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SHORT COMMUNICATION

Clonal expansions of CD8$^+$ T cells in latently HSV-1-infected human trigeminal ganglia

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Abstract Herpes simplex virus type 1 latency in trigeminal ganglia (TG) is accompanied by a chronic immune cell infiltration. The aim of this study was to analyse the T-cell receptor β-chain repertoire in latently HSV-1 infected human TG. Using complementarity-determining region 3 spectratyping, 74 expanded β-chain sequences were identified in five TG. No clone appeared in more than one subject. Similar clones were present in the right and the left TG of two subjects. This indicates that these T cells are primed in the periphery and recognise the same antigen in the TG of both sides.

Keywords HSV-1 · Latency · CD8$^+$ T cells · Human · Trigeminal ganglia

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Introduction

Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA virus, which, after productive infection of the mucosa, enters the local nerve endings and establishes lifelong latency in the sensory neurons of the trigeminal ganglia (TG) (Baringer and Swoveland 1973). The only prominent viral transcript during latency is the latency-associated transcript (LAT) (Stevens et al. 1987). Other viral transcripts are only expressed on a very low level during latency in human TG (Derfuss et al. 2007). Nevertheless, HSV-1 latency in human TG is accompanied by a prominent immune response (Theil et al. 2003). Most of the infiltrating immune cells are CD8$^+$ T cells which are key players in the control of viral infections.

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These $CD8^+$ T cells are believed to control the latency state of HSV-1 in an antigen-specific, T-cell receptor (TCR)-mediated and non-cytolytic manner as shown in the mouse model (Knickelbein et al. 2008). In mice, most TG infiltrating $CD8^+$ T cells are specific for HSV-1 with about 50% recognising a specific epitope on HSV-1 glycoprotein B (Khanna et al. 2003; St Leger et al. 2011). The specificity of T cells infiltrating human TG remains to be elucidated.

The TCR is a heterodimeric cell surface protein, consisting of one α and one β chain. Each of these chains is composed of a variable (V), a joining (J) and a constant (C) region. The β chain further contains a diversity (D) region in between the V and the J region. During rearrangement of the TCR α and β chains, random nucleotides are inserted or deleted by the nucleotide transferase at the V-(D)-J junctions, thereby generating a hypervariable region, termed complementarity-determining region 3 (CDR3). Recognition of antigenic peptides bound to major histocompatibility complex (MHC) molecules is mediated by three CDRs. Two CDRs are germline-encoded. The main contribution to recognition of the antigenic peptide, however, comes from the hypervariable, non-germline-encoded CDR3 loop. Because random nucleotides are inserted at the CDR3, CDR3 lengths of different T-cell clones may vary from zero to more than ten amino acids. CDR3 lengths of polyclonal T-cell populations follow a Gaussian distribution, whereas the outgrowth of particular clones is indicated by a preferred CDR3 length. CDR3 spectratyping is a PCR-based method that measures the length distributions of the β chains. It allows the analysis of TCR repertoire diversity and the identification of prominent clones (Pannetier et al. 1995). Here, we applied CDR3 spectratyping of TCR β chains to T-cell infiltrates in several human TG to analyse the local T-cell repertoire and to search for public T-cell clones, shared between individuals.

Results

Prominent T-cell infiltrates in latently HSV-1 infected human TG

To verify the correlation between HSV-1 latency and the infiltration of T cells into the TG, ganglia sections were stained for CD3 and the HSV-1 or VZV infection state was determined by nested PCR. Immunohistochemical staining of the human TG sections revealed higher numbers of infiltrating T cells in TG positive for HSV-1 by nested LAT RT-PCR. The two LAT negative cases showed significantly lower numbers of T cells in immunohistochemistry (Fig. 1a, b). On average, the number of $CD3^+$ T cells in latently HSV-1 infected TG was 39.54 cells per 0.1 mm^2, whereas uninfected TG only contained 11.64 cells per 0.1 mm^2 (Fig. 1c and Table 1). There was no statistically significant difference in the T-cell counts between VZV infected vs. uninfected TG ($p>0.05$ Mann–Whitney U test) (Fig. 1a–c).

Clonally expanded T-cell populations in latently infected human TG

TG of four subjects were analysed by CDR3 spectratyping (subjects 01 to 04). For two individuals, TG of both sides were assessed (subjects 01 and 03). These TG had well-preserved RNA, were positive for LAT and showed T-cell infiltrates. An HSV-1 non-infected TG from subject 04 was used as a control sample.

CDR3 spectratyping was carried out for each TG. We used 26 Vβ-specific primers combined with 13 Jβ-specific primers, which resulted in 338 PCR products per TG. PCR products were separated on a polyacrylamide gel to obtain the overall distribution of CDR3 lengths. Mono- or oligoclonal expansions are indicated by single peaks over a polyclonal Gaussian background (Fig. S1 in the Electronic supplementary material). CDR3 spectratyping revealed 22 single peaks from the right TG and 26 peaks from the left TG of subject 01. In the one TG analysed from subject 02, 20 peaks were found. In subject 03, 22 peaks from the right TG and 18 peaks from the left TG were identified. From the negative control, subject 04, 14 peaks were obtained. PCR products from all obtained peaks were sequenced. Only if readable sequences were acquired, clones were considered as expanded.

In all TG, several clonally expanded TCR β chains were detected. In total, we identified 74 β-chain sequences of clonal expansions in the latently infected ganglia and 10 expanded β chains in the negative control (Table S1 in the Electronic supplementary material). In subject 01, 34 out of 338 possible Vβ/Jβ combinations showed clonal expansions. For the other subjects 02 and 03, 15 and 25 out of 338 possible Vβ/Jβ combinations were clonally expanded. Table 2 shows identical or homologous amino acid sequences of TCR β chains that were found in the left and the right TG of subjects 01 and 03. The clones with homologous CDR3 sequences which were identified in both ganglia belonged to identical Vβ families. The CDR 1 and 2 regions are germline-encoded within the V gene segments of the β chain and mainly mediate contact to the MHC. TCRs featuring the same Vβ segment already share two of three CDRs and are therefore more homologous than TCRs with different Vβ segments. The CDR3, which is most relevant for peptide binding, is either identical or homologous in the clonally expanded TCRs listed in Table 2. The amino acids at positions 3 to 5 after the conserved cystein are considered to play a major role in recognition of the

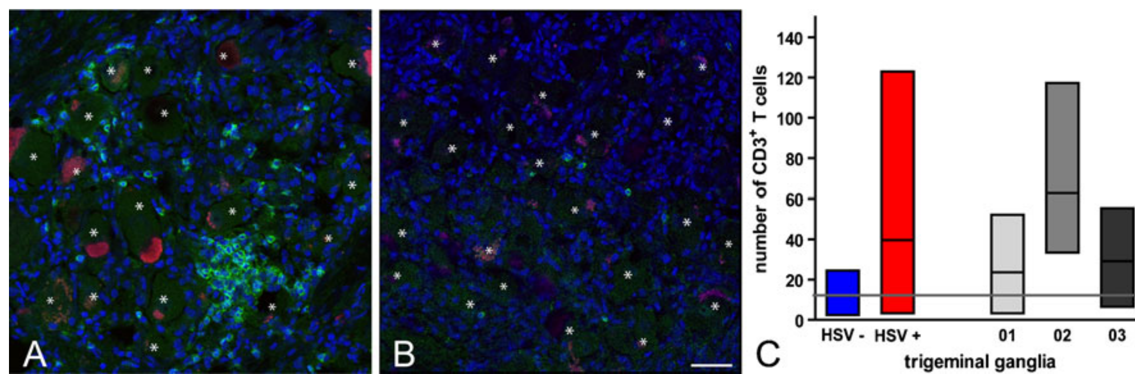

Fig. 1 Prominent T-cell infiltrates in latently HSV-1 infected human TG. **a**, **b** Representative micrographs of latently HSV-1 infected (**a**) and uninfected (**b**) human TG sections stained for CD3 (*green*). *Scale bar* 50 µm. The *red signal* is autofluorescence of lipofuscin. Neurons are indicated by *asterisks*. **c** Numbers of $CD3^+$ T cells per 0.1 mm^2 in non-infected (*blue*; $n=2$) vs. HSV-1 infected (*red*; $n=5$) TG. *Grey bars* show T-cell counts for those TG used for CDR3 spectratyping (01 to 03). *Bars* depict the range and mean of the T-cell numbers. The *grey line* indicates the mean T-cell count of HSV-1 negative TG

antigen presented by MHC class I (Rudolph et al. 2006). Therefore, TCRs with common motifs in these amino acids recognise similar antigens. Interestingly, in subject 03 for each of the two identical clones occurring in both TG, homologous clones sharing the same Vβ segment and amino acids with similar properties in their CDR3 were identified.

An inter-individual comparison of the TCR repertoires obtained from TG showed that no clone appeared in more than one subject. Hence, all clones were private to each subject. This difference between individuals reflects the heterogeneous HLA background, although subjects shared some HLA class I alleles (Table 1). However, in each of the two individuals where both TG were analysed, two matching clonally expanded TCR β chains were found on each side. These TCRs not only displayed identical amino acid sequences, but also identical nucleotide sequences. The absence of clones shared by other analysed subjects excludes the possibility that the identical clones in both TG were detected due to contaminations.

Localisation of clonally expanded TCRs in human TG

CDR3 spectratyping of RNA isolated from TG analyses all TCR molecules with no differentiation of $CD8^+$ or $CD4^+$ T cells. However, in latently HSV-1 infected TG $CD8^+$ T cells dominate the immune cell infiltrate (Theil et al. 2003). To morphologically identify expanded T-cell clones, sections of TG showing clonal expansions of T cells within the Vβ1 family were double-stained for Vβ1 and CD8. Clones featuring the Vβ1 chain were present quite frequently in the TG of subjects 02 and 03. Among the $CD8^+$ T-cell infiltrates surrounding neurons, several cells expressing the Vβ1 chain could be identified, whilst not all T cells surrounding one neuron expressed the same Vβ chain. Figure 2 shows representative micrographs of subjects 02 and 03 with clusters of $CD8^+$ T cells. Some of these T cells also express the Vβ1 chain. The vast majority of cells that stained positive for Vβ1 also stained positive for CD8. Moreover, all identified $V\beta1^+$ T cells were located adjacent to neurons.

Table 1 Overview of tissue samples used in the present study

Subject	Gender	Age	HSV-1	VZV	$CD3^+$T cells	HLA-A	HLA-B	HLA-C
01	m	56	+	–	23.6	0101	0801	0304
							1501	0701
02	m	41	+	+	62.7	0101	0801	0602
						0301	3701	0701
03	m	78	+	+	29.2	2402	0801	0701
						3001	4006	1502
04	m	62	–	+	12.5	nd.	nd.	nd.
05	m	36	–	+	10.7	nd.	nd.	nd.
06	f	17	+	+	76.9	nd.	nd.	nd.
07	f	61	+	–	24.0	nd.	nd.	nd.

The HSV-1 and VZV infection state plus the average T-cell count per 0.1 mm² are listed. The HLA class I alleles expressed by the three HSV-1 infected subjects used for CDR3 spectratyping are also stated

nd not done, *m* male, *f* female

Table 2 Similar and identical β chains in the right and left TG of subjects 01 and 03

Subject	side	Vβ	NDN			Jβ	FU
01	right	1	C A S	T L T G G A G	Y N E Q F F G P G	2.1	198
01	right	1	C A S S V	A V N	T D T Q Y F G P G	2.3	261
01	left	1	C A S S V	G G P	N Q P Q H F G D G	1.5	185
01	left	11	C A S S	L S R T G V	N Y G Y T F G S G	1.2	723
01	right	11	C A S S	L S R T G V	N Y G Y T F G S G	1.2	540
01	right	13.2	C A S	S P S Q G G H	Q P Q H F G D G	1.5	239
01	left	13.2	C A S	T W S G R S	Y G Y T F G S G	1.2	250
01	right	23	C A S S	L R Q	S Y E Q Y F G P G	2.7	303
01	left	23	C A S S	L R Q	S Y E Q Y F G P G	2.7	953
03	left	11	C A S S E	W V S G S	E Q Y F G P G	2.7	798
03	right	11	C A S S E	W V S G S	E Q Y F G P G	2.7	1138
03	left	11	C A S S E	Y W G T G	T G E L F F G E G	2.2	191
03	right	17	C A S S	P D R A G	G Y T F G S G	1.2	326
03	left	17	C A S S	P D R A G	G Y T F G S G	1.2	466
03	right	17	C A S S	P G H L	Y E Q Y F G P G	2.7	744

The amino acid sequence of the CDR3 is listed. Peak height is given in fluorescent units (FUs). CDR3 sharing amino acids with similar properties in positions 3 to 5 after the conserved cysteine are surrounded by *dashed lines* (i.e. subject 01 Vβ1 position 3: T and S are both amino acids with polar, neutral side chains, sharing similar hydropathy and L and V in position 2 both possess hydrophobic non-polar, neutral side chains). TCRs identical in their amino acid structure are surrounded by *double-lined boxes*

Discussion

We show here, that latently HSV-1 infected human TG show increased numbers of infiltrating T cells compared to HSV-1 uninfected TG. CDR3 spectratyping of the TCR β chain revealed several clonally expanded T-cell clones with certain β chains. These expansions were private to the assessed individuals, and no public T-cell responses could be identified, even though some HLA alleles were shared between individuals. However, it might be possible that particular TCR chains from expanded clones that were detected in one patient were present in other patients, but escaped detection because they were hidden in the polyclonal background. For several infections or autoimmune diseases as well as tumours (Dong et al. 2010; Junker et al. 2007; Schwab et al. 2009; Skulina et al. 2004; Pellkofer et al. 2009; Puisieux et al. 1994; Miles et al. 2011) biases in the T-cell receptor repertoire were identified. Some of these studies showed several T-cell clones appearing in different anatomical sites. A former study examining the TCR usage in HSV-2-specific $CD8^+$ T cells derived from blood showed a strong bias in the TCR repertoire with public TCR usage (Dong et al. 2010). Such pervasive clonal expansions are usually interpreted as being driven by sustained activation through a persisting antigen. One could speculate that lifelong exposure to HSV-1 antigens may shape the T-cell receptor repertoire in human TG. Infiltrating T cells in latently HSV-1-infected TG do not seem to exhibit public TCR usage. However, clones that had homologous or even identical amino acid sequences could be identified in both TG of two individuals. T cells with homologous TCRs are likely to respond to similar antigens. In subject 01 as well as subject 03, two clones with identical amino acid sequences were detected in both TG. These were even identical in their nucleotide sequence, which may suggest that these clones originated from the same T cell. This T cell probably encountered its respective antigen in the periphery, proliferated and afterwards migrated into both TG. HSV-1-specific T cells present

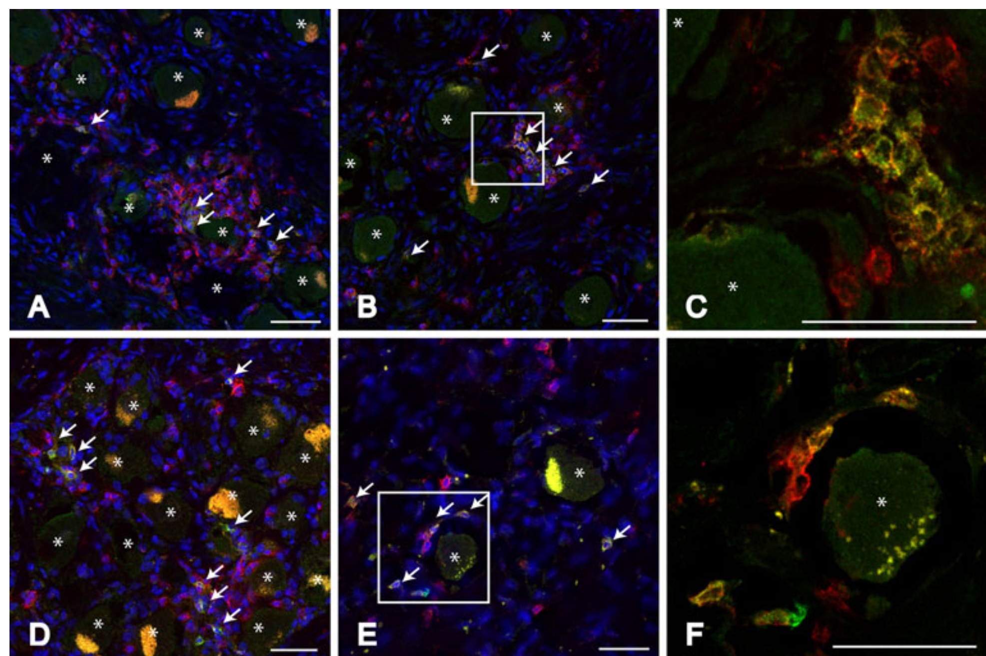

Fig. 2 Clusters of expanded T cells in human TG. Representative micrographs of human TG sections stained for CD8 (*red*) and Vβ1 (*green*) from S02 (**a**, **b**, **c**) and S03 (**d**, **e**, **f**). C and F are enlargements of **b** and **e**, respectively. $CD8^+$ $V\beta1^+$ T cells are indicated by *white arrows* in micrographs **a**, **b**, **d** and **e**. *Scale bars* 50 μm. The *yellow signal* within the neurons originates from lipofuscin. Neurons are indicated by *asterisks*

in human TG could be produced in the periphery during primary infection, may be also a reactivation event, and subsequently migrated to the latently infected tissue.

Staining for expanded Vβ chains revealed that the expanded T cells mostly belong to the $CD8^+$ subset, and that only some T cells in one cluster surrounding a neuron share the same Vβ chain. The presence of different clones in T-cell clusters around neurons could indicate that not all T cells react to the same antigen. Therefore, the TCR repertoire present in human TG seems to be more heterogeneous than in mice. This further suggests that some of the infiltrating T cells might represent unspecific bystander T cells, which were attracted by the inflammatory milieu, like already proposed by Verjans et al. in 2007. This phenomenon has also been seen in the mouse model (van Lint et al. 2005) where both specific and non-specific T cells persist in ganglia harbouring latent HSV-1.

Taken together, our data show that the TCR repertoire of infiltrating T cells in human TG is complex and differs between individuals. However, single identical T-cell clones can be found in both TG of the same individual, indicating presence of the same antigen in both TG. The fact that neuron surrounding T cells are clonally expanded adds further evidence to the significance of these immune cells in latency and reactivation.

Material and methods

The Ethics Committee of the Medical Faculty of the Ludwig Maximilian University of Munich approved the use of human TG autopsy samples. TG of both sides were removed from seven subjects 6 to 24 h after death. None of the subjects had any history of neurological disorders or an active orolabial herpes infection. Table 1 lists gender, age, HSV-1 infection state, T-cell counts and HLA-type of the TG used in this study. Ganglia were embedded directly after removal in Jung Tissue freezing medium (Leica Microsystems, Nussloch, Germany) and were stored at −80°C. Frozen sections of 10 μm were cut for immunohistochemistry and mounted on positively charged slides (Superfrost Plus, Menzel, Braunschweig, Germany). Slides were subsequently stored at −20°C. RNA and DNA were isolated from ten 30-μm sections.

RNA extraction and HSV-1/VZV PCRs

RNA was extracted using Qiazol (Qiagen, Hilden, Germany) and the miRNeasy Mini Kit (Qiagen). The quality of the isolated RNA was analysed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) combined with the Agilent RNA 6000p Kit (Agilent Technologies). Subsequently, DNA was extracted from the organic phase. After addition of back extraction buffer and phase separation by centrifugation, DNA present in the aqueous phase was precipitated by adding isopropanol. The DNA pellet was then washed three times with 75% ethanol and solubilised with water.

To assess the HSV-1 infection state of the TG, a nested LAT RT-PCR was done as described before (Derfuss et al. 2007). Furthermore, the VZV infection state was specified by nested PCR for the ORF 63 (Outer: 5′-CGCACTGGAATGT-GACGTAT, 3′-TCCCCGTCTCGATAACAATC; inner: 5′-TGAA-GACGATAGCGACGATG, 3′-CCCGTCTGGTTCACAAGAAT).

Immunohistochemistry

Immunohistochemistry was performed for CD3, CD8 and Vβ1. CD3 was detected using the polyclonal rabbit anti-human CD3 antibody (1:500; Dako Cytomation, Glostrup, Denmark) and visualised with a biotinylated goat anti-rabbit immunoglobulin antibody (1:100; Dako Cytomation), followed by Cy2-labelled streptavidin (1:100; Dianova, Hamburg, Germany) or HRP-conjugated streptavidin (1:100; Dako Cytomation) and incubation in DAB (Dako Cytomation). A dual staining for CD8 and Vβ1 was conducted using the LT8 mouse anti-human CD8α antibody (1:50; AbD Serotec, Düsseldorf, Germany) labelled with Cy3 (FluoroLinkTM MAb Cy3 labelling kit, Amersham Biosciences, Buckinghamshire, England) and the FITC-labelled Vβ1 BL37-2 antibody (1:25; Immunotech, Marseille, France). The Vβ1 signal was enhanced with an anti-FITC Alexa Fluor 488 labelled secondary antibody (1:100; Invitrogen, Karlsruhe, Germany). Antibodies to other Vβ families prominently expanded in the assessed TG were either not available or did not give reliable staining results. Stained sections were analysed by confocal imaging.

To obtain numbers of infiltrating $CD3^+$ T cells, five randomly selected fields of view (0.123 mm^2) were counted with an objective of ×20. Data were statistically analysed using Microsoft Excel 2003 and GraphPad Prism 5.

CDR3 spectratyping

The CDR3 lengths of TCR β chains were analysed by CDR3 spectratyping as described before (Junker et al. 2007; Schwab et al. 2009). In brief, cDNA was reverse-transcribed using a Cβ-specific primer and SuperScript II reverse transcriptase (Seitz et al. 2006). For increased sensitivity, a semi-nested TCR Vβ gene family-specific PCR with 25 Vβ-specific (Monteiro et al. 1996) and two different Cβ-specific primers (SpTy-β-out (Junker et al. 2007) and Cβ-reverse (Monteiro et al. 1996)) was carried out followed by a subsequent runoff reaction for every product using fluorescence-labelled Jβ-specific primers (Puisieux et al. 1994). The obtained PCR products were separated by electrophoresis according to their CDR3 length on an ABI377 DNA sequencer (Applied Biosystems, Darmstadt, Germany). Mono- or oligoclonal expansions appear as peaks of certain CDR3 length over a Gaussian background of polyclonal T cells (Fig. S1 in the Electronic supplementary material). Peak height is measured in fluorescent units (FU), the relative signal intensity. Higher numbers indicate more prominent clonal expansions. Peaks over 180 FU were considered as an actual aberration from the polyclonal background. Finally, Vβ-Jβ amplification products of expanded clones were reamplified and directly sequenced. Only clones for which we obtained a readable sequence were considered as clonally expanded.

The Vβ nomenclature described in Arden et al. (1995) is used throughout this manuscript. The subjects chosen for spectratyping were HLA typed by the Labor für Immungenetik und Molekulare Diagnostik, Munich, Germany.

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Conflict of interest The authors declare that they have no conflict of interest.

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