Identification of Viral Antigens Recognized by Ocular Infiltrating T Cells from Patients with Varicella Zoster Virus-Induced Uveitis

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PURPOSE. Varicella zoster virus (VZV) is a common cause of infectious uveitis associated with an intraocular inflammatory response involving virus-specific T cells. In the current study, the functional characteristics and the antigen specificity of VZV-reactive T cells recovered from intraocular fluid (IOF) samples of five patients with VZV were determined.

METHODS. B-cell lines were infected with a comprehensive panel of recombinant vaccinia viruses expressing 11 individual VZV open reading frames (ORFs), or alternatively pulsed with a panel of recombinant vaccinia viruses expressing 11 individual ORFs. The T-cell responsiveness of the IOF-derived VZV-specific T cells toward APCs was monitored by interferon (IFN)-γ enzyme-linked immunosorbent spot-forming assays on bulk T-cell cultures and subsequently T-cell clones (TCCs). The cytokine-secretion profile and cytotoxicity of the VZV-specific TCCs was determined by ELISA and flow cytometry, respectively.

RESULTS. T-cell reactivity to VZV proteins encoded by ORF4, -10, -14, -18, -29, -31, -61, -62, -63, -67, and -68 was demonstrated, but specificity varied individually. T-cell epitopes on ORF62 and -68 were delineated. The TCCs secreted IFNγ, but relatively low levels of interleukin-4 and -5, in response to VZV antigen-presenting APCs. The TCCs induced antigen-specific cytotoxic T-cell activity.

CONCLUSIONS. The results suggest that the intraocular VZV-specific T-cell response in the patients with VZV analyzed is directed to a broad spectrum of VZV antigens, including the latency-associated VZV proteins from ORFs 4, 29, 63, and 66, as well as those from ORF62 and -68. The VZV-specific T-cell response, encompassing Th0/1-like cytokotoxic effector memory CD4+ T cells, is directed to a broad spectrum of VZV proteins. These data are consistent with the role of VZV-infected T cells recovered from affected eyes of five patients with VZV uveitis. The data indicate that the intraocular VZV-specific T-cell response, encompassing Th0/1-like cytokotoxic effector memory CD4+ T cells, is directed to a broad spectrum of VZV proteins. These data are consistent with the role of VZV-infected T cells recovered from affected eyes of five patients with VZV uveitis. The data indicate that the intraocular VZV-specific T-cell response, encompassing Th0/1-like cytokotoxic effector memory CD4+ T cells, is directed to a broad spectrum of VZV proteins. These data are consistent with the role of VZV-infected T cells recovered from affected eyes of five patients with VZV uveitis. The data indicate that the intraocular VZV-specific T-cell response, encompassing Th0/1-like cytokotoxic effector memory CD4+ T cells, is directed to a broad spectrum of VZV proteins. These data are consistent with the role of VZV-infected T cells recovered from affected eyes of five patients with VZV uveitis. The data indicate that the intraocular VZV-specific T-cell response, encompassing Th0/1-like cytokotoxic effector memory CD4+ T cells, is directed to a broad spectrum of VZV proteins. These data are consistent with the role of VZV-infected T cells recovered from affected eyes of five patients with VZV uveitis.

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Supported in part by the Stichting Wetenschappelijk Onderzoek Oogziekenhuis (SWOO 2001-01) and the Prof. Dr. Henkes Stichting (JCMM). PRK was supported in part by USPHS Grants EY09397, EY08098, and EY15291 and funds from Research to Prevent Blindness Inc.

Submitted for publication January 8, 2007; revised March 7 and 28, 2007; accepted May 16, 2007.

Disclosure: J.C.M. Milikan, None; P.R. Kinchington, None; G.S. Baarsma, None; R.W.A.M. Kuipers, None; A.D.M.E. Osterhaus, None; G.M.G.M. Verjans, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

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specific CD4+ Th1/Th0-like cells in the intraocular inflammatory response in VZV uveitis.

**METHODS**

**Subjects and Clinical Specimens**

Heparinized peripheral blood (PB) and IOF samples were obtained from five patients with VZV uveitis during ocular surgery for diagnostic (patients 1–3, 5) or therapeutic (e.g., vitreitis or retinal detachment; patients 4 and 6) purposes. The volume of the aqueous humor (AH) and vitreous fluid (VF) samples obtained was approximately 200 μL and 500 to 1000 μL, respectively. One patient with HSV-1 uveitis (patient 6) was included as the control. Details on the clinical status of the patients, results of diagnostic tests performed on the IOF specimens, and the generation of the IOF-derived T-cell lines (TCLs) by mitogenic stimulation of T cells recovered from the surplus IOF specimens of these patients with uveitis have been described in detail recently (Table 1).14,15 The volume of the surplus AH and VF samples commonly used to generate the IOF-derived TCL was 50 to 75 μL and 300 to 600 μL, respectively. The patients’ human leukocyte antigen (HLA) class II profile was typed by standard diagnostic PCR at four-digit resolution at the Sanquin Blood Center (Rotterdam, The Netherlands). The present study was performed according to the Declaration of Helsinki and approved by the Institutional Review Board, and written informed consent was obtained from all patients.

**Cell Lines and Clones**

The isolation of peripheral blood mononuclear cells (PBMCs), generation of B-cell lines (BLCLs) by transformation with Epstein-Barr virus, were performed as described previously.15 T-cell clones (TCC) were generated from the IOF-TCL by limited 10-fold dilution.15 The T-cell mitogen phytohemagglutinin-L (1 μg/mL; Roche Applied Science, Mannheim, Germany) in the presence of 3H-thymidine (2 μCi/mL; Amersham International, Buckinghamshire, UK) was used for proliferation studies of the respective TCC. Optimal HLA allele binding peptides were selected according to predictions based on the antigen recognized and the HLA allele defined as the restriction element for the respective TCC. Optimal HLA allele binding peptides were selected using criteria reviewed recently.24 In short, the overall best-scoring peptides (n = 6) predicted by three T-cell epitope prediction software programs, namely RANKPEP,25 ProPred,26 and SYFPEITHI,27 were selected for synthesis.

To establish the HLA-restricting loci, we used predefined optimal concentrations of supernatants of hybridoma cells secreting antibodies to public specificities of HLA-DR (clone L243), HLA-DP (clone B7.21),

**Viruses and Viral Antigens**

Antigens of the VZV reference strain Dumas and HSV-1 strain KOS were harvested from virus-infected primary human embryonic lung fibroblasts (HEL cells) showing >90% cytopathic effect. Infected cells or mock-infected cells, referred to as mock-infected, were lysed in three freeze-thaw cycles, after which the antigen preparations were cleared from cell debris by high-speed centrifugation. UV-irradiated (2.5 × 10^5 mW/mm²), and stored in aliquots at −135°C. The recombinant vaccinia viruses (rVV) containing complete VZV ORFs, in frame VZV ORF62 deletion mutants, or no insert (rVV control; previously described as poly 186)15 were used for infection of autologous BLCLs (Table 2). The generation of the rVV expressing ORF-4, -10, -29, -62, -63, and -68 have been described previously.19-23 The rVV expressing ORF18 and -51 and additional ORF62 subdomains were derived in the same fashion, with the exception that the ORF62 subdomains were engineered to contain an initiating in-frame ATG for translation. All rVV were plaque purified to homogeneity, and protein expression was verified by immunoblotting with polyclonal antisera. The rVV stocks prepared in CV-1 cells were cultured and titered on RK13 cells as described previously.15

**IFN-γ Enzyme-Linked Immunosorbent Spot-Forming (IFN-γ ELISPOT) Assay**

The BLCL were infected overnight with the rVV at a multiplicity of infection 10, incubated overnight with a predefined optimal concentration of UV-irradiated VZV, HSV-1 or mock antigen preparations.14,15 Mock antigen preparations were similarly prepared from uninfected HEL cells. Alternatively, uninfected BLCLs were pulsed for 1 hour with VZV-specific 20- or 15-mer synthetic peptides in RPMI-1640 medium (2 μM per peptide), as described previously.16 The 20-mer peptides (Sigma-Aldrich, Zwijndrecht, The Netherlands), with 10 amino acid (Aa) overlap, covered the VZV ORF62 region encoding residues 733 to 1056. The 15-mer peptides (Pepscan, Lelystad, The Netherlands) were selected according to predictions based on the antigen recognized and the HLA allele defined as the restriction element for the respective TCC. Optimal HLA allele binding peptides were selected using criteria reviewed recently.24 In short, the overall best-scoring peptides (n = 6) predicted by three T-cell epitope prediction software programs, namely RANKPEP,25 ProPred,26 and SYFPEITHI,27 were selected for synthesis.

To establish the HLA-restricting loci, we used predefined optimal concentrations of supernatants of hybridoma cells secreting antibodies to public specificities of HLA-DR (clone L243), HLA-DP (clone B7.21),
enabled discrimination of target cells from effector T cells. Cell death BLCLs by the fluorescent dye PKH-26, referred to as target cells, was repeated at least two times.

The cytotoxic function of the TCC was analyzed by flow cytometry, and HLA-DQ (clone SPV3) as blocking antibodies, to distinguish between HLA-DR, -DP, and -DQ-restricted antigen recognition of the CD4⁺ TCC tested, respectively. In addition, partially HLA class II-matched BLCLs (i.e., allogeneic BLCLs expressing one or several HLA class II alleles shared with the patient TCC analyzed) were incubated with mock and VZV protein lysate and used to identify the HLA class II allele, the so-called HLA restriction element, presenting the antigenic peptide to the TCC.

The IFN-γ ELISPOT assays were performed in triplicate, according to the manufacturer’s description, with monoclonal antibodies (mAbs; Mabtech AB, Hamburg, Germany), as described previously. In short, T cells (5 x 10⁵/well) were incubated with APCs (3 x 10⁵/well), prepared as described earlier, at 37°C in anti-IFN-γ precoated 96-well silent-screen plates (Nunc, Fisher Emergo B.V. Landmeer, The Netherlands). After incubation for 6 hours, the cells were washed away and the secondary anti-IFN-γ mAb and subsequently the streptavidin-alkaline-phosphatase mAb were added to the wells. Spots were visualized by adding NBT/BCIP (nitroblue tetrazolium; 5-bromo-4-chloro-3-indolyphosphate substrate; Kirkegaard and Perry, Gaithersburg, MD). Resulting IFN-γ spots were counted with an automated ELISPOT reader (Sanquin Reagents, Amsterdam, The Netherlands). Each experiment was repeated at least two times.

Flow Cytometric Analyses
The TCCs were phenotyped by flow cytometry using the following fluorochrome-conjugated Mabs according to the manufacturers’ instructions: anti-CD3 (allophycocyanin; APC; Dako, Heverlee, The Netherlands); anti-CD4 (fluorescein isothiocyanate; FITC; Dako); anti-CD8 (peridinin chlorophyll A protein; PERCP; BD Biosciences, Alphen aan den Rijn, The Netherlands); and anti-CD11a-FITC, anti-CD45RA-FITC, anti-CD69-FITC, anti-CCR7 (phycoerythrin [PE]), anti-CD5-PE, anti-CD28-PE, anti-CD4-PE/CPC, anti-CD5-APC, anti-CD27-APC, and anti-CD45RO-APC (all from Dako).

T cells recognize the MHC/peptide-complex on the APCs by T-cell receptor αβ (TCRαβ). The TCRαβ, unique for each T-cell, is composed of a variable region and a constant region. The variable gene region of the α and β chain are made up by in-frame joining of individual variable (Vα or Vβ); joining (Jα or Jβ); or in the case of the β chain, diversity gene segments (Dβ). A multiparametric analysis tool (IOTest Beta Mark Mab kit; Beckman Coulter, Marseilles, France), containing Mabs directed to approximately 70% of all known TCR variable genes (TCRβ), was used to determine the TCRβ gene segment usage of the TCCs.

The cytotoxic function of the TCC was analyzed by flow cytometry, as described recently. In this assay, membrane labeling of autologous BLCLs by the fluorescent dye PKH-26, referred to as target cells, enabled discrimination of target cells from effector T cells. Cell death was monitored by the ability of the dye Topro-3 to bind DNA on entry into cells permeable due to target cell death. The advantage is that both cell populations can be monitored separately, and no radioactive reagents are needed. In brief, labeling of the BLCLs with the red fluorescent dye PKH-26, which integrates into the cellular membranes, was performed according to the manufacturer’s descriptions at a final concentration of 2.5 μM (Sigma-Alrich). Subsequently, the labeled target cells were incubated with medium or the relevant peptide. The effector T cells and targets cells (10⁴ cells/well) were cultured in duplicate for 6 hours at 37°C, at an effector-target (E/T) ratio of 10:1 or 30:1. To identify dead cells, Topro-3 was added (final concentration 25 nM; Invitrogen-Molecular Probes, Breda, The Netherlands) in the presence of 2.5 μM EDTA, incubated for 20 minutes at 37°C, put on ice, and then immediately used for flow cytometric analyses.

Cytokine ELISA Assays
T-cell stimulation assays containing both 5 x 10⁵/TCC and VZV or mock antigen–pulsed APCs were incubated for 2 days at 37°C, and cell-free supernatants were harvested, aliquoted, and stored at -20°C. Secretion levels of the following cytokines were measured by standard sandwich ELISA kits: IFN-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, and IL-5 (U-CyTech, Utrecht, The Netherlands). Assay detection sensitivities were 30 pg/mL for IFN-γ, 2 pg/mL for TNF-α and IL-5, and 34 pg/mL for IL-4.

RESULTS
Identification of Viral Antigens Recognized by IF0-Derived TCLs from Patients with Herpetic Uveitis
We recently described the selective infiltration of VZV-specific T cells in IOF samples from patients with VZV, but not those with HSV-1 uveitis. In the current study, we addressed the antigen specificity of the intraocular VZV-specific T cells in patients with VZV uveitis in more detail. The IOF-TCL, generated by mitogenic stimulation of T cells recovered from IOF samples of patients with clinically and diagnostically proven VZV (n = 5) or HSV-1 (n = 1) uveitis (Table 1), were assayed for virus-specific T cells. In agreement with previous studies, the IOF-TCL reacted to the causative virus (Fig. 1). To
determine the VZV antigens recognized, we used rVV-mediated expression of 11 different VZV proteins in BLCL (Table 2). T-cell reactivity to all VZV proteins tested for was observed, but varied between patients. In contrast to the patient with HSV-1 uveitis (patient 6), the IOF-TCL of each of the patients with VZV uveitis reacted to multiple VZV proteins (Fig. 1).

**Antigen Specificity and Cytokine Secretion of IOF-Derived VZV-Specific TCCs**

To analyze the intraocular VZV-specific T-cell response at the clonal level, the T cells of the IOF-derived TCLs of three representative patients were cloned by limiting dilution. A substantial number of the TCCs isolated were VZV-specific: 28 (15%) of 187 from patient 1, 20 (9%) of 228 from patient 2, and 10 (3%) of 347 from patient 3. Most of the TCCs were CD4+.

Representative VZV-specific responses of these TCC are shown in Table 3. All VZV-specific TCCs of patient 1 were directed to ORF62 and expressed the same TCRBV—namely TCRBV5S1 (Table 3; data not shown), suggesting that these TCCs are identical. The target antigens of 12 of 20 TCC from patient 2 could be identified and the TCCs recognized ORFs 4, 29, 31, 62, 63, or 68. Unfortunately, despite using a TCRBV repertoire mAb kit that covers approximately 70% of all known human TCRBVs, we could not define the TCRBV usage by the two ORF29 (TCC 71 and 132) and four ORF62 (TCC 36, 77, 211 and 213) reactive TCCs of patient 2. Four of 10 TCCs of patient 3 were typed for a specific VZV antigen: three ORF62- and one ORF4-specific TCC. TCRBV analyses on the three ORF62-specific TCCs of patient 3 revealed that TCC53 expressed a different TCRBV than did TCC7 and -146 (Table 3; data not shown).

The secretion of the cytokines IL-4, IL-5, TNF-α, and IFN-γ by the CD4+ VZV-specific TCC was determined, to define their Th phenotype. On antigenic stimulation, all TCCs secreted significant amounts of IFN-γ. Whereas some TCC secreted predominantly the classic Th1 cytokines TNF-α and IFN-γ (e.g., TCC7 and -145), others secreted both the Th1 and Th2 cytokines IL-4 and IL-5 simultaneously (e.g., TCC29 and -53; Table 3). Based on their cytokine secretion profiles, the TCCs were considered to be either Th1- or Th0-like, the latter characterized by secretion of both Th1 and -2 cytokines.

**Definition of T-Cell Epitopes and HLA Restriction of IOF-Derived VZV-Specific TCCs**

A panel of eight rVV-expressing in-frame-deletion mutants of ORF62 were used to delineate the antigenic region of ORF62 recognized by the ORF62-specific TCCs. All TCCs from patient 1 reacted to the rVV expressing ORF62 Aa 733-1056, but did not recognize the rVV expressing Aa 386-823. This finding delimits residues 823-1056 as the antigenic region (TCC199 is shown as a representative TCC; Table 4). The same region contained the epitopes recognized by TCC 77 and 213 of patient 2. In the case of patient 3, TCC7 and -53 both reacted to the ORF62 Aa 166-385 region (Table 4). Synthetic 20-mer peptides that spanned the ORF62 Aa 733-
### Table 3. Protein Specificity and Cytokine Secretion Levels of VZV-Reactive TCCs Recovered from Intraocular Fluid Samples of Three Study Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>TCC No.</th>
<th>Phenotype</th>
<th>VZV-ORF‡</th>
<th>Mock</th>
<th>VZV</th>
<th>rVV-CTRL</th>
<th>rVV-VZV</th>
<th>Cytokine Secretion Levels (pg/mL)†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>199</td>
<td>CD4</td>
<td>ORF 62</td>
<td>52 ± 10</td>
<td>321 ± 9</td>
<td>41 ± 7</td>
<td>333 ± 1</td>
<td>IFN-γ &gt;3000, TNF-α ND, IL-4 ND, IL-5 UND</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>CD8</td>
<td>ORF 24</td>
<td>24 ± 13</td>
<td>217 ± 99</td>
<td>17 ± 12</td>
<td>324 ± 36</td>
<td></td>
</tr>
<tr>
<td>77</td>
<td>CD8</td>
<td>ORF 62</td>
<td>12 ± 5</td>
<td>283 ± 38</td>
<td>28 ± 16</td>
<td>273 ± 86</td>
<td>ND, ND ND ND</td>
<td></td>
</tr>
<tr>
<td>211</td>
<td>CD4</td>
<td>ORF 62</td>
<td>26 ± 2</td>
<td>230 ± 9</td>
<td>2 ± 5</td>
<td>317 ± 167</td>
<td>ND, ND ND ND</td>
<td></td>
</tr>
<tr>
<td>213</td>
<td>CD4</td>
<td>ORF 62</td>
<td>21 ± 5</td>
<td>283 ± 13</td>
<td>14 ± 10</td>
<td>597 ± 27</td>
<td>890 ± 120, 100 ± 99</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>CD4</td>
<td>ORF 4</td>
<td>14 ± 10</td>
<td>136 ± 16</td>
<td>39 ± 9</td>
<td>507 ± 35</td>
<td>ND, ND ND ND</td>
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<tr>
<td>55</td>
<td>CD4</td>
<td>ORF 4</td>
<td>89 ± 102</td>
<td>239 ± 44</td>
<td>151§</td>
<td>342§</td>
<td>ND, ND ND ND</td>
<td></td>
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<tr>
<td>156</td>
<td>CD4</td>
<td>ORF 63</td>
<td>10 ± 1</td>
<td>240 ± 10</td>
<td>17§</td>
<td>235§</td>
<td>ND, ND ND ND</td>
<td></td>
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<tr>
<td>71</td>
<td>CD4</td>
<td>ORF 29</td>
<td>19 ± 6</td>
<td>273 ± 3</td>
<td>28 ± 14</td>
<td>407 ± 39</td>
<td>904 ± 7, 9 UND</td>
<td></td>
</tr>
<tr>
<td>132</td>
<td>CD4</td>
<td>ORF 29</td>
<td>14 ± 5</td>
<td>277 ± 43</td>
<td>11 ± 5</td>
<td>350 ± 4</td>
<td>1065 ± 12, 7 UND</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>CD4</td>
<td>ORF 31</td>
<td>30 ± 38</td>
<td>224 ± 1</td>
<td>77 ± 73</td>
<td>560 ± 45</td>
<td>ND, ND ND ND</td>
<td></td>
</tr>
<tr>
<td>196</td>
<td>CD4</td>
<td>ORF 51</td>
<td>11 ± 6</td>
<td>376 ± 127</td>
<td>25§</td>
<td>308§</td>
<td>ND, ND ND ND</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>CD4</td>
<td>ORF 68</td>
<td>21 ± 10</td>
<td>314 ± 28</td>
<td>23 ± 25</td>
<td>442 ± 14</td>
<td>841 ± 111, 10</td>
<td>5</td>
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<tr>
<td>3</td>
<td>CD4</td>
<td>ORF 62</td>
<td>6 ± 4</td>
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<td>13 ± 11</td>
<td>304 ± 51</td>
<td>842 ± 130, 558</td>
<td>60</td>
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<tr>
<td>53</td>
<td>CD4</td>
<td>ORF 62</td>
<td>8 ± 1</td>
<td>444 ± 34</td>
<td>3 ± 1</td>
<td>293 ± 51</td>
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<td>60</td>
</tr>
<tr>
<td>146</td>
<td>CD4</td>
<td>ORF 62</td>
<td>1 ± 1</td>
<td>258 ± 1</td>
<td>7 ± 9</td>
<td>255 ± 14</td>
<td>ND, ND ND ND</td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>CD4</td>
<td>ORF 4</td>
<td>3 ± 1</td>
<td>384 ± 61</td>
<td>5 ± 1</td>
<td>416 ± 93</td>
<td>452 ± 17, 426</td>
<td>521</td>
</tr>
</tbody>
</table>

* Mean number ± SD of IFN-γ spot-forming cells per 5000 T cells in response to mock- and VZV-antigen-pulsed B cells, or infection with rVV expressing the indicated VZV ORF, or no insertion (rVV-CTRL).
† Net cytokine secretion levels in response to VZV minus mock-antigen-pulsed B cells. UND, undetectable; ND, not determined.
‡ VZV ORFs recognized by the respective TCCs.
§ TCC reactivity to indicated ORF demonstrated in monoplo.

1056 region were used to locate the epitope recognized by the ORF62-specific TCCs from patients 1 and 3 (Fig. 2). The epitope of TCC 199 of patient 1 was located between ORF62 residues 914-960 (ARRRLVLDAVALERWPRDG). Of note, TCC7 and -53 of patient 3, expressing different TCRBV and using different HLA-DR alleles as the HLA-restriction element, both reacted to the same 20-mer peptide located at ORF62 residues 911-930 (ALNARGVLLLISTRDLAFA; Fig. 2).

The HLA class II restriction of the CD4 + TCC was defined in two ways. First, blocking mAbs directed to public specificities of HLA-DR, -DQ and -DP were added to the assays to define the HLA restriction loci involved. Second, partially HLA class II-matched BLCLs were used to identify the actual HLA allele involved as restriction element. At least five different HLA class II alleles restricted the VZV-specific CD4 + TCC responses (Table 5). The HLA restricting allele for the TCC of patient 1, TCC199, presented as a representative TCC, was HLA-DQB1*0202. This is because the number of IFN-γ spot-forming cells detected on incubation with autologous BLCL pulsed with VZV antigen was blocked significantly by the anti-HLA-DQ mAb (30%) and not by the anti-HLA-DR or -DP mAbs (data not shown). Furthermore, BLCLs matched for HLA-DQB1*0201, but not those matched for HLA-DQB1*0301, were recognized by the TCC (Table 5). Responses of the remaining ORF62-reactive TCC7 and -53, and an ORF68-specific TCC29, were all preferentially inhibited by the anti-HLA-DR mAb (data not shown). Subsequent experiments, using partially HLA-DR-matched BLCLs, identified the restricting allele DRB1*1404 and DRB1*0703 for TCC7 and -53, respectively, of patient 3. All ORF62-reactive TCCs of patient 2 were HLA-DRB1*0301 restricted, and the restriction element of TCC 29 was defined as HLA-DRB1*1501 (Table 5; data not shown).

### Table 4. Localization of the Epitope-Containing Region in VZV ORF62 Recognized by the Intraocular ORF62-Reactive CD4 + TCCs

<table>
<thead>
<tr>
<th>rVV-Expressing ORF62 Region</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCC 199</td>
<td>134 ± 93</td>
<td>245 ± 62</td>
<td>383 ± 20</td>
</tr>
<tr>
<td>TCC 7</td>
<td>73</td>
<td>0 ± 2</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>TCC 53</td>
<td>54 ± 3</td>
<td>86 ± 3</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>TCC 77</td>
<td>296 ± 59</td>
<td>297 ± 61</td>
<td>ND</td>
</tr>
<tr>
<td>TCC 213</td>
<td>323 ± 78</td>
<td>324 ± 79</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Mean number ± SD of IFN-γ spot-forming cells per 5000 T cells in response to autologous B cells infected with recombinant vaccinia viruses (rVV) expressing the indicated region of ORF62. ND, not determined. Aa, amino acid.
During the past decade, several approaches, including three-dimensional structural analyses of HLA class II molecules, pool sequencing of HLA class II–binding peptides, and identification of natural peptide, as presented herein, have facilitated the definition of motifs of peptides potentially binding to HLA class II alleles and the development of in silico T-cell epitope prediction software. Based on the ORF68 peptides predicted by three different computer prediction programs, and comparing probability ranking and co-occurrence in the output of the individual programs, a set of six potential peptides was synthesized and tested. This process resulted in the identification of the epitope of TCC29 in patient 2 localized at ORF6 residues 474-489 (LYVFVVYFNGHVEAV; Fig. 2).

### Cytotoxic T-Cell Activity of IOF-Derived VZV-Specific TCCs

The cytotoxic T-cell potential of the CD4⁺ TCCs was examined using a recently developed flow cytometric assay. Specific-cell lysis of autologous BLCLs, pulsed with the relevant peptides, revealed that the TCC analyzed exert cytotoxic properties. At an E:T ratio of 10:1, specific cell lysis varied between 36% and 51% (Table 6), whereas TCC199 of patient 1 showed 36% and 51% specific cell lysis.

### Table 5. Determination of the Restricting HLA Allele of the Intraocular CD4⁺ VZV-Specific TCCs

<table>
<thead>
<tr>
<th>Patient</th>
<th>TCC No.</th>
<th>VZV ORF*</th>
<th>BLCL Used as APC†</th>
<th>HLA Class II Type‡</th>
<th>Δ IFN-γ SFC§</th>
</tr>
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<tr>
<td>1</td>
<td>199</td>
<td>ORF62</td>
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<td>DQB1*0301; 0202</td>
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<td>VAVY</td>
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<td>MGAR</td>
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* VZV ORFs recognized by the respective TCCs.
† Names of the B-cell lines used to define the HLA restriction element of the respective TCCs.
‡ Donor’s HLA class II matching allele.
§ Mean net number of IFN-γ spot-forming cells per 5000 T cells in response to antigen-pulsed B cells.
Intraocular Fluid-Derived VZV-Specific TCC
Express an Effector Memory Phenotype

T cells with specificities for persistent viruses vary in phenotype and function. Human memory T cells, characterized as CD45RA⁻CD45RO⁺ T cells, can be assigned to two broad classes based on the lymph node (LN)-homing potential, reflected in their expression of the LN-homing receptors CCR7 and CD62L, immediate effector function, and ability to proliferate after subsequent encounter of the cognate antigen. In contrast to central memory T cells (T CM), effector memory T cells (T EM) are CCR7⁻CD62L⁻ and are enriched in peripheral tissues. Repetitive antigenic stimulation of T EM results in a more differentiated T EM phenotype characterized by down-regulation of the costimulatory molecules CD27 and CD28. Conversely, the expression of the natural killer (NK) marker CD57 and the integrin CD11a, which together with CD18 forms a heterodimer CD11a/CD18 (LFA-1) that interacts with CD54 (ICAM-1) on the APC and enabling T cells to enter peripheral tissues, are enhanced on chronically stimulated T EM cells. CD57 expression on T cells and NK cells is a general marker of proliferative inability, a history of more cell divisions, and short telomeress. To determine whether IOF-derived VZV-specific CD4⁺ TCC display markers indicative of a relatively early or more differentiated effector memory phenotype, we examined the expression of the aforementioned markers on a representative panel of TCC of patient 1 (TCC 199), patient 2 (TCC29, -71, and -213) and patient 3 (TCC7 and -53). All TCC analyzed expressed the following phenotype: CD45RA⁻CD45RO⁺CD27⁻CD28⁻CD57⁻CD11a⁻, inferring that the TCCs analyzed express a less differentiated T EM phenotype (data not shown).

**DISCUSSION**

In the present study, we determined the specificity and functional characteristics of the VZV-specific T-cell pool recovered from IOF samples obtained from affected eyes of five patients with VZV uveitis. By using a comprehensive panel of rVVs that encode 11 different VZV proteins, we found that the intraocular VZV-specific T-cell response in the patients with VZV uveitis studied was polyclonal and multispecific. Analyses of the IOF-derived VZV-specific T cells at the clonal level revealed that they were cytolytic and expressed a Th1/0-like effector memory phenotype.

Intraocular IFN-γ flow cytometric and IFN-γ ELISPOT assays in recent studies have defined the VZV T-cell responder frequency in PBMCs of VZV-immune adults as approximately 1 in 10,000 PBMCs. In comparison, the IOF-TCL described herein contained relatively higher numbers of VZV-reactive T cells, suggesting local enrichment of VZV-reactive T cells in the IOF samples of the patients with VZV uveitis studied. The data are in line with our previous studies on IOF-TCL obtained from HSV-1 and patients with *Toxoplasma gondii* uveitis, demonstrating a high number of T cells toward the causative agent. The T-cell mitogen PHA, used to generate the IOF-TCL, was chosen as the best alternative to limit selective outgrowth of T-cell subpopulations. Nonetheless, we cannot rule out the option that the culture conditions may have influenced the repertoire of the IOF T-cell pool. Limiting dilution assays on freshly isolated IOF T cells may overcome this potential problem and should be considered in future studies.

Whereas most of the IOF-TCL (excluding patient 5; data not shown) contained both T-cell subsets, the VZV-specific TCC generated from these TCL were mainly CD4⁺. This bias is most likely attributable to the use of BLCLs pulsed with VZV antigen as APCs for the first screening of the TCC for VZV reactivity after limiting dilution. This procedure of APC preparation favors presentation of viral peptides via the HLA class II pathway. T-cell reactivity toward a specific VZV protein could be defined in the IOF-TCL and subsequently at the clonal level for a substantial number of the VZV reactive TCC. Whereas no preference was seen for VZV structural or nonstructural proteins, reactivity toward the major transcriptional regulatory proteins, which are also latency-associated, was well represented: ORF4, -29, -63, and particularly -62 were common target antigens in all patients with VZV uveitis. Compared with HSV, only a few studies have documented epitopes recognized by VZV-specific TCCs.

We have identified three new CD4⁺ T-cell epitopes: one within ORF68 and two separate epitopes within ORF62, with the latter in close proximity to each other. Noticeably, the ORF62 epitope from residues 911-930 was recognized in the context of two different HLA-DR alleles by two genetically different TCCs derived from the same eye.

The recognition of the latency-associated VZV proteins ORF4, -29, -62, and -63 may be due to a restricted expression of these VZV proteins within the affected ocular tissue or may merely reflect the locally circulating VZV T-cell repertoire. Latency-associated VZV proteins are continuously expressed during latency in human sensory ganglia and, as a result, may provide a persistent pool of potential T-cell antigens. Although uveal latency cannot formally be ruled out, it is more conceivable that restricted expression of these proteins and other immediate early and early VZV proteins, has already commenced before viral reactivation by the local innate immune response. The alternative explanation is that the collective VZV target proteins identified herein are relatively immunodominant, due to their length or abundant expression within infected cells. Infection-mediated breakdown of the blood–retina barrier would enable circulating VZV-specific memory T cells to enter the eye and target the VZV-infected uveal tissue. Studies on systemic and localized CD4⁺ T-cell responses toward HSV-1 and -2 support the latter assumption. For obvious reasons, distinction between these options is not possible in humans. Clinical specimens can be obtained only when the disease has progressed beyond the initiation phase and nearly all patients are receiving antiviral and immune-suppressive therapy at the time of surgery. Studies on an experimental monkey uveitis model of simian varicella virus (SVV), the closest relative of VZV, may provide more insight in the viral and host factors involved in this sight-threatening disease.

In summary, the data presented are consistent with selective infiltration of VZV-specific T cells, involving potentially cytolytic CD4⁺ T cells with a Th0/1-like effector memory phenotype, into the ocular compartment of patients with VZV uveitis.
uveitis. The intraocular VZV-specific T-cell pool appeared to be directed toward various VZV proteins, including the regulatory and latency-associated VZV proteins, with ORF62 appearing as a common T-cell target in all the patients studied. Future studies combining VZV T-cell antigen recognition with immunohistologic analyses of ocular tissue specimen should be conducted, to provide more insight into the role of the VZV-specific T-cell response in the pathogenesis of VZV uveitis in humans. The SVV uveitis monkey model should be used in this line of research, given the constraints encountered in analyzing human specimens.

Acknowledgments

The authors thank Frits Koning (LUMC, Leiden, The Netherlands) for providing the anti-HLA class II hybridomas.

References


