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Letter to the Editor

Circulating functional T cells specific to human herpes virus 6 (HHV6) antigens in individuals with chromosomally integrated HHV6

Dear Sir,

Strenger *et al.* [1] have recently reported that, in individuals with chromosomally integrated human herpes virus 6 (ciHHV6), a significant emergence of HHV6 (U54 protein) -specific immune responses (mainly interferon-γ (IFN-γ)-producing $CD8^+$ effector memory T lymphocytes) can be observed after short-term peripheral blood mononuclear cell expansion, namely 9-day culture with interleukin-2 (IL-2) and IL-7 proliferative cytokines, using U54-derived peptides as antigenic stimulation. We read with interest the authors' speculations, that this finding represents, first, a further (indirect) evidence of active viral protein expression in $ciHHV6^+$ individuals, and that, in turn, such increased T-cell cytotoxic responses against HHV6 antigen-bearing cells could be implied in autoimmune-like pathological features, speculatively associated with ciHHV6. Here we briefly report and discuss our immunological data observed in another series of individuals with or without ciHHV6, obtained by performing similar antigen-specific assays (for U54 and U90 HHV6 proteins), but using not-expanded peripheral blood mononuclear cell samples to directly investigate the frequencies of circulating HHV6-specific T cells.

After approval by the local ethics committee (protocol no. 1835-63/11) and having obtained informed consent, we collected and analysed one to five peripheral blood samples from 12 proven $ciHHV6^+$ subjects (described in Table 1) and from 27 ciHHV6-negative HHV6-seropositive healthy controls (total of 30 and 31 samples tested, respectively). We performed both IFN-γ-Elispot assay (Mabtech, Nacka Strand, Sweden) and flow cytometry-based cytokine secretion assays (CSA; Miltenyi Biotech, Bologna, Italy) for different cytokines (IFN-γ, tumour necrosis factor-α (TNF-α), IL-2, IL-10, each also with memory T-cell profiling for CD62L/CCR7 expression, as previously reported) [2,3], using uncultured peripheral blood mononuclear cells stimulated for 18 h (IFN-γ-Elispot) or 3 h (CSA) with HHV6-specific peptide pools (PepMix, JPT, Berlin, Germany), derived from either U54 (tegument protein) or U90 (immediate early protein). Both assays were performed according to the manufacturer's instructions and as previously described [2,3]. For all cases and controls, at least one blood sample was analysed by IFN-γ-Elispot and by CSA for all the cytokines mentioned above (except for TNF-α in the control group).

Main results of the immunological analyses are summarized in Table 2. In general, our data clearly show that HHV6-specific T lymphocytes are also readily measurable in the blood of $ciHHV6^+$ individuals, without *ex vivo* T-cell expansion. In particular, U54-specific IFN-γ^+ T cells were detectable at least in one sample from 11 out of 12 $ciHHV6^+$ subjects (92%). Moreover, in line with data reported by Strenger *et al.* [1], other U54-specific cytokine-producing T-cell responses were also detectable, although less frequently (TNF-α: five out of seven $ciHHV6^+$ cases available for analysis, 71%; IL-2: two of six, 33%; IL-10: four of six, 66%) and at lower magnitudes (Table 2).

However, according to both Elispot and CSA methods, the frequencies of circulating U54-specific IFN-γ^+ T cells were not significantly different between the $ciHHV6^+$ group and $ciHHV6^-$ controls. Also, with regard to U90-specific analyses, we detected positive samples in most $ciHHV6^+$ individuals (10/11, 91%), again without a significant increase in the magnitude of IFN-γ^+ responses, compared with $ciHHV6^-$ controls. Of note, towards both U54 and U90, median values of the specific IFN-γ-producing T cells tended to be even higher (about two- to three-fold) in the control group (Table 2). Interestingly, by comparison, in a series of $ciHHV6^-$ patients affected with HHV6-associated chronic/recurrent benign lymphadenopathy, we showed very high mean magnitudes of circulating IFN-γ^+ T lymphocytes specific to U54 (0.81%) and U90 (2.82%), and this is well consistent with the presence of an active immune process linked to HHV6 reactivation from typical latency [2].

Concerning IFN-γ^+ memory T-cell profiles, we also found that $CD62L^-$ effector memory $CD8^+$ T lymphocytes, specific to either U54 or U90, represented the prevalent circulating subsets in the $ciHHV6^+$ group. In particular, U90-specific IFN-γ^+ effector memory $CD8^+$ T cells constituted the only memory pool with a median magnitude (0.27%) higher than in the control group (0.11%) (p 0.393). Instead, in this latter group, $CD8^+/CD62L^+$ central memory appeared to be the prevalent phenotype of specific T-cell response. Indeed, in agreement with the study by Strenger *et al.* [1], it is conceivable that, upon antigen re-challenge, specific memory pools of circulating T cells with cytotoxic effector memory $CD8^+$ phenotype may be those able to promptly react and have a proliferative burst.

We also demonstrated the presence of circulating T cells able to secrete IL-10 after U54/U90 antigenic stimulation, so disclosing that HHV6-specific suppressive responses may be revealed, at lower magnitude, along with the cytotoxic ones.

Taken together, the findings by Strenger *et al.* [1] and by our group suggest that, in $ciHHV6^+$ individuals, HHV6-specific cytotoxic T-cell responses are significantly more reactive (i.e. more pronouncedly expandable) upon specific prolonged stimulation, whereas in basal conditions, these specific effector T cells are still

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Table 1
Main features of the enrolled individuals with proven chromosomally integrated human herpes virus 6 (ciHHV6)

ID no.	Age	Sex	Integrated virus	Site of integration	Clinical information	Family
1	40	M	ciHHV6B	17p ter	familial Mediterranean fever	A
2	10	F	ciHHV6B	17p ter	healthy, daughter of no. 1	A
3	5	F	ciHHV6B	17p ter	healthy, daughter of no. 1	A
4	67	F	ciHHV6B	17p ter	healthy, mother of no. 1	A
5	71	M	ciHHV6B	17p ter	healthy, father of no. 1	A
6	49	M	ciHHV6B	19p ter	multiple sclerosis	B
7	12	M	ciHHV6B	19p ter	healthy, son of no. 6	B
8	63	F	ciHHV6B	n.a.	non-Hodgkin's lymphoma	C
9	88	M	ciHHV6B	n.a.	Waldenström macroglobulinaemia	D
10	37	M	ciHHV6A	17p ter	retinitis pigmentosa	E
11	61	M	ciHHV6A	17p ter	healthy, father of no. 10	E
12	68	M	ciHHV6A	n.a.	myelodysplastic syndrome	F

In this study, we analysed 12 individuals (mean age 47 years, range 5–88 years, M/F 4/8) with proven ciHHV6 by positive HHV6 PCR on hair follicles, according to HHV6 Foundation recommendations (http://hhv-6foundation.org/clinicians/cihhv-6-testing), by using a commercially available diagnostic kit (Real Time Q-PCR, Nanogene, Milano, Italy) [2]. In addition, the site of integration was investigated using fluorescent *in situ* hybridization (the fragments ZVB70 and ZVH14 were used as probes) [4], and/or chromosome-specific PCR with primers derived from subtelomere regions of chromosomes 2p, 11q, 17p, 18q and HHV-6 left/right direct repeats [5]. As controls, we analysed 27 HHV6 seropositive individuals (mean age 40 years old, range 10–77 years, M/F 10/17), in which ciHHV6 was excluded by negative HHV6 PCR on hair follicles. Abbreviation: n.a., not available (chromosomal integration not found in 2p, 11q, 17p, 18q).

Table 2
Main immunological data on circulating cytokine-producing T cells specific to human herpes virus 6 (HHV6) antigens (either U54 or U90 proteins) in individuals with (ciHHV6$^+$) and without (ciHHV6$^-$) chromosomal integration of HHV6

		ciHHV6$^+$		Controls (ciHHV6$^-$)	
		U54 median (range)	U90 median (range)	U54 median (range)	U90 median (range)
Elispot					
IFN-γ SFCs/10^6 PBMCs		22 (8–242)	21 (8–60)	60 (10–1150)	55 (15–380)
Cytokine secretion assays					
IFN-γ$^+$ T cells (%)	CD4$^+$ and CD8$^+$	0.15 (0.02–1.42)	0.17 (0.11–0.63)	0.26 (0.20–0.48)	0.63 (0.28–1.23)
	CD4$^+$	0.07 (0.02–0.72)	0.12 (0.02–0.23)	0.12 (0.06–0.15)	0.26 (0.15–0.35)
	CD8$^+$	0.17 (0.03–0.36)	0.16 (0.09–0.40)	0.26 (0.10–0.33)	0.51 (0.36–0.88)
	CD4$^+$ EM	0.04 (0.01–0.11)	0.03 (0.01–0.05)	0.09 (0.04–0.14)	0.12 (0.06–0.20)
	CD4$^+$ CM	0.04 (0.01–0.71)	0.10 (0.03–0.18)	0.03 (0.01–0.10)	0.13 (0.08–0.19)
	CD8$^+$ EM	0.14 (0.02–0.45)	0.27 (0.01–0.39)	0.17 (0.01–0.21)	0.11 (0.06–0.26)
	CD8$^+$ CM	0.02 (0.01–0.25)	0.08 (0.01–0.15)	0.11 (0.01–0.28)	0.45 (0.25–0.62)
IL-2$^+$ T cells (%)	CD4$^+$ and CD8$^+$	0.02 (0.01–0.03)	0.03 (0.02–0.03)	0.04 (0.02–0.14)	0.12 (0.02–0.16)
TNF-α$^+$ T cells (%)	CD4$^+$ and CD8$^+$	0.18 (0.05–0.74)	0.18 (0.06–0.18)	n.d.	n.d.
IL-10$^+$ T cells (%)	CD4$^+$ and CD8$^+$	0.06 (0.03–0.06)	0.11 (0.04–0.19)	0.10 (0.04–0.24)	0.09 (0.06–0.17)

Median frequencies (and range) of responding T cells are separately reported for IFN-γ-Elispot assay and CSA assays for different cytokines. Elispot results are expressed as number of spot-forming cells (SFCs) out of 1 million of peripheral blood mononuclear cells (PBMCs). CSA results are expressed as percentages (%) of different functional subsets out of total T lymphocytes (CD4$^+$ and CD8$^+$), and out of total CD4$^+$ or total CD8$^+$ T cells. Statistical analyses were conducted using Mann–Whitney *U*-test; p values <0.05 were considered statistically significant. Comparisons of HHV6-specific T-cell frequencies between ciHHV6 positive and negative groups were not statistically significant (p >0.05) for all subsets. No patients received specific treatment during 3 months before the collections of blood samples used for immunological analyses. Abbreviations: n.d., not done; EM, effector memory (CD62L$^-$, CCR7$^-$); CM, central memory (CD62L$^+$, CCR7$^+$); IFN-γ, interferon-γ; IL-2, interleukin-2; TNF-α, tumour necrosis factor-α.

found, but at lower levels, similar to those observed in HHV6-seropositive subjects without viral integration. However, differences between these two studies could also be related to the use of different immunological methods (intracellular staining versus CSA and Elispot), as well as to variability between unrelated small cohorts. These data well support the notion that a complete immune tolerance toward HHV6, by means of classic thymic selection, is not occurring in ciHHV6$^+$ individuals. However, it is possible that, *in vivo*, some peripheral tolerance mechanisms, such as those mediated by regulatory T cell and T helper type 2 subsets, may act to maintain harmless levels of 'auto-reactive' cytotoxic T cells targeting HHV6-derived epitopes, which are potentially exposed ubiquitously in ciHHV6$^+$ individuals. We speculate that the regulatory arm of immunity could have a role in the prevention of excessive immune activations against HHV6 antigen-bearing cells, which, in turn, could be primarily involved in the development of various autoimmune events possibly described in ciHHV6$^+$ patients. Unfortunately, when trying to identify distinct clinico-immunological subgroups among ciHHV6$^+$ individuals enrolled in our study, no statistical analyses were feasible, due to the low number of cases.

In conclusion, it would be interesting to compare frequencies of HHV6-specific cytokine-producing T cells between ciHHV6$^+$ 'patients' (with pathological conditions that are possibly ciHHV6-related) and ciHHV6$^+$ healthy individuals (with no associated abnormalities), focusing on functional memory T-cell profiles reactive to different HHV6 antigens. Of course, larger cohorts of ciHHV6$^+$ individuals are required to address this issue, also considering that ciHHV6 patients typically display transient or subtle autoimmune-like features, which frequently remain unrecognized and for which the triggers remain unknown. Clinico-immunological monitoring should be set up to assess whether an enhanced cytotoxic immunity against HHV6 antigens is detectable in a significant concomitance with pathological manifestations associated with ciHHV6.

Transparency declaration

The authors declare no competing financial interests related to this manuscript.

Authorship contribution

PB, GR and DV conceived and designed the experiments; PB, DV, GR, CQ and RE performed the experiments; PB, GR, DV, IL and RE analysed the data; GR, PB and DV wrote the paper; VC, MMa, AP and EC provided well-characterized blood samples and related data; and FF, MMo, FN, RM, PC, DC, TT, LP and ML critically revised the manuscript:

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