



T Cell Responses to Human Herpesvirus-8 (HHV-8) in Patients with Multicentric Castlemán's disease (MCD)

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ABSTRACT

Purpose: MCD is a devastating HHV-8 related lymphoproliferative disorder in HIV-infected patients. We aimed to evaluate the role of HHV-8 T cell responses in the pathophysiology of MCD.

Methods: Eleven HIV/HHV-8 co-infected asymptomatic carriers (AH) and 11 patients with MCD, all seropositive for HHV-8, were matched for CD4 cell count. T cell responses to HHV-8 were first screened in ELISpot IFN- γ assays using 56 peptides: predicted HLA-A2, 15-mer peptides overlapping by 10 AA covering K12 protein and exons 1 to 3 in K15, and 4 previously described epitopes in lytic glycoproteins GpH, Gp35/37 and GpB. HHV-8 specific T cells were then analysed if possible in 5-color flow cytometry using surface staining for CD45RA, CCR7, CD27 and CD8 molecules and intracellular staining for IFN- γ .

Results: T cell responses against HHV-8 could be detected in 6/11 (54.5%) MCD patients and in 7/11 (63.6%) AH patients in ELISpot IFN- γ assay. The median sum of HHV-8 specific T cells detected was equivalent in both groups: 110 (0-1420) and 125 (0-490) SFC/10⁶ PBMCs, respectively (p=0.662). HHV-8 specific T cell numbers were not correlated to CD4 cell counts (p=0.818, r=0.229), neither to HHV-8 viral load in PBMCs (p=0.396, z=0.848, non parametric Spearman test). Repertoire of T cell responses was not very different in both groups of patients and responses were observed against all protein tested: K12 (3 MCD and 4 AH responders), K15 (4 MCD and 1 AH), LANA (2 MCD and 0 AH), and lytic glycoproteins (3 MCD and 4 AH). Five/6 MCD and 6/7 AH responders in ELISpot were tested positively in cytometry after stimulation with appropriate peptides and IFN- γ intracellular staining. We found significantly more CCR7 CD27 CD45RA CD8⁺IFN- γ specific T cells in MCD than in AH patients (p=0.045, non parametric Mann-Whitney test). In opposite, the control of the disease is associated in AH patients with more CCR7 CD27 CD45RA CD8⁺IFN- γ specific T cells (p=0.092), and the differentiation phenotype of specific CD8⁺ T cells was similar to those observed for asymptomatic EBV chronic infection, suggesting the same CD8⁺ T cell profile is involved in the control of both EBV and HHV-8 chronic infection.

Conclusion: Patients with HHV-8 related MCD disclose equivalent frequencies of HHV-8 specific T cells but have significantly more terminal effectors CCR7 CD27 CD45RA CD8⁺IFN- γ T cells than AH patients. These results suggest that MCD is not related to a quantitative impairment but is associated with an accelerated differentiation of HHV-8 specific CD8⁺ T cell population.

OBJECTIVES

(¹) To analyse the role of T cell responses in HHV-8-related MCD. In this purpose, frequencies and differentiation phenotype of HHV-8-specific T cells were compared in patients with controlled chronic HHV-8 infection (chronically asymptomatic HHV-8-infected patients, co-infected with HIV), and in patients with HHV-8-related MCD.
(²) To perform for the first time a phenotypic analysis of HHV-8-specific CD8⁺T cells with regard to classic differentiation markers of CD8⁺ cells (CCR7, CD27, CD45RA), with comparison to EBV-specific T cells.

PATIENTS CHARACTERISTICS

TABLE I. Patients characteristics									
Code	MCD (n=11)				AH (n=11)				CD4 Status
	HHV-8	HHV-8	CD4	CD4	HHV-8	HHV-8	CD4	CD4	
	VL	VL	mm ³	VL	VL	mm ³	VL	mm ³	mm ³
Med	+	E	-	365	260	Prog	210	<10	279
Niv	+	E	-	<10	180	Prog	238	ND	273
lqo	+	E	ND	171	LTPN	9008	<10	258	
Ram	+	E	E	ND	92	Prog	212	<10	258
Neg	+	E	E	ND	34	Prog	204	<10	254
Fel	+	R	E	1068	492	LTPN	8021	11333	694
Ibm	+	R	-	<10	327	LTPN	12001	<10	488
Rap	+	R	-	837	305	Prog	180	<10	361
Uag	+	R	-	12770	2146	LTPN	2002	<10	1895
Eug	+	R	R	51	789	LTPN	11007	<10	709
Median				260					323

MCD, Multicentric Castlemán's disease; AH, Asymptomatic HIV-8 carrier.
HIV co-infected: HHV-8 VL, HHV-8 viral load (copies/5 x 10⁶ PBMCs); E, Evolution; R, Remission; KS, Kaposi's sarcoma; LTPN, Long-term non-progressor for HIV infection; Prog, Progressor for HIV infection; ND, Not Determined.

Eleven patients with Multicentric Castlemán's disease (MCD) were enrolled (Table I). Nine patients had HIV-related MCD, and 2 patients had classic MCD. Six had evolutive MCD, and 5 were in remission status. All patients had biopsy-proven MCD on peripheral lymph node analysis. Five patients had currently evolutive or past history of Kaposi's sarcoma. Median CD4 cell count in this group was 260/mm³.
Eleven HIV-8 infected patients asymptomatic for HHV-8 infection (AH) were matched to MCD patients for CD4 cell counts. Six patients were progressor for HIV infection (Prog) undergoing antiretroviral therapy, and without HHV-8-related disease (MCD, Kaposi's sarcoma, or Primary effusion lymphoma), and 5 patients were asymptomatic long term non-progressors for HIV infection (LTPN) without antiretroviral therapy. In this group, median CD4 T cell count was 323/mm³.

METHODS

ELISpot IFN- γ assays. Overall, 56 HHV-8 peptides were synthesized (Epytop, Nimes, France). 4 previously described HHV-8 T epitopes FLNWOQLNLV (GpH)-ORF22 (Gp35/37), ELTDLAFSGSY (Gp35/37-244) and LLYLVCPRCKRPPK (Gp35/37-245) on Gp35/37-ORF K12, and LMWYELSKI on GpB-ORF8 (Gp246); 15-mer peptides overlapping by 10 amino acids (aa) spanning K12 sequence (containing the LLNGWRWRLL peptide previously described), the first 3 exons of K15protein, and 9 9- and 10-mer peptides predicted to bind HLA-A2 in LANA-1 (Table 2). Twenty EBV optimal peptides ranging from 8 to 11mers on EBNA-3A, B, C, BMLF-1, LMP-2 and BZLF-1 proteins, previously described and matched different HLA molecules were tested in 2 pools. ELISpot IFN- γ assays were performed as previously described [Sun Y, J Immunol Methods, 2003;272:23-34]. Seven blood donors, HHV-8 negative, were tested in ELISpot assay and disclose no response. Positive threshold for patients was 50 SFC/10⁶ PBMCs, that is blood donors responses plus 2 standard deviation.

METHODS CONTINUED

TABLE 1. HHV-8 specific responses (n=11)		TABLE 2. HHV-8 specific responses (n=11)	
Peptide	Sum of spot forming cells (SFC) (n=11)	Peptide	Sum of spot forming cells (SFC) (n=11)
HLA-A2	110	FLNWOQLNLV	110
ELTDLAFSGSY	120	LLYLVCPRCKRPPK	120
LLYLVCPRCKRPPK	130	LMWYELSKI	130
...

Intracellular IFN- γ staining. One million of PBMCs were stimulated with relevant peptides or pool of peptides at 5 μ g/ml for 18 hours. PBMCs incubated with PHA or medium alone served as positive and negative control, respectively. Five μ g/ml BFA (Sigma-Aldrich) were added in the last 16 h. At the end of incubation, cells were harvested, washed and stained with anti-CD8-PCT, CD45RA-ECDC (Beckman-Coulter®), anti-CCR7-PE (R&D), and CD27-FITC (BD) for 15 min, fixed, permeabilised and stained internally with

APC-conjugated IFN- γ mAb (BD®). A total of at least 75,000 events in CD8 lymphocytes were measured for each analysis. Results of the staining for CD45RA, CCR7, and CD27 were analysed within the CD8⁺IFN- γ gated population. The background has been subtracted to all data. Results were considered as positive for IFN- γ when above 0.1% of CD8⁺ cells.

RESULTS

1) ELISpot IFN- γ assays

T cell responses against HHV-8 could be detected in 6/11 (54.5%) MCD patients (1 classic and 5 HIV-related MCD) and in 7/11 (43.6%) AH patients in ELISpot assay (Figure 1). The median sum of HHV-8 specific T cells detected was equivalent in both groups: 110 (0-1420) and 125 (0-490) SFC/10⁶ PBMC respectively (p=0.662, non parametric Mann-Whitney test). HHV-8 specific T cell numbers were not correlated to CD4 cell counts (p=0.8204, r=0.227) (Figure 2). Repertoire of T cell responses was not very different in both groups of patients and responses were mainly against K12 protein (4 MCD and 4 AH responders) and lytic glycoproteins (3 AH and 3 MCD), and in a less extent against LANA (1 AH and 2 MCD), and K15 (1 AH and 4 MCD) (Figure 3). These results suggest that MCD patient disclose equivalent frequencies of functional HHV-8-specific T cells than AH patient, with a similar repertoire of responses against latent and lytic viral proteins.

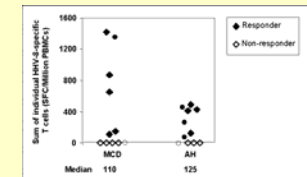


Figure 1. Sum of HHV-8 specific T cells in asymptomatic HHV-8 carriers (AH), and multicentric Castlemán's disease (MCD) patients, measured in ELISpot IFN- γ assay using 56 HHV-8-specific peptides.

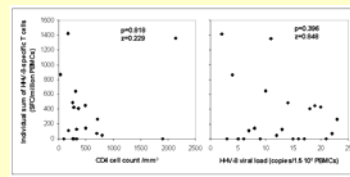


Figure 2. No correlation between CD4 cell counts and frequency of HHV-8-specific T cells in peripheral blood in 22 patients (Spearman non parametric test: p=0.818, z=0.229), nor between HHV-8 viral load in PBMCs and frequency of HHV-8-specific T cells in peripheral blood in 17 patients (p=0.396, z=0.848)

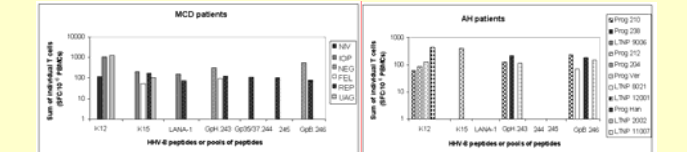


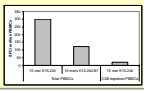
Figure 3. Repertoire of HHV-8-specific T cells in MCD and AH patients who disclose detectable HHV-8 T cell responses in ELISpot IFN- γ . Each color represent a patient, and identical colors have been used for CD4 matched-patients within groups. Only positive responses above 50 SFC/10⁶ PBMCs are depicted.

RESULTS CONTINUED

2) Identification of a new 10-mer B7 CD8 epitope in K15 protein

Peptides from pools of HHV-8 peptides inducing positive responses were tested separately in ELISpot assay, allowing the description of new epitopes in AH patients (Abstract A-162, CROI 2005). Here we report the identification of a 10-mer B7 CD8 epitope in K15 protein (Figure 5).

Figure 5. 15-mer peptide K15.240 induced significant positive responses with total PBMCs of classic MCD UAG Patient exhibiting A2 A30 / B7 B44 phenotype. 15-mer K15.240 sequence was tested in an HLA- linkage prediction software. A 10-mer K15.240 peptide (HLA B7) with a high binding score was synthesized and induced significant positive response with total PBMCs when tested. After CD8 depletion the response was lost asserting the CD8 phenotype of IFN- γ secreting cells.



3) Differentiation phenotype of HHV-8-specific CD8+ T cells

HHV-8 CD8⁺ T cell phenotypes could be analysed in 5/6 MCD ELISpot responders, and in 6/7 AH ELISpot responders with intracellular IFN- γ staining after stimulation with appropriate peptides (Table 3). We found significantly more CCR7 CD27 CD45RA CD8⁺IFN- γ HHV-8 specific T cells in MCD than in AH patients (p=0.045, Figures 6, 7). We also found more CCR7 CD27 CD45RA HHV-8 specific T cells in AH than in MCD patients, but the difference was not statistically different (median 4.1 and 1.6% respectively, p=0.092). These results suggest that HHV-8 specific CD8⁺ T cells in AH patients are less differentiated (more early and intermediate effector memory CCR7 CD27⁺ CD8⁺ T cells) than MCD patients who disclose higher percentages of terminal effector CCR7 CD27⁻ HHV-8-specific CD8⁺ T cells. This difference was not found for EBV-specific CD8⁺ T cells which disclose similar differentiation profile in AH and MCD patients (Figure 6). Besides, EBV-memory CD8⁺ T cells are mainly early and intermediate memory T cells in AH patients controlling the disease, as observed for during asymptomatic HHV-8 chronic infection (Figure 6).

Table 3. Frequencies of HHV-8-CD8⁺ specific T cells in AH and MCD patients measured by IFN- γ intracellular staining. All responders in ELISpot IFN- γ assay were analyzed by IFN- γ intracellular staining after stimulation with appropriate virus-specific peptides or pool of peptides. Results are expressed as percentage of CD8⁺ cells, and background (non stimulated cells) have been subtracted to all data. Only positive results are given and positive threshold was 0.1% of CD8⁺ cells.

Patient	Percentage of HHV-8 specific CD8 ⁺ T cells with IFN- γ intracellular staining	
	Terminals	Early + Intermediate
MCD patients		
CAS NEG	GpH 243-GpB 246	1.02
CAS POS	K15 240	0.20
CAS NEG	K15 240	0.11
CAS POS	LANA 250-K15 201-K12 202-K15 240	0.24
CAS POS	GpB 246	0.24
CAS POS	LANA 250-K15 201-K12 202-K15 240	0.24
CAS POS	LANA 250	0.01
AH patients		
ALT 12001	LANA 250	0.01
ALT 1001	GpH 243-GpB 246	0.08
ALT 11007	GpH 243-GpB 246	0.14
BM 212	K15 202-K15 240	0.14
BM 210	K15 202-GpH 243-GpB 246	0.09
BM 1000	GpH 246	0.19
BM 1000	GpB 246	0.14

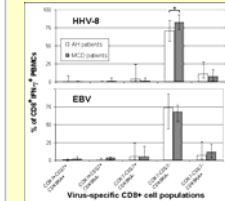


Figure 6. HHV-8 and EBV-specific CD8⁺ T cells differentiation phenotype after peptide stimulation measured by IFN- γ intracellular staining in MCD and AH patients. Six AH and 5 MCD patients were studied for HHV-8-specific CD8⁺ T cells, and 6 AH and 5 MCD patients were studied for EBV-specific CD8⁺ T cells. Phenotypes of virus-specific CD8⁺ T cells on CD8⁺IFN- γ gated population were classified according to classic models of CD8⁺ T cell differentiation [Appay et al., Sem. Immunol. 2004;16:205]. Histograms are median values in each group and limits are minimum and maximum individual values. Non-parametric Mann-Whitney test was used to compare percentages of different populations, and only significant differences are depicted (*p=0.045).

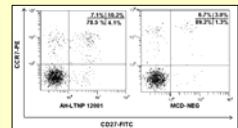


Figure 7. Characterization of HHV-8-specific CD8⁺ T cells in an AH and an MCD representative patient. Results were analyzed here for CCR7 and CD27 staining, on CD8⁺IFN- γ gated population. HHV-8-specific peptides used for stimulation were (K12 202 + LANA 250) in the AH patient, and (GpH 243 + GpB 246) in the MCD patient. There is a shift from CD27⁺ cells towards CD27⁻CD8⁺IFN- γ T cells in the MCD patient when compared to the AH patient, asserting that HHV-8-specific CD8⁺ T cells are more differentiated in the MCD patient.

CONCLUSION

HHV-8 and EBV-specific CD8⁺ T cells have the same differentiation phenotype within AH patients, with a great majority of terminal effector cells, suggesting that EBV and HHV-8 asymptomatic chronic infections are controlled by the same differentiated virus-specific CD8⁺ T cells. Patients with HHV-8 related multicentric Castlemán's disease (MCD) disclose equivalent frequencies of HHV-8 specific T cells but have significantly more terminal effectors CCR7⁻CD27⁻CD45RA-CD8⁺IFN- γ specific T cells than asymptomatic HHV-8 carriers (AH). These results suggest that MCD is not related to a quantitative impairment but is associated to an accelerated differentiation of HHV-8 specific CD8⁺ T cell population.