TCR Bias and HLA Cross-Restriction Are Strategies of Human Brain-Infiltrating JC Virus-Specific CD4+ T Cells during Viral Infection

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Virus-specific CD4+ T cells play a central role in control of viral pathogens including JC polyoma virus (JCV) infection. JCV is a ubiquitous small DNA virus that leads to persistent infection of humans with no clinical consequences. However, under circumstances of immunocompromise, it is able to cause an opportunistic and often fatal infection of the brain called progressive multifocal leukoencephalopathy (PML). PML has emerged as a serious adverse event in multiple sclerosis patients treated with the anti–VLA-4 mAb natalizumab, which selectively inhibits cell migration across the blood–brain barrier and the gut’s vascular endothelium thus compromising immune surveillance in the CNS and gut. In a multiple sclerosis patient who developed PML under natalizumab treatment and a vigorous immune response against JCV after Ab washout, we had the unique opportunity to characterize in detail JCV-specific CD4+ T cell clones from the infected tissue during acute viral infection. The in-depth analysis of 14 brain-infiltrating, JCV-specific CD4+ T cell clones demonstrated that these cells use an unexpectedly broad spectrum of different strategies to mount an efficient JCV-specific immune response including TCR bias, HLA cross-restriction that increases avidity and influences in vivo expansion, and a combination of Th1 and Th1-2 functional phenotypes. The level of combinatorial diversity in TCR– and HLA–peptide interactions used by brain-infiltrating, JCV-specific CD4+ T cells has not, to our knowledge, been reported before in humans for other viral infections and confirms the exceptional plasticity that characterizes virus-specific immune responses.

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immune reconstitution inflammatory syndrome (PML-IRIS), which leads to efficient destruction of JCV-infected oligodendrocytes and astrocytes (34, 35). Recently, the analysis of a diagnostic brain biopsy from an MS patient who developed PML-IRIS under natalizumab treatment allowed us to study for the first time, to our knowledge, the role of brain-infiltrating, JCV-specific CD4+ T cells during JCV infection of the CNS. Our results demonstrated that JCV-specific CD4+ T cells with Th1 and Th1-2 phenotype are probably the most critical element among the CNS-infiltrating T and B cells. IFN-γ released by JCV-specific CD4+ T cells is most likely responsible for the proinflammatory state in the CNS with macrophage activation, upregulation of HLA class II, and efficient Ag presentation, whereas IL-4 leads to the activation and expansion of memory B cells/plasmablasts and intrathecal production of virus-specific Abs (36).

Virus-specific T cell responses are often characterized by the biased use of particular TCR V regions (37, 38). The V region of the α-chain of the TCR (TRAV) is encoded by V and J gene segments, whereas the V region of the β-chain of the TCR (TRBV) is encoded by V, D, and J gene segments. There are three regions of hypervariability known as CDRs, which are responsible for TCR diversity. The most variable CDR region is CDR3. CDR3 variation results from somatic recombination of V, D, and J gene segments and addition of non-template-encoded nucleotides at V, D, and J junctions. TCR bias can result from the selection of a single TRAV and/or TRBV region but little conservation in the CDR3 regions or can also involve conserved amino acids in CDR3 sequences (37). Several examples of TCR bias in human CD8+ T cells during persistent but also acute viral infections have been reported (39–43). TCR bias can confer an advantage in virus-specific immune responses by selecting virus-specific CD8+ T cells with higher avidity (41, 44) and with optimal TCR structural characteristics for a given peptide–MHC complex topology (45–47). In addition to the peptide–MHC complex recognized, other factors that influence TCR bias are the Ag load and the spectrum of TCRs available in the naive T cell pool. Regarding CD4+ T cells, TCR bias has been reported in some animal models (48–50), but never in human CD4+ T cells during viral infection.

The interaction between αβ-TCRs and peptide–HLA class II complexes is generally conceived as being highly specific for one or a small set of peptides and restricted by a unique HLA class II molecule. However, some examples of human T cells that are able to recognize different peptides in the context of one or several restriction elements have been described by us and others (51–56). The weak recognition of self-peptide–self-HLA complexes during positive selection in the thymus is a prerequisite for the development of T cells that are able to recognize foreign peptide–self-HLA complexes and implies that the T cell repertoire contains cross-reactive T cells that are in principle autoreactive and able to induce autoimmune diseases such as MS (57). Some examples of myelin basic protein-specific T cell clones (TCCs) as well as data from in vivo clonally expanded cerebrospinal fluid-infiltrating T cells from MS patients that are able to recognize different peptides in the context of different HLA class II molecules have been reported (52–54). However, cross-reactivity (i.e., the ability to recognize multiple peptides) and HLA cross-restriction (the ability to use multiple restriction elements to recognize the same and/or different peptides) do not seem to be features exclusively found in autoreactive T cells, as it has been reported that TCCs specific to foreign pathogens such as HSV type 2 (55), influenza A virus (56), and Mycobacterium tuberculosis (51) are also able to recognize different peptides in the context of different restriction elements. Although most studies have focused on understanding the structural aspects of cross-restriction, hardly any studies have addressed its functional relevance in the context of the respective disease. During an infection, T cell recognition of a specific peptide together with two or more HLA class II molecules could ensure more efficient activation, if the different HLA molecules are expressed on the APCs. In addition, under conditions where expression of one restriction element is limited (e.g., the brain), cross-restriction could provide a safeguard and would still allow T cell activation. However, in addition to mounting a more efficient immune response against infectious organs, cross-restriction may increase the risk for autoimmunity. A putative physiological role of cross-restriction in facilitating T cell activation was supported by our previous observation using TCCs that were in vivo clonally expanded in the cerebrospinal fluid of an MS patient during relapse, which allowed us to demonstrate a correlation between the stimulatory potential of peptides and their ability to be presented by different restriction elements (52).

In the current study, we aimed at dissecting the strategies used by CD4+ T cells to mount efficient JCV-specific immune responses. For this purpose, we have analyzed 14 brain-infiltrating JCV major capsid protein (VP1)-specific TCCs with respect to TCR bias, their ability to use different HLA class II restriction elements, and with respect to how cross-restriction influences T cell avidity, clonal expansion, and their cytokine profile.

Materials and Methods

Ethics statement

This study was approved by the local ethics committee (Ethik Kommission der Ärztekammer Hamburg, protocol no. 2758), and informed consent was obtained from the patient.

Brain-infiltrating, VP1-specific TCCs and APCs

Brain-infiltrating, VP1-specific TCCs were generated as previously described (36). Briefly, brain tissue from a relapsing remitting-MS patient, who developed PML-IRIS under natalizumab treatment and who expressed the HLA class II molecules DRB1*11:03, DBR1*15:01, DRB3*02:02, DRB5*01:01, DQA1*01:02, DQA1*05, DQB1*03:01, and DQB1*06:02, was cut into small pieces and disrupted by incubation in a solution containing 1 mg/ml collagenase A (Roche Diagnostics, Penzberg, Germany) and 0.1 mg/ml DNase I (Roche). Brain-derived mononuclear cells were then separated using a Percoll density gradient centrifugation (GE Healthcare, Munich, Germany) and expanded as bulk populations by stimulation with 1 μg/ml PHA-L (Sigma, St. Louis, MO), 20 U/ml hrIL-2 (Tecin; Roche Diagnostics), and allogeneic irradiated PBMCs. Although our culture conditions favored the expansion of CD4+ over CD8+ T cells, the relative composition of CD4+ T cells remained stable as demonstrated by staining with mAbs against TCR variable chains VB1–VB22 (36). VP1-specific TCCs were generated by seeding brain-derived PHA-expanded T cells with autologous irradiated PBMCs and VP1 protein. After 48 h of culture, plates were split into mother and daughter plates. Proliferation was measured in daughter plates, and VP1-responsive cultures were identified in mother plates. TCCs were established from these VP1-responsive cultures by limiting dilution, and fine specificity was determined using 64 (13–16 mer) peptides overlapping by 5 aa and covering the JCV VP1 protein (Peptides and Elephants, Potsdam, Germany) (36).

The following APCs were used: autologous PBMCs; autologous EBV-transformed B cell lines; PBMCs from a donor expressing HLA class II molecules DRB1*11:03, DRB1*08:01, DRB3*02:02, DQA1*04:01, DQA1*05, DQB1*03:01, and DQB1*04:02; and bare lymphocyte syndrome (BLS) cells transfected with single HLA class II molecules DR2a (DRA1*01:01, DRB5*01:01), DR2b (DRA1*01:01, DRB1*15:01), and DQw6 (DQA1*01:02, DQB1*06:02) kindly provided by G. Nepom and W. Kwok (University of Washington, Seattle, WA).

PCR and sequencing of TCR rearrangements

The TRAV chain repertoire was assessed using a primer set published by Han et al. (58), and the TRBV chain primer set was based on a publication of Currier and coworkers (59). Primers were obtained from Biomers (Ulm, Germany), and sequences are provided in Supplemental Table I. PCR amplification was performed in a 50-μl reaction volume containing 1× HotStart...
PCR Buffer, 200 μM deoxynucleotide triphosphate, 2.5 mM MgCl₂, 1.25 μM C3 primer, 1.25 U Maxima HotStart Taq DNA Polymerase (all reagents were provided by Fermentas, St. Leon-Rot, Germany), 1.25 μM forward primer, and 100 ng cDNA. The cycling conditions were as follows: initial denaturation for 4 min at 95°C and 35 cycles of 95°C for 30 s, primer annealing at 60°C for 20 s, and primer extension at 72°C for 60 s, terminated by a final extension at 72°C for 10 min. The PCR product was validated by electrophoresis in a 1.5% agarose gel.

Sequencing of PCR products was performed by incubating 5 μl PCR product with 10 U exonuclease I (no. EN0581; Fermentas) and 1 U FastAP Thermosensitive alkaline phosphatase (no. EF0651; Fermentas) in a reaction volume of 6.5 μl for 15 min at 37°C. The reaction was terminated by heating for 15 min at 85°C. After the addition of 5 μl of C3 primer, direct sequencing of the PCR products was carried out with fluorescent dideoxy terminators, and analysis was performed on an ABI 3130 Genetic Analyzer and with Sequence Analysis v 5.4 software (Applied Biosystems, Foster City, CA). TCR gene designations are in accord with IMmunogeneTics (IMGT) nomenclature (http://www.IMGt.org).

CDR3 spectratyping
To assess in vivo clonal expansion, high-resolution TRAV and TRBV CDR3 length (CDR3-L) spectratyping was performed for each TCC and brain-derived PHA-expanded mononuclear cells. Five microliters of each specific PCR product was subjected to five cycles of run-off reactions in a final volume of 20 μl containing 0.1 μM C1-FAM-labeled primer, 200 μM of each deoxynucleotide triphosphate, and 0.5 μl PFU DNA polymerase in 1× PFU buffer containing MgSO₄ (all reagents were provided by Fermentas). Amplification was conducted in a GeneAmp PCR System 9700 (PerkinElmer, Rodgau-Jürgensheim, Germany), with cycle conditions as follows: denaturation at 95°C for 10 min, 5 cycles of 2 min at 95°C, 2 min at 60°C, and 10 min at 72°C. Subsequently, the run-off products were cleaned with Microclean (Biofidal), and thereafter, ~1 μl (0.1–3) of each reaction was mixed with 10 μl formamide containing GeneScan LIZ 600 Size Standard (Applied Biosystems) in a 96-well plate. After denaturation at 95°C for 5 min followed by incubation at 4°C for 5 min, the samples were analyzed using a 3130 Genetic Analyzer (Applied Biosystems). The number of peaks, peak size (expressed in bp), peak height, and area under the curve (AUC) were calculated using Gene Mapper (version 4.1) and peak scanner software (Applied Biosystems). The percent contribution of each TCC’s TRBV or TRAV CDR3-L peak in a CDR3-L spectrum was calculated according to the formula %AUC Vn = (AUC Vn / ΣAUC all V) × 100. Because for each TCC two different %AUC values were obtained corresponding to TRBV and TRAV, to estimate conservatively we considered always the lowest one, and if several TCCs shared the same TRBV or TRAV CDR3-L, the %AUC was divided by the number of known TCCs contributing to the peak.

Proliferative assays and cytokine production
To determine whether TCCs were able to recognize specific peptides in the context of the different autologous HLA class II molecules, 2 × 10⁶ T cells were seeded in duplicate with 10 μM specific peptide and 5 × 10⁶ irradiated BLS cells transfected with DR2a (DRA1*01:01, DRB5*01:01), DR2b (DRA1*01:01, DRB1*15:01), and DQw6 (DQA1*01:02, DQB1*06:02) as APCs. Because BLS transfected with the other autologous HLA class II molecules DRB1*11:03, DRB3*02:02, DQA1*05, and DQB1*03:01 were not available, we used as APCs to test recognition in these restriction elements 10 × 10⁶ irradiated PBMCs from a donor expressing the following HLA class II molecules: DRB1*11:03, DRB1*08:01, DRB3*02:02, DQA1*04:01, DQA1*05, DQB1*03:01, and DQB1*04:02. To estimate TCC avidity, 2 × 10⁵ T cells were seeded in duplicate with 5 × 10⁶ irradiated autologous EBV-transformed B cells and different concentrations of the specific peptide (0.01–10 μM). To test the peptides with alanine substitutions, 2 × 10⁵ T cells were seeded in duplicate with 5 × 10⁶ irradiated autologous EBV-transformed B cells and 10 μM peptides. Proliferation was measured by methyl-[3H]thymidine (Amersham Biosciences, Buckinghamshire, U.K.) incorporation. The stimulation index (SI) was calculated as SI = mean (duplicates cpm peptide)/mean (cpm background). IFN-γ and IL-4 release was determined by ELISA following the manufacturer’s protocol (Biosource, Camarillo, CA) in culture supernatants of TCCs stimulated for 48 h with 10 μM peptide using as APCs autologous EBV-transformed B cell lines.

Statistical analysis
Statistical analysis was performed using GraphPad Prism V5.02. After testing for Gaussian distribution (Kolmogorov–Smirnov test), either Student t test or Mann–Whitney U test was used for comparison of two groups of nonpaired samples. For comparison of more than two unpaired sample groups, ANOVA for nonparametric (Kruskal–Wallis) data and post hoc test (Dunn’s test) was performed.

Results
In vivo clonal expansion of VP1-specific CD4⁺ TCCs
In a previous study, we had established—from an MS patient who had developed PML-IRIS under natalizumab treatment—several brain-infiltrating, JCV-specific CD4⁺ TCCs that recognized different peptides of the major capsid protein of JCV, VP1 (36). Toward this aim, brain-derived, PHA-expanded mononuclear cells were seeded with autologous PBMCs and VP1 protein. Growing wells containing VP1-specific T cells were identified, and VP1-specific single cell-derived cultures were generated by limiting dilution. Clonality of these VP1-specific T cells was confirmed by TCR analysis. From a total of 21 VP1-specific cultures, 14 different TCCs were identified after sequence analysis of the TCR α- and β-chains (Table I). The fine specificity of these 14 TCCs was determined using overlapping peptides spanning the VP1 sequence (36) (Supplemental Table I). All TCCs recognized only one VP1 peptide with the exception of TCC-7, which recognized three peptides that differ in a single amino acid and represent common mutations of the same amino acid sequence (36). Five TCCs (TCCs 1–5) were specific for peptide VP1₃₄₅ (the number denotes the first amino acid). Notably, these five TCCs used the same TRBV and TRAV, but different TRBJ, TRBD, and TRAJ genes and had accordingly different CDR3 sequences (Table I). TCC-6 was specific for peptide VP1₃₄₅, TCC-7 for three VP1₇₄₄ peptides with common mutations (VP1₇₄₄₁, VP1₇₄₄₂, and VP1₇₄₄₃). TCC-8 for peptide VP1₉₆, TCC-9 for peptide VP1₄₄₅, and TCC-10 for peptide VP1₂₉₂. Two TCCs (TCC-11 and TCC-12) were specific for peptide VP1₃₁₆, and two other TCCs (TCC-13 and TCC-14) were specific for peptide VP1₃₅₅. The number of single cell-derived cultures corresponding to each TCC provides an approximate estimate of its relative frequency in the brain infiltrate. TCCs 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, and 12 were represented by a unique single cell-derived, growing well suggesting that the expansion of these TCCs in brain tissue was comparable. TCC-7, which was represented by five growing wells, was probably the most frequent TCC in the brain infiltrate followed by TCC-12 represented by three growing wells and TCC-13 represented by two growing wells (Table I).

The relative frequency of the different TCCs in the brain tissue was also assessed by CDR3 spectratyping. We quantified the in vivo clonal expansion of the 14 TCCs in the brain infiltrate by determining the percent contribution (expressed as AUC) of the TRAV and TRBV CDR3-L peaks from individual TCCs within the CDR3-L spectrum of brain-derived, PHA-expanded mononuclear cells (Fig. 1A). For each TCC, we obtained two percentages corresponding to TRAV and TRBV, and, to estimate more conservatively rather than overestimate their expansion, we considered the lowest one. Using this approach, we found that TCC-7, TCC-11, TCC-12, and TCC-13 were in vivo clonally expanded as they had a %AUC higher than 20% (Fig. 1B). Confirming our assumption that the number of single cell-derived, growing wells generated for each TCC provides an approximate estimate of its relative frequency in the brain, three of these TCCs (TCC-7, TCC-12 and TCC-13) were also the TCCs for which more growing wells were obtained (see above and Table I).

TCR bias in brain-infiltrating, VP1-specific CD4⁺ TCCs
As mentioned above, VP1₃₄₅-specific TCCs expressed TRAV13-1*02 and TRBV20-1*01 sequences that were almost identical with the exception of the CDR3 regions and the associated J
Table I. Characterization of TCCs

<table>
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<th>TCC</th>
<th>TRBV</th>
<th>TRBJ</th>
<th>TRBD</th>
<th>CDR3</th>
<th>TRAV</th>
<th>TRAJ</th>
<th>CDR3</th>
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<td>TRBD2*01</td>
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<td>TRAV13-1*02</td>
<td>TRAJ17*01</td>
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<td>TRBD1*01</td>
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<td>TRBD1*01</td>
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</table>

The three VP134 peptides on the left correspond to common mutations. Each square represents a single cell-derived culture. Single cell-derived cultures containing identical TCCs are shown in black.
segments (Fig. 2). Additionally, in four of these TCCs (TCC-1, TCC-3, TCC-4, and TCC-5), there is one amino acid exchange downstream of the CDR2 region of TRA V13-1 at position 54 differing from the germline-encoded sequence. Specifically, the germline amino acid isoleucine (I; nucleotide codon ATA) has been exchanged by valine (V; nucleotide codon GTA) as a consequence of a nucleotide point mutation. Overall, the amino acid composition of the CDR3\textsubscript{b}-chains of these TCCs was more heterogeneous than the composition of the CDR3\textsubscript{a}-chains (Fig. 2).

To determine whether these five TCCs used the same primary TCR contacts for VP134 peptide recognition, we tested peptides with single alanine substitutions. Using these alanine-scan peptides, we identified the charged amino acid glutamic acid in position 41 (E41) as the main TCR contact for all TCCs, as the substitution of this amino acid by a nonconservative amino acid like alanine completely abrogated recognition of peptide VP134 (Fig. 3A). Phenylalanine at position 43 (F43) seems to be an additional TCR contact for TCC-2 and TCC-4, glutamic acid in position 38 (E38) for TCC-3 and TCC-4, and isoleucine at position 37 (I37) and proline at position 46 (P46) for TCC-1 and TCC-5. The recognition patterns of the five TCCs indicated that TCC-1 and TCC-5 are the two TCCs showing the strongest similarity (Fig. 3B). Notably, TCC-1 and TCC-5 have identical CDR3\textalpha amino acid sequences although they use different non-germline-encoded nucleotide codons (Fig. 3C).

Cross-restriction is a feature of some brain-infiltrating, VP1-specific CD4\textsuperscript{+} TCCs and is associated with higher avidity

To dissect the different strategies used by brain-infiltrating CD4\textsuperscript{+} T cells to recognize the major capsid protein of JCV, VP1, we characterized the above-mentioned 14 brain-infiltrating, VP1-specific TCCs regarding HLA class II restriction. The ability of these TCCs to recognize the specific VP1 peptides in the context of different autologous HLA class II molecules is shown in Fig. 4. Nine of these 14 TCCs (64%; TCCs 4, 6, 7, 8, 9, 11, 12, 13, and 14) were cross-restricted, whereas only five TCCs (36%; TCCs 1, 2, 3, 5, and 10) were restricted and recognized the peptide in the context of a single HLA class II molecule (Figs. 4, 5A). These results suggest that cross-restriction is a feature of some VP1-specific CD4\textsuperscript{+} T cells.

FIGURE 1. In vivo expansion of brain-infiltrating, VP1-specific CD4\textsuperscript{+} TCCs. (A) TRAV and TRBV CDR3-L spectratypes corresponding to TCCs and brain-derived, PHA-expanded mononuclear cells. Histograms represent the relative CDR3-L distributions of brain-derived, PHA-expanded mononuclear T cells (top rows) and of each TCC (bottom rows). Fluorescence intensity is listed on the y-axis and the electrophoresis time resolving in-frame rearrangements of TRAV and TRBV CDR3 at 3-nt intervals on the x-axis. Numbers indicate peak size (expressed in bp) and allow identification of the correct alignment. (B) The graph represents the percent contribution (expressed as %AUC) of the CDR3 of TCCs to all CDR3s with the same TRAV or TRBV chain in brain-derived, PHA-expanded mononuclear cells. For each TCC, only the lowest %AUC from the two obtained corresponding to TRBV or TRAV is represented. If several TCCs shared the same TRAV or TRBV CDR3-L (e.g., TRBV20 shared by TCCs 1, 2, 3, and 5), the %AUC was divided by the number of known TCCs contributing to the peak, in this case 4. The specific VP1 peptide recognized by each TCC is indicated, and TCCs recognizing identical peptide are grouped.
Two TCCs (TCC-8 and TCC-9) representing also 22.5% of the cross-restricted TCCs were classified as intermediate cross-restricted because although they were able to recognize the specific peptide in the context of four different restriction elements, the response was markedly different between the different class II molecules (Figs. 4, 5A). Finally, five TCCs (TCCs 6, 11, 12, 13, and 14) representing 55% of the cross-restricted TCCs were classified as high cross-restricted as they were able to recognize the specific peptide in the context of at least four different restriction elements (Figs. 4, 5A). Notably, all TCCs with the same peptide specificity showed identical or similar restriction. The five TCCs specific for peptide VP134 were all DQB1*06:02-restricted. TCC-4 in addition showed recognition in the context of another autologous HLA class II molecule (Figs. 4, 5B). The two TCCs specific for peptide VP1319 were both highly cross-restricted as well as the two TCCs specific for peptide VP1335 (Figs. 4, 5B).

Autologous APCs express on their surface all autologous HLA class II molecules, and because highly cross-restricted TCCs can recognize peptides bound to all these restriction elements, we examined whether cross-restriction influences T cell avidity. We measured the reactivity, expressed as SI, of the 14 TCCs at two different concentrations (1 and 0.1 mM) of the corresponding specific peptide (Fig. 4). The proliferative capacity of the highly cross-restricted TCCs (TCCs 6, 11, 12, 13, and 14) to 1 and 0.1 mM peptide (mean SI ± SD to 1 mM peptide = 17.5 ± 9.2 and to 0.1 mM peptide = 8.4 ± 2.6) was significantly higher than the proliferative capacity of the TCCs restricted by a single HLA class II molecule (mean SI ± SD to 1 mM peptide = 4.8 ± 4, p = 0.0317, and to 0.1 mM peptide = 1.1 ± 0.9, p = 0.0079; Fig. 5C). Notably, three of the five highly cross-restricted TCCs (60%; TCCs 11, 12, and 13) were in vivo clonally expanded, whereas none of the restricted TCCs was (Fig. 5D), suggesting that high cross-restriction can facilitate in vivo expansion of T cells by increasing avidity. Supporting this notion, the reactivity of in vivo clonally expanded TCCs to 0.1 mM of specific peptide was higher than the reactivity of TCCs that were not expanded, although differences did not reach statistical significance (Fig. 5E).

IFN-γ and IL-4 production by brain-infiltrating, VP1-specific CD4+ TCCs

In a previous study, we reported the ability of some VP1-specific CD4+ T cells to secrete simultaneously IFN-γ and IL-4, which we termed a Th1-2 phenotype (36). Cytokine release by the 14 brain-infiltrating, VP1-specific CD4+ TCCs after in vitro stimulation with specific peptide was determined by ELISA (Fig. 6A). Notably, for the three VP1 peptides the Th1-2 phenotype could not be associated with the response to any specific VP1 peptide (Fig. 6B). Actually, for the three VP1 peptides for which different specific TCCs were identified, it is interesting to note that both Th phenotypes were present in each case. Two of the five TCCs specific for VP134 were Th1-2 and...
three had a Th1 phenotype. Regarding the two TCCs specific for peptide VP1319, one was Th1-2 and the other Th1, and the same applied for the two TCCs specific for peptide VP1335. Notably, 50% of the Th1-2 TCCs generated (TCCs 7, 11, and 13) were in vivo clonally expanded, whereas only one TCC (TCC-12) representing 12.5% of the Th1 TCCs generated was in vivo expanded (Fig. 6C). The Th1-2 phenotype could not be associated with highly cross-restricted or restricted TCCs (Fig. 6D), nor with TCCs with high or low avidity (Fig. 6E).

**Discussion**

JCV is a ubiquitous virus that only causes PML under circumstances of immunocompromise and especially impaired CD4+ T cell function (13–15). The lack of PML development in immunocompetent healthy individuals, who are persistently infected with JCV, implies the existence of very efficient JCV-specific immunity. In MS patients, who developed PML under natalizumab treatment, cessation of mAb therapy reestablishes CNS immune surveillance leading to PML-IRIS, an inflammatory response in the brain that rapidly eliminates JCV-infected cells. In a previous study, we had the opportunity to examine in great detail a diagnostic brain biopsy from an MS patient who developed PML under natalizumab treatment and a vigorous immune response that was most likely directed against JCV-infected oligodendrocytes and astrocytes after Ab cessation (36). Analysis of brain inflammation demonstrated abundance of CD4+ T cells highly specific for peptides from several JCV proteins, particularly the major capsid protein VP1, and with a Th1 and a Th1-2 functional phenotype. In the current study, we analyzed in detail the Ag recognition patterns of 14 brain-infiltrating, VP1-specific CD4+ TCCs from this patient to dissect the strategies used by VP1-specific CD4+ T cells to achieve highly efficient JCV recognition and elimination from the brain. We found that brain-infiltrating, VP1-specific CD4+ TCCs used a great variety of strategies to mount efficient JCV-specific immune responses including TCR bias, HLA cross-restriction that increases avidity and influences in vivo expansion, and a combination of Th1 and Th1-2 functional phenotypes.

One important characteristic of JCV-specific immunity and in particular of the VP1-specific T cell responses is the broad spectrum of epitopes that are recognized (36). JCV VP1 is probably the
most important target of the cellular and humoral immune response, and therefore it is not surprising that the brain-infiltrating CD4+ TCCs that we generated using VP1 whole protein recognized eight different VP1 epitopes. Taking into account the number of different TCCs identified for each peptide as well as the relative frequency of each TCC in the brain infiltrate, we identified VP134, VP174, VP1319, and VP1335 as immunodominant peptides. These four peptides were also identified as immunodominant in our previous study by testing bulk PHA-expanded, brain-derived mononuclear cells with overlapping VP1 peptides (36). Notably, recognition of these four VP1 peptides showed marked differences. Peptide VP134 was recognized by five TCCs, which were not expanded in vivo. These expressed almost identical TCR \( \alpha \)- and \( \beta \)-chains but different CDR3 regions and associated J segments. In contrast, a unique but highly in vivo-expanded TCC recognized three natural variants of peptide VP174 that differ in a single amino acid. VP1319 and VP1335 peptides each were recognized by two different TCCs that were in vivo clonally expanded. These differences in JCV recognition by CD4+ T cells suggest that different TCCs use different strategies to generate an efficient JCV immune response (Fig. 7).

Virus-specific CD8+ T cell responses are frequently characterized by TCR bias. Although several examples of TCR bias in human CD8+ T cells during viral infection have been reported (39–43), to our knowledge TCR bias has never been shown for human CD4+ T cells during viral infections. In this study, we have identified five brain-derived CD4+ TCCs specific for VP134 peptide with almost identical TCR \( \alpha \)- and \( \beta \)-chains but different CDR3 regions and the associated J segments. To our knowledge, these data provide the first evidence in humans that TCR bias also occurs in CD4+ T cells during viral infection. Some studies have suggested that TCR bias can result from the preferential selection of T cells with high avidity (41, 44). Selection of higher-avidity TCCs probably is not the factor determining TCR bias in these five TCCs as their avidity was overall low compared with other brain-derived CD4+ VP1-specific TCCs. Other studies suggested that the structure of the peptide–MHC complex could be the determining factor for TCR bias (45, 46). The peptide–HLA complex (VP1 34–DQA1*01:02/DQB1*06:02) recognized by these TCCs has a particular topography with many charged amino acids that might influence TCR bias. Peptide VP134 (amino acid sequence VDSITEVECFLTPEM) contains four negatively charged residues.

**FIGURE 4.** Cross-restriction and avidity of brain-infiltrating, VP1-specific CD4+ TCCs. Proliferative responses expressed as SIs of 14 brain-infiltrating, VP1-specific CD4+ TCCs to the corresponding specific peptide (10 \( \mu \)M) in the context of different autologous HLA class II molecules (left columns) and to two different concentrations of the specific peptide (1 and 0.1 \( \mu \)M) presented by autologous EBV-transformed B cell lines (right columns). Graphs show the mean SI ± SEM. The color indicates the level of cross-restriction: white indicates restriction, light gray indicates low cross-restriction, dark gray indicates intermediate cross-restriction, and black indicates high cross-restriction. TCCs are grouped by specificity, and specific peptide is shown.
amino acids and one aromatic. From testing these TCCs with alanine-scan peptides, we showed that these charged and aromatic amino acids are relevant for TCR recognition. Glutamic acid in position 41 (E41) has been identified as the main TCR contact for all TCCs. Other amino acids such as glutamic acid in position 38 (E38) and phenylalanine at position 43 (F43) seem to be additional TCR contacts. Regarding DQA1*01:02/DQB1*06:02 molecules, superposition of the crystal structures corresponding to DRA1*01:01/DRB1*15:01, DRA1*01:01/DRB5*01:01, and DQA1*01:02/DQB1*06:02 through their α1 and β1 domains showed that although six of eight potential contacts are identical between DRA1*01:01 and DQA1*01:02 with the only difference

FIGURE 5. Cross-restriction, avidity, and in vivo clonal expansion. (A) Pie chart representing the percentage of restricted and cross-restricted TCCs and pie chart representing the percentage of cross-restricted TCCs showing low, intermediate, and high cross-restriction. (B) Schematic representation of the distribution of restricted, low cross-restricted, intermediate cross-restricted, and high-cross-restricted TCCs according to the specific peptide. The circle size illustrates the number of TCCs specific for each peptide, and the color indicates the level of cross-restriction: white, restriction; light gray, low cross-restriction; dark gray, intermediate cross-restriction; gray, intermediate cross-restriction; and black, high cross-restriction. (C) Proliferative responses of five high cross-restricted, brain-derived, VP1-specific CD4+ TCCs (TCCs 6, 11, 12, 13, and 14) and five restricted (TCCs 1, 2, 3, 5, and 10) to 1 μM (left graph) and 0.1 μM (right graph) peptides presented by autologous EBV-transformed B cell lines. Graphs show the mean SI ± SEM. *p = 0.0317, **p = 0.0078. (D) Graph shows the percentage of high cross-restricted and restricted TCCs that were in vivo clonally expanded. (E) Proliferative responses of four in vivo-expanded TCCs (TCCs 7, 11, 12, and 13) and 10 not expanded (TCCs 1, 2, 3, 4, 5, 6, 8, 9, 10, and 14) to 0.1 μM peptide presented by autologous EBV-transformed B cell lines. Graphs show the mean SI ± SEM.
in the side-chain conformation of N57α (single letter code for amino acid is used), two important replacements, DRA1*01:01 A61α replaced by DQA1*01:02 R61α and DRA1*01:01 A68α replaced by DQA1*01:02 H68α, resulted in the introduction of two positive charges at TCR-contacting positions in DQA1*01:02.

With respect to the β-chains, four potential TCR-contacting residues are identical among the DRB1*15:01, DRB5*01:01, and DQB1*06:02 molecules, and two replacements in DQB1*06:02 resulted in conserved charge or minor change (52). The side chains of all these amino acids in the VP134–DQA1*01:02/DQB1*06:02 complex most likely confer a particular topography to this complex responsible for TCR bias. It has been reported that some peptide–MHC complexes induced TCR bias broadly shared between different individuals, which is referred to as “public” T cell repertoires (60). To determine whether the TCR bias induced by VP134–DQA1*01:02/DQB1*06:02 complex is private or public will require further analysis of additional PML patients sharing DQA1*01:02/DQB1*06:02 expression. As a final important point that underscores the biological relevance of peptide VP134, it is interesting to note that peptide VP134 contains the JCV epitope VP136 that is one of the two previously described CD8+ T cell epitopes in the context of HLA-A*02:01 (61). This notion is supported by our observation that the patient, from whom the above VP134-specific CD4+ TCCs had been isolated, also showed brain-infiltrating HLA-A*02:01/VP136 tetramer-positive CD8+ T cells in the brain during PML-IRIS (36).

HLA restriction of T cells in response to specific peptide stimulation is a fundamental concept in immunology. However, exceptional cases of TCCs that are able to recognize autoantigens but also peptides from foreign pathogens in the context of several restriction elements have been reported (51–56). In this study, we demonstrate that HLA cross-restriction is a feature of some brain-infiltrating, VP1-specific CD4+ T cells, as nine of the 14 TCCs generated in vitro were able to recognize the specific VP1 peptide in the context of more than one HLA class II molecule. For the three peptides for which more than one specific TCC was generated, it is interesting to note that these TCCs specific for a particular peptide showed the same pattern of restriction. The two TCCs specific for peptide VP1319 were both highly cross-restricted as well as the two TCCs specific for peptide VP1335. The five TCCs specific for peptide VP134 were all DQA1*01:02/DQB1*06:02 restricted; only TCC-4 showed in addition recognition with at least another autologous HLA class II molecule. The two TCCs specific for peptide VP1319 had different TCR β-chains but shared the same TRAV9-2β-01-04 chain, and despite expressions of different CDR3α regions, these regions shared 58% of the amino acid sequence. This similarity in the TRAV and CDR3α regions suggests an important contribution of the TCR α-chain in HLA class II–peptide complex
The five TCCs specific for peptide VP134 not only shared the TCR α- but also the β-chain and differed only in the CDR3 regions and associated J segments. In these five TCCs, the amino acid composition of the CDR3β was more heterogeneous than the composition of the CDR3α regions supporting an important role of CDR3α-chain in HLA-class II–peptide complex recognition. Furthermore, TCC-4, the only TCC able to use a second HLA class II molecule in addition to DQA1*01:02/DQB1*06:02, showed the highest variation in the CDR3α region. The structural basis of cross-restriction in the two TCCs specific to VP1335 peptide that did not share any TCR chain requires further investigation.

Autologous APCs express on their surface all autologous HLA class II molecules. Consequently, TCCs that are able to recognize peptides in the context of different HLA class II molecules could reach the activation threshold at lower peptide concentrations than TCCs restricted by a single HLA class II molecule, suggesting a putative physiological role of cross-restriction in facilitating T cell activation. In a previous study (52), we demonstrated for a cerebrospinal fluid-derived, in vivo-expanded TCC from an MS patient a correlation between the stimulatory potential of peptides and their ability to be presented on different restriction elements. Supporting these data and the above hypothesis, we found in the current study that highly cross-restricted, brain-infiltrating, VP1-specific CD4+ TCCs showed a higher avidity than TCCs restricted by a single HLA class II molecule. In addition, we observed that 60% of the highly cross-restricted TCCs were in vivo clonally expanded whereas none of the restricted TCCs was, suggesting that high cross-restriction can facilitate in vivo expansion of T cells by increasing avidity for HLA–peptide complexes. Cross-restriction appears to be an efficient strategy used by brain-infiltrating CD4+ TCCs described in this study to recognize JCV, as all five TCCs specific for three of the four immunodominant peptides, particularly of VP154, VP1319, and VP1335, were cross-restricted and four of them in vivo clonally expanded.

As already mentioned above, in our previous work we demonstrated that JCV-specific CD4+ T cells with Th1- but also Th1-2 functional phenotypes are crucial in JCV immunity (36). Simultaneous production of IFN-γ and IL-4 most likely induced a widespread expression of HLA class II molecules in the brain and a strong intrathecal Ab response against JCV. The stability of this Th1-2 functional phenotype was confirmed at the clonal level by intracellular cytokine staining, cytokine secretion, and transcription factor expression. In this study, we have analyzed the functional phenotype of the 14 brain-infiltrating, VP1-specific CD4+ TCCs and observed a comparable representation of both functional phenotypes. Although we do not know the context and signals that lead to this Th1-2 differentiation, it is conceivable that these cells were reprogrammed in the brain by a mechanism similar to that reported in an infection model with lymphocytic choriomeningitis virus (62). In agreement with a putative protective role of these Th1-2 CD4+ VP1-specific T cells, it is interesting to note that for the three VP1 peptides for which different specific TCCs were identified, always both Th functional phenotypes were present. Coexistence of Th1 and Th1-2 CD4+ T cells with identical specificity may represent another strategy used by brain-infiltrating, VP1-specific CD4+ T cells to generate an efficient JCV immune response.

In this study, the exceptional opportunity to get access to JCV-infected human brain during acute JCV infection allowed us for the
first time, to our knowledge, to analyze in detail brain-infiltrating, JCV-specific CD4+ T cells in humans. Our results demonstrate that to achieve a highly efficient JCV immune response, brain-infiltrating CD4+ TCCs use an unexpectedly broad spectrum of different strategies including TCR bias, cross-restriction that in-...other disorders caused by JC virus; in HLA-A2+ progressive multifocal leukoencephalopathy patients with a CTL response specific for a commonly recognized JC virus epitope. J. Immunol. 168: 399–404.


