross-species T cell reactivity (Fig. 2, Table II). TCC 7 recognized its cognate IE62_918–927 epitope and cross-reacted—only at high concentrations—with the orthologous canid herpesvirus 1 peptide (LLLSTKDLAF). TCC 53 recognized only its cognate IE62_918–927 T cell epitope. Collectively, the data indicated that two VZV-specific CD4 TCC recognize a peptide that is highly conserved among primate alphaherpesvirinae.

The VZV IE62 peptide binds to multiple HLA-DR alleles and is a common target of memory T cells in blood of HSV-1– and VZV-coinfected healthy individuals

In contrast to MHC class I, the binding motifs of MHC class II molecules are highly degenerative enabling certain peptides, so-called promiscuous peptides, to bind to multiple MHC class II alleles (42, 43). On the basis of the disparate HLA-DR allele usage of the IE62_918–927-reactive TCC 7 and 53, we determined the HLA-DR promiscuity of the IE62 epitope in detail by silico binding analyses. Notably, peptide IE62_915–929 was predicted to bind with high affinity to multiple HLA-DR alleles, including HLA-DR*0101, -DR*0301, -DR*0401, -DR*0701, -DR*1101, -DR*1301, and -DR*1501 (data not shown). Next, we determined the binding affinity of the peptides IE62_915–926, IE62_918–926, IE62_919–927, and IE62_918–929 for HLA-DR*0101, -DR*0201, -DR*0301, and -DR*0401 using an in vitro competition assay (39). Except for
IE62_919–927 binding to HLA-DR*0301, all peptides bound with high (<1 μM) or intermediate (1–10 μM) affinity to HLA-DR*0101, -DR*0201, -DR*0301, and -DR*0401 molecules (Table III).

On the basis of the HLA-DR promiscuity and the conservation of the IE62 T cell epitope among αHHV, we hypothesized that the peptide is a target of memory T cells in blood of HSV-1– and VZV-coinfected individuals. PBMC of 17 HSV-1– and VZV-seropositive healthy adults, and as control CBMC of five donors, were incubated with the IE62 919–929 peptide to enrich for peptide-specific memory T cells. Donors were selected for expression of the following predicted epitope-binding HLA-DR alleles: HLA-DR*0101, -DR*0301, -DR*0401, and -DR*1501, which have a combined frequency of ∼35% in the world population, irrespective of ethnic background (44). The resulting short-term T cell cultures were assayed for peptide-specific T cell reactivity by IFN-γ ELISPOT. Five of 17 HSV-1– and VZV-seropositive healthy donors, including three HLA-DR*0401 POS and two HLA-DR*1501 POS donors, responded to IE62 918–929 (Fig. 3). No T cell responses to IE62 915–929 were observed in five cord blood donors, demonstrating the specificity of the observed T cell responses. Overall, these data demonstrated that the identified VZV IE62 epitope is HLA-DR promiscuous and is a common target Ag of memory CD4 T cells in blood of HSV-1– and VZV-coinfected healthy individuals.

The complete conservation of the identified CD4 T cell epitope among αHHV suggests that the respective VZV-specific TCC recognize both HSV-1– and HSV-2–infected cells. Surprisingly, both TCC 7 and 53 recognized HSV-1– but not HSV-2–infected autologous BLCL (Fig. 4A). Because VZV does not infect BLCL efficiently (data not shown), we used BLCL pulsed with a VZV protein lysate as APC. In contrast to HSV-1 and VZV, HSV-2 Ag–pulsed BLCL were not recognized (Fig. 4A; data not shown).

Epitope-flanking residues affect Ag processing and subsequent presentation of the cognate peptide to T cells (45–47). Alignment of the HSV ICP4 protein sequences revealed 9-aa substitutions and 1-aa insertion in the epitope-flanking region of HSV-2 compared with HSV-1 ICP4 (Fig. 4B). To determine whether these epitope-flanking residues were involved in the differential T cell recognition of HSV ICP4, fragments of HSV-1 ICP4 (aa 945–1054) and HSV-2 ICP4 (aa 972–1082) encompassing the αHHV-conserved T cell epitope plus 54 (HSV-1) or 55 (HSV-2) N-terminal flanking residues and 46 C-terminal residues were cloned into an expression vector. The TCC were cultured with autologous BLCL nucleofected with plasmids encoding HSV-1 ICP4945–1054 and HSV-2 ICP4972–1082 (Fig. 4C). Notably, both TCC recognized BLCL that expressed the HSV-1 and HSV-2 ICP4 protein fragments implicating that the failure to recognize the αHHV-conserved CD4 T cell epitope in

### Table I. TCR ωβ gene usage and CDR3 region of TCC 7 and 53

<table>
<thead>
<tr>
<th>TCC</th>
<th>TCR V</th>
<th>TCR D</th>
<th>TCR J</th>
<th>Amino Acid Sequence CDR3 Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>TRAV3*01</td>
<td>NA</td>
<td>TRAJ6*01</td>
<td>CAVRDMDTGGSYIPTF</td>
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<tr>
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<td>TRBV5-1*01</td>
<td>TRBD2*01</td>
<td>TRBJ2-5*01</td>
<td>CASSSSGQETQYF</td>
</tr>
<tr>
<td>53</td>
<td>TRAV23/DV6*01</td>
<td>NA</td>
<td>TRAJ27*01</td>
<td>CAAGGSTNAKSTF</td>
</tr>
<tr>
<td>53</td>
<td>TRBV6-5*01</td>
<td>TRBD1*01</td>
<td>TRBJ1-5*01</td>
<td>CASPDGALGKNQPHF</td>
</tr>
</tbody>
</table>

*TCR V, D, and J gene annotation of the TRA and TRB chains are according to Ref. 40. NA, not applicable.
the context of HSV-2 infection was not due to proximal epitope-flanking amino acid polymorphisms.

**VZV/HSV-1 cross-reactive CD4 T-cells control VZV but not HSV-1 infection in human RPE cells**

To determine the functional properties of VZV/HSV-1 cross-reactive CD4 T-cells, we assayed the capacity of TCC 53 to inhibit VZV and HSV-1 replication in target cells relevant to the pathogenesis of ARN: human RPE cells (22–24). Under physiological conditions and in cell culture, human RPE cells do not express HLA-DR. The expression of inflammatory cytokines, including IFN-γ and TNF-α, in affected eyes of uveitis patients shape-resident RPE cells to potent APC (48–50). This can be mimicked in vitro by stimulating human primary RPE cell cultures with IFN-γ for 3 d, resulting in increased HLA-I and the induction of HLA-II and the T cell costimulatory molecules CD40 and CD54 surface expression (17, 49, 50). First, we determined whether IFN-γ stimulation of human RPE influenced productive HSV-1 and VZV infection in vitro. At 24 h postinfection, IFN-γ–stimulated RPE infected with EGFP-expressing HSV-1 and VZV expressed IE (HSV-1 ICP0), early (VZV ORF66), and late viral proteins (HSV-1 VP16 and VZV gE; Fig. 5A). Herpesvirus genes are expressed in a coordinated temporal fashion, such that the detection of late proteins implicates that viral DNA replication has occurred (1, 2). Thus, EGFP expression is a valid marker to detect a productive HSV-1 and VZV infection in IFN-γ–stimulated human primary RPE cells.

Next, the capacity of TCC 53 to control VZV and HSV-1 replication in human RPE cells in vitro was determined (Fig. 5B). TCC 53 significantly inhibited VZV replication in HLA-DR matched but not mismatched RPE cells. The data are in line with our previous studies, demonstrating that IFN-γ–stimulated human primary RPE cells efficiently process and present the TCC's cognate VZV peptide in an HLA-DR–restricted fashion (17). Contrastingly, TCC 53 did not inhibit HSV-1 replication in both HLA-DR–matched and–mismatched RPE cells. To demonstrate that this differential effect was not due to the TCC and RPE cell line used, the experiments were repeated with a different TCC/RPE combination. We used TCC 44, a CD4 TCC originally obtained from a vitreous fluid sample of a patient with HSV-1 ARN (patient number 1 in Ref. 21), which recognizes HSV-1, but not HSV-2 and VZV (Ref. 20; data not shown). Analogous to TCC 53, TCC 44 did not inhibit HSV-1 replication in RPE cells (Fig. 5C), demonstrating that HSV-1–reactive CD4 T cells are unable to control HSV-1 infection in human RPE cells.

**VZV/HSV-1 cross-reactive CD4 T cells inhibit VZV replication in human RPE cells via soluble factors and potentially direct cell–cell contact**

Previously, we have shown that TCC 53 secretes the antiviral cytokine IFN-γ in response to VZV Ag-pulsed BLCL and exerts cytotoxic activity toward IE62901–930–pulsed BLCL (18). In this study, we determined whether the inhibition of VZV replication in human RPE cells by TCC 53 involved soluble factors and/or direct cell–cell contact. TCC 53 was cultured with mock-
IE62_{15-929}-pulsed autologous BLCL in Transwell inserts placed in wells containing HLA-DR-mismatched VZV-infected human RPE cells (Fig. 6A). The polyester membrane of the Transwell inserts contained 0.4-μm pores, which allows diffusion of soluble factors but prohibits migration of T cells to the lower RPE cell compartment (data not shown). Under these conditions, TCC 53 partially inhibited VZV replication in RPE cells (i.e., relative reduction of 40% at 72 h postinfection) suggesting that soluble factors (e.g., cytokines) secreted by peptide-stimulated T cells are involved (Fig. 6A). Indeed, recombinant human IFN-γ—the most abundant cytokine secreted by TCC 53 (18) (Fig. 7)—significantly reduced VZV replication in human RPE cells when VZV-infected cells were treated with 100 pg/ml and 1000 pg/ml IFN-γ in a dose-dependent manner: relative reduction of 16 and 27% at 72 h postinfection, respectively. Contact-dependent T cell cytotoxicity can be mediated by death receptors such as FasL and TRAIL or by release perforin and grB in the immunological synapse between TCC and RPE (51). TCC 53 did not express FasL or TRAIL nor was FasL or TRAIL expression induced upon culture with IE62_{15-929}-pulsed autologous BLCL (Fig. 6C). By contrast, TCC 53 expressed perforin and grB at steady-state levels (Fig. 6D). The mean fluorescence intensity of grB expression increased 3-fold in response to IE62_{15-929}-pulsed autologous BLCL (Fig. 6D).

Table III. Binding affinity of VZV protein IE62-derived peptides to different HLA-DRB1 alleles

<table>
<thead>
<tr>
<th>Location</th>
<th>Amino Acid Sequence</th>
<th>DRB1*0101</th>
<th>DRB1*0201</th>
<th>DRB1*0301</th>
<th>DRB1*0401</th>
</tr>
</thead>
<tbody>
<tr>
<td>915–926</td>
<td>RGVLLLSTRDLA</td>
<td>0.04</td>
<td>0.3</td>
<td>0.2</td>
<td>0.006</td>
</tr>
<tr>
<td>919–926</td>
<td>LLLSTRDLA</td>
<td>0.1</td>
<td>4.0</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>919–927</td>
<td>LLLSTRDLAFAG</td>
<td>1.0</td>
<td>10</td>
<td>&gt;20</td>
<td>0.3</td>
</tr>
<tr>
<td>918–929</td>
<td>LLLSTRDLAFAG</td>
<td>0.06</td>
<td>1.1</td>
<td>0.08</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*aLocation of N- and C-terminal residues (amino acid) of the respective peptide in VZV IE62.

*bAmino acid sequence according to a reference VZV protein IE62 sequence (GenBank accession number NP_040184.1).

*cBinding affinity of each peptide is expressed as the IC_{50}, referred to as the test peptide concentration (μM) at which binding of the HLA-DR allele–specific standard fluorescence-labeled peptide is reduced to 50% of its maximal value. IC_{50} < 1 μM: high binding affinity; IC_{50} 1–10 μM: intermediate binding affinity; IC_{50} 10–100 μM: weak binding affinity; IC_{50} > 100 μM: no binding affinity (38).

FIGURE 3. Detection of VZV peptide IE62_{15-929}-reactive CD4 T cells in peripheral blood (PB)– and cord blood (CB)–derived mononuclear cells (MC) of HLA class II typed healthy adults and newborns, respectively. PBMC and CBMC, isolated from HSV-1– and VZV-coinfected individuals (n = 17) and naive CB donors (n = 5), were stimulated with peptide IE62_{15-929} for 1 wk in vitro and subsequently analyzed for reactivity to peptide IE62_{15-929} by IFN-γ ELISPOT. The donors’ HLA-DR genotypes presented are those able to bind the IE62 peptide with high affinity (see Table III). Data are presented as mean ± SEM number of IFN-γ SFC/10^{4} T cells. *Short-term T-cell cultures with more than or equal to two times IFN-γ SFC in response to IE62_{15-929} compared with medium.

FIGURE 4. TCC 7 and 53 recognize VZV and HSV-1– but not HSV-2–infected B cells. (A) TCC were cultured for 6 h with autologous EBV-transformed B-cells (BLCL) infected overnight with HSV-1 and HSV-2, or pulsed with protein lysates of mock and VZV-infected Vero cells. T cell reactivity was determined by IFN-γ ELISPOT. (B) Alignment of the HSV-1 and HSV-2 ICP4 orthologous protein sequences of the VZV IE62_{18-927} T cell epitope (boxed residues) and their flanking regions (GenBank accession numbers ACM62283 and NP_044530; http://www.ncbi.nlm.nih.gov/genbank/, respectively). The location of N- and C-terminal residues of the respective protein sequences are indicated in brackets. Dashes and asterisks indicate identical amino acids and insertion introduced to align the protein sequences, respectively. (C) TCC were cultured with autologous BLCL pulsed with peptide or medium. Alternatively, BLCL were nucleofected with the vectors pEGFP-N1/HSV-1:ICP4 945–1054 (encoding HSV-1 ICP4 residues 945–1054), pEGFP-N1/HSV-2:ICP4 972–1082 (encoding HSV2 ICP4 residues 972–1082), or pEGFP-N1/CTRL (no insert) as negative control. T cell reactivity was determined by IFN-γ ELISPOT. (A and C) Data are presented as mean ± SEM number of IFN-γ SFC/10^{4} T cells.
EGFP-VP16–infected RPE cells were cultured with (N)HLA-DR–matched IFN-γ (0.05, **SEM. Differences in the number of EGFP POS RPE cells with TCC compared to the TCC’s HLA-DR restricting allele. Data are presented as mean ± SEM. Differences in the number of EGFP POS RPE cells with TCC compared with no TCC added were analyzed by the paired Student t test. *p < 0.05, **p < 0.01.

**FIGURE 5.** VZV/HSV-1 cross-reactive CD4 T-cells inhibit VZV but not HSV-1 infection in human RPE cells. (A) IFN-γ-stimulated RPE cells were infected with rEGFP expressing VZV (VZV.EGFP-ORF66) and HSV-1 (HSV-1.EGFP-VP16) strains for 24 h and stained for VZV glycoprotein E (gE; red) or HSV-1 protein ICP0 (red). Nuclei were stained with DAPI (blue). EGFP is fused to VZV protein ORF66 or HSV-1 protein VP16 (green). Original magnification ×400. (B) VZV.EGFP-ORF66 or HSV-1.EGFP-VP16 infected IFN-γ-stimulated RPE cells were cultured with (○) or without (■) the VZV/HSV-1 cross-reactive CD4 TCC 53. (C) HLA-DR–matched IFN-γ-stimulated VZV.EGFP-ORF66 or HSV-1. EGFP-VP16–infected RPE cells were cultured with (○) or without (■) the solely HSV-1–specific TCC 44. (B and C) At the indicated times the percentage of EGFP-positive RPE cells (i.e., CD3NEGHLA-DRPOS cells) was determined by flow cytometry. HLA-DR (mis)matched RPE cells refer to the TCC’s HLA-DR restricting allele. Data are presented as mean ± SEM. Differences in the number of EGFPPOS RPE cells with TCC compared with no TCC added were analyzed by the paired Student t test. *p < 0.05, **p < 0.01.

**Discussion**

HSV and VZV are closely related endemic αHHV that establish latency in sensory neurons and reactive intermittently to cause recrudescent disease (1, 2). On the basis of the extensive homology between the HSV and VZV proteomes, cross-reactive adaptive immune responses are anticipated. These responses are of potential interest for vaccine development to confer cross-protection but may also be involved in immunopathogenesis. Whereas various αHHV cross-reactive B cell target Ags have been identified (52–54), T cell immunity directed to multiple αHHV is restricted to HSV-1 and HSV-2 (29, 55, 56). The current study reports three main findings. First, we report on the first HLA-DR promiscuous VZV/HSV-1 cross-reactive CD4 T cell epitope, located within VZV IE62 and HSV-1 ICP4, which is immunoprevalent in HSV-1– and VZV-infected healthy individuals. Second, despite complete conservation of this epitope in HSV-2 ICP4, HSV-2–infected cells were not recognized by the cross-reactive CD4 TCC. Third, VZV/HSV-1 cross-reactive CD4 TCC selectively controlled VZV but not HSV-1 infection in human RPE cells, suggesting that HSV-1 actively inhibits CD4 T cell activation.

Immunogenicity of pathogen-derived proteins is determined by various factors including the host T cell repertoire, Ag abundance, size and structure, antigenic competition, and Ag processing (57–59). VZV IE62 and HSV ICP4 are abundantly expressed viral proteins that are transported from the nucleus to the cytoplasm for incorporation in the tegument of the virion during lytic infection (27, 60–62). Both viral proteins are large (~1300 aa long) and contain a DNA binding and a large C-terminal transactivator domain that are both highly conserved among αherpesvirinae of diverse animal species (28, 40, 63). Previous studies showed that viral tegument proteins, including VZV IE62 and HSV-1 ICP4, are immunoprevalent targets of αHHV-specific CD4 and CD8 T cells in blood of healthy HSV-1– and/or VZV-infected adults (29, 64–66), lesions of genital herpes patients (67), affected eyes of herpetic uveitis and keratitis patients (18–20, 68), and at the site of HSV-1 latency in human trigeminal ganglia (69). The identification of a HLA-DR promiscuous and broadly recognized CD4 T cell epitope, located within VZV IE62 and peptide-induced IFN-γ secretion and potentially perforin/granzyme-mediated cytotoxicity are involved in TCC 53-mediated control of VZV replication in human RPE cells.

HSV-1 infection inhibits CD4 T cell activation by human RPE cells

Deficient CD4 T cell control of HSV-1 infection in RPE cells may be due to a virus-mediated inhibition of T cell recognition of infected cells. To test this hypothesis, we determined IFN-γ levels, as a marker of T cell activation, in conditioned medium of TCC 53 cocultured with HSV-1– and VZV-infected RPE cells (Fig. 7A). Notably, the TCC secreted high levels of IFN-γ in response to VZV- but not HSV-1–infected HLA-DR–matched RPE cells. Similar data were obtained with TCC 44 (Fig. 7B), indicating that this effect was not TCC, target protein and RPE cell line specific.

The inhibitory effect may be attributed to the inability of RPE cells to process and present the cognate peptide or may involve an HSV-1–specific evasion strategy that counteracts CD4 T cell activation by the infected RPE cells. To differentiate between both options αHHV Ag pulsed RPE cells were used as APC. In contrast to HSV-1–infected RPE cells, HSV-1 Ag pulsed RPE cells activated TCC 53 efficiently (Fig. 7C). As expected both VZV-infected and VZV Ag–pulsed RPE cells were recognized by TCC 53. Collectively, the data indicate that HSV-1 infection selectively inhibited CD4 T cell activation by RPE cells (Fig. 7) but not BLCL (Fig. 4A).
HSV-1 ICP4 underscores the immunogenicity of both viral proteins as T cell targets. The identified VZV IE62 CD4 T cell epitope was highly conserved among alphaherpesvirinae of multiple animal species, with complete conservation in VZV, HSV-1 and HSV-2. Nonetheless, the epitope-specific CD4 TCC 7 and 53 did not recognize the non-αHHV orthologous peptides at low concentrations. All nonreactive peptides diverged from the αHHV conserved epitope at HLA-II anchor residues P4, P6, and P9 (43, 57). Possibly, the peptides are unable to form stable peptide:HLA-DR complexes or do not functionally engage with the TCR of both TCC. Despite complete conservation of the IE62/ICP4 epitope among αHHV, both TCC failed to recognize HSV-2–infected BLCL.

**HSV-1 ICP4, underscores the immunogenicity of both viral proteins as T cell targets.**

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**FIGURE 6.** VZV/HSV-1 cross-reactive CD4 T cells inhibit VZV replication in human RPE cells via soluble factors and potentially direct cell–cell contact. (A) TCC 53 was cultured with autologous BLCL, pulsed with medium (●) or peptide IE62<sub>915-929</sub> (○), in Transwell inserts (0.4-μm pore size) placed in wells containing HLA-DR–mismatched human RPE cells infected with VZV.EGFP-ORF66. Gray shading indicates medium level. (B) IFN-γ–stimulated VZV.EGFP-ORF66–infected RPE cells were cultured with the indicated times, the percentage of EGFP<sup>POS</sup> RPE cells (i.e., CD3<sup>NEG</sup>HLA-DR<sup>POS</sup> cells) was determined using flow cytometry. Data are presented as mean ± SEM. Differences in the number of EGFP<sup>POS</sup> RPE cells with TCC compared with no TCC added were analyzed by the paired Student t test. *p < 0.05. (C) Surface expression of TRAIL and FasL by TCC 53 was determined using flow cytometry at 24 h after coculture with medium- or VZV peptide IE62<sub>915-929</sub>–pulsed autologous BLCL. (D) Intracellular expression of perforin and granzyme B by TCC 53 was determined using flow cytometry at 8 h after coculture with medium- or VZV IE62<sub>915-929</sub>–pulsed autologous BLCL. (C and D) T cells were gated on viable CD3<sup>POS</sup>CD4<sup>POS</sup> cells, and quadrants were set with reference to isotype controls. Percentages of cells in each quadrant are provided.
is affected by the context of the respective Ag via its tertiary structure and protease susceptibility in epitope-flanking residues (42, 43, 45–47). Compared to HSV-1 ICP4, the epitope-containing domain of HSV-2 ICP4 includes several amino acid substitutions. However, nuclease-digest BLCL expressing a HSV-2 ICP4 fragment, including the εHHV-conserved T cell epitope along with ~50 N- and C-terminal residues, were readily recognized by both TCC, indicating that the proximal epitope-flanking polymorphic residues in HSV-2 ICP4 did not affect processing and presentation of the cognate peptide to the TCC. HSV-1 and HSV-2 ICP4 proteins are highly homologous (70), suggesting that differential protease susceptibility because of more distal residues rather than the overall tertiary ICP4 structure are most likely involved in the inability to present the cognate epitope by HSV-2–infected BLCL.

ARN is a potentially blinding inflammatory eye disease that is predominantly caused by ocular HSV and VZV infections (8–10). Most ARN patients have pre-existing serum IgG toward the initiating herpesvirus, indicating that reactivation of latent virus rather than primary infection triggers the disease (71). Reactivated virus may reach the retina by axonal transport via the optic nerve (71), explaining the increased risk of developing ARN following herpetic encephalitis (72). Whereas under normal physiological conditions RPE cells are involved in creating and maintaining ocular immune privilege, inflammatory reactions evoked by intracellular infections shape RPE cells into potent APC to coordinate local T cell responses (48–50). Given the potential uveitogenic role of intracellular virus-specific T cells in herpetic uveitis patients and corresponding animal models (11–16), the functional properties of the ocular-derived IE62/ICP4 cross-reactive TCC toward HSV-1– and VZV-infected human RPE was analyzed in detail. In agreement with our previous study (17), VZV infection was controlled in human RPE cells by the IE62/ICP4 cross-reactive CD4 TCC in vitro. The T cell effector mechanisms involved are IFN-γ secretion and potentially cytolytic of infected RPE cells by the perforin/grB pathway (17).

In contrast, the same cross-reactive CD4 TCC did not recognize and consequently failed to control HSV-1 infection in RPE cells. Because the TCC recognized both HSV-1–infected autologous BLCL and HSV-1 Ag–pulsed RPE cells, the data suggest that productive HSV-1 infection selectively renders RPE cells unable to activate CD4 T cells. Currently, two HSV–1–specific immune evasion strategies have been reported that reduce, but do not completely abrogate, CD4 T cell recognition of infected cells (73–75). The first mechanism involves HLA–II downregulation on infected cells through the combined actions of the virion host shutoff protein, γ34.5 protein, and gB (74, 75). The second pathway is mediated by HSV-1 ICP22 protein that impairs Ag presentation but not Ag processing (73). The high HLA–II surface expression on IFN-γ–stimulated RPE cells, used as APC throughout the current study, was only modestly reduced upon HSV–1 infection (data not shown). Consequently, the HSV-1 ICP22 pathway or a novel immune evasion mechanism is potentially involved in the inhibition of CD4 T cell recognition of HSV–1–infected RPE cells (73).

In conclusion, we report on the first HLA–DR promiscuous and immunoprevalent VZV/HSV–1 cross-reactive CD4 T cell epitope located within VZV Iε62 and HSV ICP4. The data warrant further studies on the applicability of both proteins, preferentially the conserved C-terminal domain that contains the εHHV-conserved CD4 T cell epitope, in future vaccines to confer εHHV-specific and possibly εHHV cross-protective T cell immunity. Furthermore, the use of ocular-derived VZV/HSV–1 cross-reactive TCC revealed that HSV–1 infection selectively rendered human RPE but not B cells invisible to CD4 T cell control. Studies are in progress to identify the HSV–1 protein, potentially ICP22 (73), and the mechanism involved in the CD4 T cell immune evasion strategy recognized.

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