

# No evidence for circulating HuD-specific CD8<sup>+</sup> T cells in patients with paraneoplastic neurological syndromes and Hu antibodies

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## Abstract

**Aim** In paraneoplastic neurological syndromes (PNS) associated with small cell lung cancer (SCLC) and Hu antibodies (Hu-PNS), Hu antigens expressed by the tumour hypothetically trigger an immune response that also reacts with Hu antigens in the nervous system, resulting in tumour suppression and neuronal damage. To gain more insight into the hypothesized CD8<sup>+</sup> T cell-mediated immune pathogenesis of

these syndromes, we searched for circulating HuD-specific CD8<sup>+</sup> T cells in a large cohort of Hu-PNS patients and controls.

**Patients and methods** Blood was tested from 43 Hu-PNS patients, 31 Hu antibody negative SCLC patients without PNS and 54 healthy controls. Peripheral blood mononuclear cells (PBMC) were stimulated with HuD protein-spanning peptide pools (15-mers) and individual HuD-derived peptides (9-mers) and analysed by cytokine flow cytometry and interferon- $\gamma$  ELISPOT-assays. Additionally, HuD-based Class I HLA multimers were used to visualize HuD-specific CD8<sup>+</sup> T cells. **Results** No HuD-specific CD8<sup>+</sup> T cells could be detected in the blood of Hu-PNS patients or controls.

**Conclusions** Our results do not support a role for HuD-specific CD8<sup>+</sup> T cells in Hu-PNS. Further studies should focus on the detection of circulating HuD-specific CD4<sup>+</sup> T cells and examine the antigen specificity of T cells in affected tissues.

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## Introduction

Paraneoplastic neurological syndromes (PNS) are considered as naturally occurring, successful anti-tumour immune responses in humans [6]. However, this tumour immunity goes along with autoaggression against the nervous system, resulting in severe neurological dysfunction [9]. The mechanisms responsible for the anti-tumour response and neuronal damage are poorly understood. Antigens expressed by the tumour that are normally restricted to neurons (so-called

onconeural antigens) hypothetically trigger an immune response that cross-reacts with the same antigens in the nervous system [7]. One of the most frequently involved tumours is small cell lung cancer (SCLC) and approximately 50% of patients with PNS and SCLC have high-titre Hu antibodies (Hu-PNS). Hu antibodies are directed against a family of neuron specific, mRNA binding proteins, of which HuD is the best-documented member. Consistent expression of HuD in all SCLC suggests that HuD plays a central role in triggering the immune response [15, 17].

High titres of Hu antibodies in serum and cerebrospinal fluid suggested a pathogenic role for these antibodies, that could, however, never be proven in animal models [3, 22]. Furthermore, expression of Hu antigens is exclusively intracellular, and it is therefore difficult to understand how such antibodies could target tumours or neurons [5]. In addition, pathological examination of PNS neuronal tissue demonstrated localized inflammatory cell infiltrates, containing B cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, in the proximity of overt neuronal cell damage [2, 13, 18, 25]. The presence of an oligoclonal CD8<sup>+</sup> T cell infiltrate in nervous tissues and tumours of Hu-PNS patients further suggests an immunopathogenic role for such cells [18, 25].

Some authors report the presence of HuD peptide-specific CD8<sup>+</sup> T cells in the blood of these patients, but also in that of apparently healthy controls (HC) [19, 20, 24]. In addition, the presence of circulating HuD-specific CD4<sup>+</sup> T cells has been suggested [1].

Here, we have investigated the presence of circulating HuD-specific T cells in a large cohort of Hu-PNS patients and controls. Despite a multifaceted approach mainly geared towards the detection of HuD-reactive CD8<sup>+</sup> T cells and, to a somewhat lesser extent, CD4<sup>+</sup> T cells, no such cells were detected in the blood of Hu-PNS patients and controls.

## Materials and methods

### Patients

Forty-three patients with high-titred Hu antibodies and a definite clinical diagnosis of PNS [12], 31 Hu antibody negative SCLC patients without neurological symptoms or signs (SCLC) and 54 apparently HC were tested. The Erasmus MC Institutional Review Board approved the study and all individuals provided written informed consent. The individuals' class I HLA alleles were typed by standard diagnostic PCR at the two-digit resolution level. Patient characteristics are shown in the Table 1. Anti-Hu IgG titres were

determined as described previously [21] and in Hu-PNS the dependence in activities of daily living was scored using the modified Rankin scale [21]. Twenty-five HC were male and 29 female, their median age was 46 years (range 17–89) and all were Hu antibody negative. No HC had received previous chemotherapy or immunosuppressive treatment and 30 HC (56%) were CMV-seropositive.

### Reagents

Ninety-three HuD protein-spanning synthetic peptides, 15-mers with 11 amino acids overlap, were pooled to constitute the HuD peptide mix (HuDmix) and smaller peptide pools [Jerini Peptide Technologies (JPT), Berlin, Germany]. For interferon- $\gamma$  enzyme-linked immunosorbent spot-forming (IFN- $\gamma$  ELISPOT) assays and the construction of HLA class-I multimers, HuD-derived 9- and 10-mers were selected based on previous studies [19, 20]. The phycoerythrin (PE)-labeled multimers and corresponding peptides used were: HLA-A\*0101-<sup>147</sup>ELEQLFSQY<sup>155</sup>, HLA-A\*0101-<sup>245</sup>RLDNLLNMA<sup>254</sup>, HLA-A\*0201-<sup>86</sup>SLGYGFVNYI<sup>95</sup>, HLA-A\*0201-<sup>248</sup>NLLNMA<sup>256</sup>YGV, HLA-A\*0201-<sup>315</sup>QLFGPFGAV<sup>323</sup>, HLA-A\*0201-<sup>362</sup>RLGDRVLQV<sup>370</sup>, and HLA-A\*2402-<sup>154</sup>QYGRIITSRI<sup>163</sup> (ProImmune, Oxford, UK). As positive and negative controls, HLA-A\*0201-<sup>495</sup>NLVPMTATV<sup>503</sup> [CMV phosphoprotein-65 (CMV-pp65); Beckman Coulter, San Diego, CA] and HLA-A\*0201 presenting an irrelevant peptide (ProImmune), respectively, were included.

To measure general T-cell responsiveness, we used phorbol myristate acetate (PMA) plus ionomycin, or phytohemagglutinin (PHA). A peptide pool containing 15-mers spanning CMV-pp65 (JPT) was used as positive- and negative antigen-specific control in CMV seropositive and seronegative individuals, respectively.

### Cytokine flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated within 12 h after venipuncture and stimulated in duplicate as described elsewhere [14]. Briefly,  $2 \times 10^6$  PBMC were incubated at 37°C in a CO<sub>2</sub> incubator for 18 h with 1  $\mu$ g/ml HuDmix, 1  $\mu$ g/ml CMV-pp65, 1  $\mu$ g/ml ionomycin plus 25 ng/ml PMA, or without antigen. After 2 h of stimulation, brefeldin A was added to one of the duplicate tubes allowing for intracellular accumulation of cytokines in activated T cells. Brefeldin A was not added to the second tube to allow detection of secreted cytokines in the supernatant. In some individuals, additional stimulation was

**Table 1** Patient characteristics at the time of study entry

	Hu-PNS	SCLC
N	43	31
Age (median, range)	64 (4–81) <sup>a</sup>	61 (40–83)
Gender (M/F)	15/28	21/10
Hu-Ab titre (median, range)	12,800 (400–204,800)	Negative
CMV serostatus (pos/neg)	29/14	17/14
Paraneoplastic neurological syndrome		NA
PSN	27	
PEM	5	
PCD	4	
PLE/ BE	3	
Pseudo-obstruction	2	
Motor neuron disease	2	
Tumour		
No tumour	8 <sup>b</sup>	0
SCLC	31	31
Limited	28	17
Extended	3	14
NSCLC	2	0
Prostate	1	0
Neuroblastoma	1	0
Prior treatment		
None	27	27
Chemotherapy ± immunosuppression	16	4
Neurological symptoms		NA
Interval onset symptoms (study entry)	5 months (2–15) <sup>c</sup>	
Interval onset symptoms (diagnosis)	4 months (1–12) <sup>c</sup>	
Progressive at study entry <sup>d</sup>	34 (79%)	
Modified Rankin score		NA
MRS = 2	7	
MRS = 3	22	
MRS = 4	10	
MRS = 5	4	

*Hu-Ab* Hu antibody, *CMV* cytomegalovirus, *pos* positive, *neg* negative, *NA* not applicable, *PSN* paraneoplastic sensory neuropathy, *PEM* paraneoplastic encephalomyelitis, *PCD* paraneoplastic cerebellar degeneration, *PLE* paraneoplastic limbic encephalitis, *BE* brainstem encephalitis, *SCLC* small cell lung cancer, *NSCLC* non-small cell lung cancer, *MRS* modified Rankin score

<sup>a</sup> One Hu-PNS patient was a 4-year-old boy with an underlying neuroblastoma. The remaining Hu-PNS patients were aged between 49 and 81 years

<sup>b</sup> No tumour mass visible on CT-scan or FDG-PET scan

<sup>c</sup> Median (ranges) of intervals are shown

<sup>d</sup> Progression of neurological symptoms was defined by the increase of at least one point on the modified Rankin scale during 2 months prior to study entry

performed in the presence of co-stimulatory monoclonal antibodies (mAb) directed against CD28 and CD49d (BD Biosciences, San Jose, CA). Stimulated PBMC

were stained and analysed using anti-CD3 conjugated with peridiny chlorophyllin (PerCP), anti-CD8 conjugated with allophycocyanin (APC), anti-interferon (IFN)- $\gamma$  conjugated with fluorescein isothiocyanate (FITC), anti-tumour necrosis factor (TNF)- $\alpha$  conjugated with PE, or appropriate isotype control mAb (all from BD Biosciences) [14]. CD4<sup>+</sup> T cells were defined as CD3<sup>+</sup>, 8<sup>-</sup>. Positive responses were defined by (1) percentage of cytokine-positive CD4<sup>+</sup> or CD8<sup>+</sup> T cells >2 times the negative control (i.e., no antigen) and (2)  $\geq 0.1\%$  of the total number of CD4<sup>+</sup> or CD8<sup>+</sup> T cells, each after subtraction of isotype control results.

#### Detection of secreted cytokines

The secretion levels of IFN- $\gamma$ , TNF- $\alpha$ , interleukin (IL)-2, IL-4, IL-5 and IL-10 were measured in supernatants using a cytometric bead array (BD Biosciences). Based on CMV data (not shown), a positive result was defined as cytokine concentration >2 times the background (no antigen) and a minimum level of 50 pg/ml.

#### IFN- $\gamma$ ELISPOT

PBMC ( $2 \times 10^5$ /well) were pre-stimulated in duplicate in 96-well plates with culture medium containing 3  $\mu$ g/ml HuD 9-mers, HuDmix, CMV-pp65, PHA, or no antigen for 1.5 h at 37°C and 5% CO<sub>2</sub> [10]. The PBMC were subsequently transferred to anti-IFN- $\gamma$ -coated ELISPOT plates (Nalge Nunc, Rochester, NY) for a further 18-h incubation. The ELISPOT assay was performed using standard protocols and automated reading (AELVIS GmbH, Hanover, Germany) [10]. The mean number of spot-forming cells (SFC) in duplicate wells was used as assay outcome. Positive results were defined by numbers of SFC/well >3 times background (no antigen) and a minimum of 15 SFC/well.

#### Detection of HuD-specific CD8<sup>+</sup> T cells using Class-I HLA multimers

Thawed PBMC were stained as described previously [11]. Following acquisition of  $1 \times 10^5$  viable (i.e., 7-amino-actinomycin-D [7AAD] negative) CD8<sup>+</sup> T cells, a positive result required a percentage of  $\geq 0.1\%$  of viable CD8<sup>+</sup> T cells binding the HuD multimer and a brightly staining HuD multimer-binding CD8<sup>+</sup> T-cell population that did not overlap with the dimly staining irrelevant multimer-binding T-cell population.

## Results

### Cytokine flow cytometry of PBMC after stimulation with HuD-derived peptides

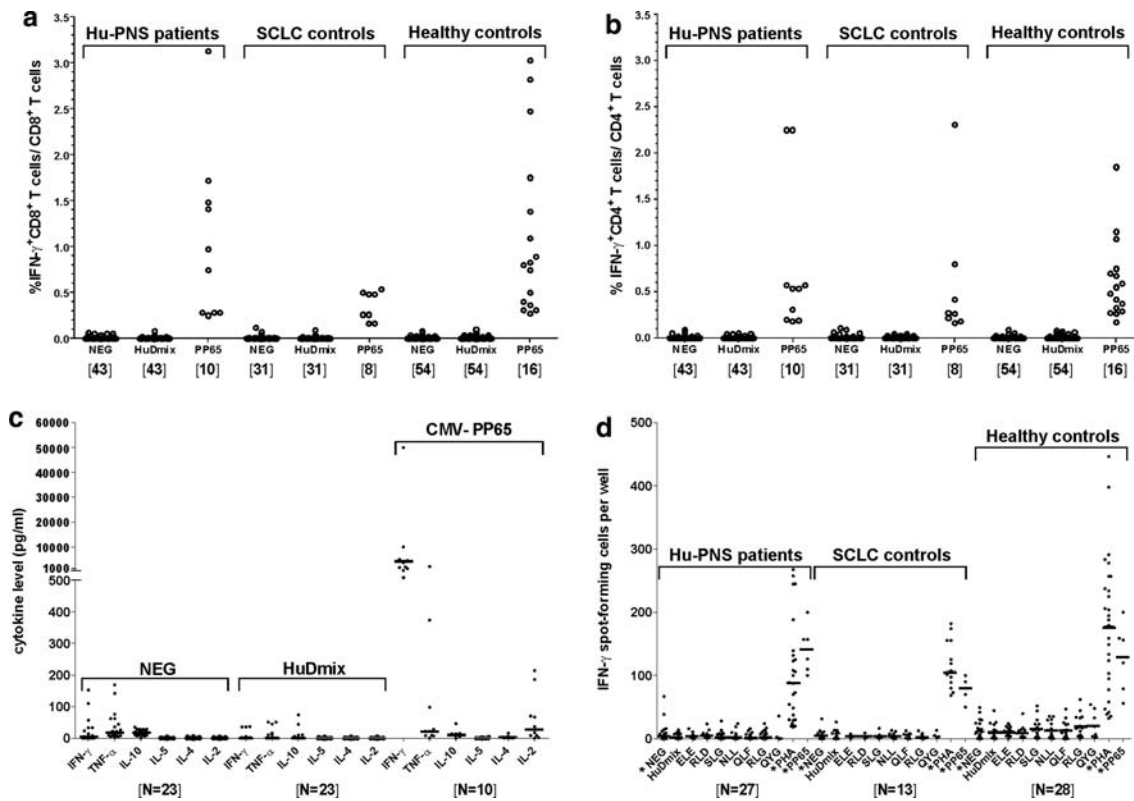
Stimulation with PMA and ionomycin induced IFN- $\gamma$  production in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells of all individuals (not shown). Whilst all CMV-seropositive individuals specifically responded to CMV-pp65, no HuD-specific T-cell reactivity was observed in any of the Hu-PNS patients, SCLC patients or HC (Fig. 1a, b). Similar results were obtained when intracellular TNF- $\alpha$  (not shown) or secreted cytokines (Fig. 1c) were measured. The use of co-stimulatory antibodies in combination with HuDmix did not result in the detection of HuD-specific T-cell reactivity (not shown).

### IFN- $\gamma$ -ELISPOT assay on PBMC stimulated with HuD-derived 9-mers

All individuals responded to PHA as determined by IFN- $\gamma$ -ELISPOT assay. In addition, all CMV-seropositive individuals responded to CMV-pp65. However, no T cell reactivity towards the previously described class-I HLA-binding HuD peptides [20] was detected in individuals with the appropriate HLA types in any of the study groups (Fig. 1d).

### Analysis of HuD-specific CD8<sup>+</sup> T cells using Class-I HLA multimers

Finally, we investigated the presence of HuD-specific CD8<sup>+</sup> T cells in PBMC using Class-I HLA multimers containing HuD-derived peptides. Whilst CMV-seropositive



**Fig. 1** Cytokine production in response to HuDmix and HLA-matched HuD 9-mers. Proportions of CD8<sup>+</sup> (panel a) and CD4<sup>+</sup> (panel b) T cells expressing intracellular IFN- $\gamma$  after stimulation with HuDmix in Hu-PNS patients, SCLC and healthy controls. Each dot represents the result observed in a single individual (panel c). After stimulation of PBMC with HuDmix and control antigens, the indicated cytokines were measured in assay supernatants. The results are shown for Hu-PNS patients only. Horizontal lines indicate median values of each group (panel d). ELISPOT assay showing the number of IFN- $\gamma$  SFC after stimulation of  $2 \times 10^5$  PBMC with HuDmix or HuD 9-mers. Each dot

represents the mean result of duplicates for each stimulus in each individual (panels a–d). Responses to CMV antigens are shown for CMV seropositive individuals only, as they were consistently negative in CMV seronegative individuals (not shown). The numbers of individuals tested are given in between brackets. NEG negative control (incubation without antigen), HuDmix HuD protein-spanning peptide pool, PP65 CMV pp-65 protein-spanning peptide pool, IL interleukin TNF tumour necrosis factor, IFN interferon, SFC spot-forming cell, PHA phytohemagglutinin. ELE, RLD, SLG, NLL, QLF, RLG and QYG designate individual HuD-based peptides

individuals with the appropriate HLA types showed distinct populations of pp65 multimer-binding CD8<sup>+</sup> T cells, no HuD-specific CD8<sup>+</sup> T cells were observed in individuals with the appropriate HLA types in any of the study groups (Fig. 2).

## Discussion

We set out to detect HuD-specific T cells in the blood of Hu-PNS patients as they are postulated to play a pivotal role in the immunopathology of this disease. Although we applied three different approaches we could not detect circulating HuD-specific T cells.

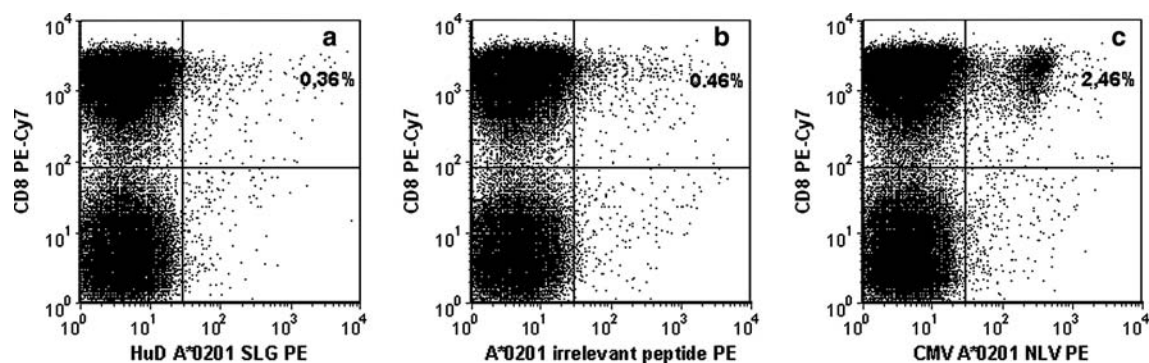
First, to induce cytokine responses in T cells, we used 15-mer protein-spanning peptide pools that have the advantage of covering the full protein sequence and of eliciting both CD8<sup>+</sup> and CD4<sup>+</sup> T-cell responses [14], as demonstrated by the CD8<sup>+</sup> and CD4<sup>+</sup> CMV-pp65-specific T-cell responses. However, no CD8<sup>+</sup> or CD4<sup>+</sup> HuD-specific T-cell responses were observed. These results are at variance with the detection of HuD-specific CD4<sup>+</sup> T-cell proliferative responses in PBMC of Hu-PNS patients by Benyahia et al. [1]. This discrepancy may be explained by differences in read-out (i.e., 3-day lymphocyte proliferation in Benyahia's study [1] vs. overnight cytokine production in ours) and the use of recombinant HuD protein [1] versus a protein-spanning 15-mer peptide pool (this manuscript).

We then studied responses to HLA class-I binding HuD peptides that were previously selected [20]. Using the same experimental setup and HuD 9-mer peptides, Rousseau et al. [20] reported HuD-specific T-cell reac-

tivity in 7/10 Hu-PNS patients and in 3/10 HC [20]. In that small study a positive response was defined as an experimental value  $\geq 2$  times above background. With that criterion, 3 PNS, 2 SCLC and 4 HC would have been classified as HuD T-cell responders in our study. However, using that cut-off we would also have detected T-cell reactivity in individuals whose Class-I HLA molecules did not have the appropriate binding motifs (data not shown). Therefore, we used more stringent cut-off levels resulting in a negative outcome. As most patients in both studies had progressive neurological disease and were tested shortly after start of symptoms and prior to therapy, differences between the study populations do not explain this discrepancy.

Finally, we could not detect HuD-specific circulating CD8<sup>+</sup> T cells using Class-I HLA multimers with the same fine specificities as defined by Rousseau et al. [20].

The absence of detectable circulating HuD-specific CD8<sup>+</sup> T cells may not be surprising. In a PCR-based study, Plonquet et al. [18] detected the same T-cell clone in neoplastic and nervous tissues, but not in blood. This finding suggests that T cells involved in the pathogenesis of Hu-PNS circulate in concentrations below detection level. An immune response taking place in the central nervous system parenchyma may deplete the circulating pool of CD8<sup>+</sup> T cells with that specificity [23]. Furthermore, vaccination studies in melanoma patients demonstrate that clinically effective anti-tumour immune responses may occur despite low levels of melanoma-specific cytotoxic T cells, i.e., below the detection limit of multimer-based assays [4, 16].



**Fig. 2** Analysis of HuD peptide-loaded, Class-I HLA multimer-binding CD8<sup>+</sup> T cells. PBMC from a CMV-seropositive Hu-PNS patient were stained with HLA-A\*0201 multimers loaded with the HuD peptide SLGYGFVNYI (*panel a*), an irrelevant peptide (*panel b*) or with the CMV-pp65 peptide NLVPMVATV (*panel c*). All data shown are obtained after selection of T cells (i.e., CD3<sup>+</sup>, low sideward scatter signals) [12]. Binding of Class-I HLA multimers (*panels a–c*, horizontal axes) was analysed in relation

to CD8 expression. In this example, the proportion of SLGYGFVNYI multimer-binding CD8<sup>+</sup> T cells (0.36%) was similar to that of irrelevant multimer-binding CD8<sup>+</sup> T cells (0.46%); binding resulted in low-intensity fluorescence signals only. In contrast, 2.46% of CD8<sup>+</sup> T cells bound the NLVPMVATV multimer resulting in high-intensity fluorescence signals from most CD8<sup>+</sup> T cells

In conclusion, we were unable to detect HuD-specific T cells in a large cohort of Hu-PNS patients and controls. However, two of our three assays were designed for the detection of CD8<sup>+</sup> T cells only. The IgG<sub>1</sub> isotype predominance of serum Hu antibodies in Hu-PNS indicates a T-helper response to the Hu antigen [13]. Therefore, further studies are warranted that focus on the detection of circulating HuD-specific CD4<sup>+</sup> T cells. In this context, regulatory CD4<sup>+</sup> T cells—which down regulate immune responses towards autoantigens and tumour-antigens—are of interest. Although the numbers of regulatory T cells are increased in cancer patients [26] and in PNS patients [8] (de Beukelaar et al. unpublished data) their (possibly impaired) function in PNS remains to be studied. Finally, examination of the antigen-specificity of T cells in affected tissues may shed further light on the role of HuD-specific T cells in the pathogenesis of Hu-PNS.

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