PARANEOPLASTIC NEUROLOGICAL SYNDROMES WITH ANTI-HU ANTIBODIES

Pathogenesis and treatment

Arjen de Jongste

The studies described in this thesis were performed at the departments of Neurology and Medical Oncology of the Erasmus University Medical Center (Rotterdam, The Netherlands) and the Inserm, U986, Diabetes & Autoimmunity Research Laboratory (Paris, France).

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Pathogenese en behandeling

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General introduction

Paraneoplastic neurological syndromes (PNS) are remote effects of cancer that are neither caused by invasion of the tumor or its metastasis, nor by infection, ischemia, metabolic and nutritional deficits, surgery or other forms of tumor treatment.[1] PNS cause severe neurological disability and carry a poor prognosis. In PNS patients, various antibodies (Abs) have been found directed at neuronal antigens that are also expressed by the associated tumors in most cases. In 2004, the term 'well characterized onconeural Ab' was defined based on (1) Abs for which there are recognisable patterns on routine immunohistochemistry and for which immunoblotting on recombinant proteins must be used to confirm their specificities; (2) the number of cases reported associated with tumours; (3) the description of well characterised neurological syndromes associated with the antibodies; (4) the unambiguous identification of the Abs among different studies, and (5) the frequency of these Abs in patients without cancer.[2] These 'well-characterized' onconeural Abs are by definition almost exclusively found in patients with cancer and include anti-Hu, Yo, CV2, Ri, Ma2 and amphiphysin.[2] Recently, the anti-Tr antigen has been identified as the transmembrane protein Delta/Notch-like epidermal growth factor-related receptor (DNER). Anti-DNER is thightly associated with Hodgkin lymphoma, and can therefore be considered a 'well-characterized' onconeural Ab, although DNER is not expressed by Hodgkin lymphoma.[3] Anti-Hu is the most frequent well characterized onconeural Ab and usually associated with paraneoplastic encephalomyelitis and sensory neuronopathy.[4] This thesis will focus on the immunopathogenesis of PNS with well-characterized onconeural Abs, in particular those with anti-Hu Abs (Hu-PNS).

IMMUNOPATHOGENESIS OF HU-PNS

Anti-Hu Abs are directed at a family of RNA-binding proteins (HuD, HuC, Hel-N1 and HuR).[5] Three of these proteins (HuD, HuC and Hel-N1) are exclusively expressed by neurons, and antibody titers against these three proteins are much higher than those against HuR indicating that the immunoreactivity against HuR represents cross-reactive antibody specificities directed against conserved sequences in the family.[6] In addition, only HuD and Hel-N1 are expressed by small cell lung cancer and small cell lung cancer cell lines.[5,7] The presence of high titers of anti-Hu Abs in the blood and cerebrospinal fluid of Hu-PNS patients led to the hypothesis that Hu-PNS are caused by an immune response triggered by Hu expression in tumor cells that is subsequently also directed at Hu-expressing neurons.[8] Additional evidence for this immune response is found in HLA-associations[9], intrathecal antibody production[10], cerebrospinal fluid (CSF) pleocytosis[11], and infiltrates of oligoclonal cytotoxic T cells around damaged neurons at autopsy.[12-15] Since immunization of animals against HuD

did result in high titer anti-Hu antibodies but no disease, it was hypothesized that the anti-Hu antibodies were not pathogenic.[16] Furthermore, these studies showed that anti-Hu antibodies did not enter neurons and bind the intracellular Hu-proteins, suggesting that the observed intraneuronal IgG accumulation in autopsy studies in humans may have resulted from a postmortem artefact.[16] Hence, it seems more likely that cytotoxic $CD8^+$ T cells are responsible for neuronal destruction in Hu-PNS, as supported by the $CD8^+$ T cell infiltrates around damaged neurons found at autopsy.[16,17]

DETECTION OF HUD-SPECIFIC T LYMPHOCYTES

The intracellular localization of HuD makes it inaccessible to autoantibodies, and therefore it is more likely that neuronal destruction in Hu-PNS is caused by HuD-specific cytotoxic T cells. Previous studies that aimed to detect circulating HuD-specific T cells showed different, often conflicting results.[18-25] First, it is uncertain whether or not HuD can elicit a specific T cell response. One study in healthy subjects and mice reported that the HuD protein can elicit a T cell response,[22] whereas another study in mice reported tolerance to the HuD protein.[23] Second, it is unclear which epitopes within the HuD protein are the targets of the hypothetical T cell response. Previously, we[24,25] could not confirm T cell responses to T cell epitopes that were identified by others.[18,22] More recently, Roberts et al.[21] reported T cell responses to the HuD-derived T cell epitopes Hu133 and Hu157 that have not been confirmed by others until now. Third, it is unclear which cytokines are produced by HuD-specific T cells. Previously, classical IFN-γ producing $CD8^+$ T cells were described,[18] whereas Roberts et al. also reported 'type 2' $CD8^+$ T cells that secreted robust amounts of the type 2 cytokines IL-4, IL-5 and IL-13.[21] The detection and further characterization of any HuD-specific T cells is potentially relevant to patients with Hu-PNS, since these cells could serve as a target for specific therapies. Detection of HuD-specific T cells could additionally be relevant to cancer patients. If we would be able to dissect the properties of the T cells that are involved in the effective antitumor immune response from the properties of T cells involved in the harmful antineuronal immune response, these insights could ultimately contribute to more effective anticancer immunotherapies.

CELLULAR COMPOSITION OF CEREBROSPINAL FLUID IN HU-PNS

In 93% of of Hu-PNS patients, signs of inflammation are present in the CSF including pleocytosis (38%), elevated protein levels (80%), or HuD-specific oligoclonal bands (59%).[10,26] Previously, we showed that CSF of Hu-PNS patients contained five times

higher numbers of $CD4^+$ T cells and $CD8^+$ T cells. The B cell numbers were 20 times higher than in non-cancer controls and cancer patients without PNS,[11] which is in line with the intrathecal antibody-production found in these patients.[10] In other neurological inflammatory diseases, it has been shown that lymphocytes in CSF might search for their specific antigen and migrate to the nervous tissue to fulfil their effector functions or return to the lymph nodes, depending on whether or not they have encountered their antigen.[27,28] Additionally, dendritic cells (DC) were shown to be present in CSF and might play a role by presententation of antigens from the CNS in the CSF, or transport these antigens further to the deep cervical lymph nodes.[29,30] Therefore, the CSF of Hu-PNS patients offers an opportunity to study the CNS inflammation early in the disease without the need for nervous tissue biopsies. Analysis of the cellular composition of the CSF may help to identify immune cells that are involved in disease pathogenesis.

TREATMENT OF HU-PNS

In patients with Hu-PNS, detection and treatment of the underlying tumor offers the best chance to stabilize their PNS.[31] However, even after tumor treatment, only 3-6% of Hu-PNS patients improve one point or more on the modified Rankin scale, while 30% deteriorate.[4,31] Median survival for Hu-PNS patients is 12 months, and 60% of the patients die from progression of their PNS, not of their tumor.[4] Hence, in addition to anti-tumor therapy, there is a clear need for immunotherapies to suppress the harmful auto-immune response in PNS and prevent further progression of neurological disability and death. There is evidence that SCLC patients with low titers of anti-Hu Abs without PNS have a better tumor prognosis[32] and spontaneous tumor regressions have been reported in Hu-PNS patients.[33] These findings indicate that the immune response against Hu may control tumor growth and that immunotherapy may potentially suppress this beneficial immune response against the associated tumor.[33] However, in a series of 51 Hu-PNS patients, no negative effect was seen of immunotherapy on the outcome of anti-tumor therapy.[31] Hence, the severity of PNS and the poor functional outcome after tumor treatment alone indicate that there is a need for effective immunotherapy in PNS.

SCOPE OF THIS THESIS

In this thesis, we aim to further unravel the immunopathogenesis of Hu-PNS in order to contribute to the development of an effective immunotherapy for Hu-PNS, and

probably other PNS with well characterized onconeural Abs. In **chapter 2**, we search for the presence of HuD-specific T lymphocytes, using techniques especially suited to detect rare antigen-specific T lymphocytes, and $CD8^+$ T lymphocytes with a 'type 2' phenotype. In **chapter 3 and 4**, we review the use of flow cytometry to study the cellular composition of CSF, and investigate the role of CMV carrier status on the distribution of T lymphocyte subsets in CSF. These chapters provide the knowledge needed for the study of T lymphocyte subsets, B lymphocyte subsets and dendritic cells in the CSF of Hu-PNS patients (**chapter 5**). In **chapter 6**, we report our efforts to further enhance the possibilities of CSF flow cytometry by investigating the usefulness of a novel CSF-stabilizing agent. In **chapter 7**, we review the available evidence on immunotherapies in Hu-PNS and other PNS with well characterized onconeural Abs. Subsequently, we report the results of a clinical trial in which we treat Hu-PNS patients with sirolimus, an inhibitor of activated T lymphocytes (**chapter 8**). The main findings of this thesis are summarized and discussed in **chapter 9**.

REFERENCES

1. de Beukelaar JW, Sillevis Smitt PA. Managing paraneoplastic neurological disorders. *The oncologist.* Mar 2006;11(3):292-305.
2. Graus F, Delattre JY, Antoine JC, et al. Recommended diagnostic criteria for paraneoplastic neurological syndromes. *J Neurol Neurosurg Psychiatry.* Aug 2004;75(8):1135-1140.
3. de Graaff E, Maat P, Hulsenboom E, et al. Identification of delta/notch-like epidermal growth factor-related receptor as the Tr antigen in paraneoplastic cerebellar degeneration. *Ann Neurol.* Jun 2012;71(6):815-824.
4. Graus F, Keime-Guibert F, Rene R, et al. Anti-Hu-associated paraneoplastic encephalomyelitis: analysis of 200 patients. *Brain.* Jun 2001;124(Pt 6):1138-1148.
5. Manley GT, Smitt PS, Dalmau J, Posner JB. Hu antigens: reactivity with Hu antibodies, tumor expression, and major immunogenic sites. *Ann Neurol.* Jul 1995;38(1):102-110.
6. King PH, Redden D, Palmgren JS, Nabors LB, Lennon VA. Hu antigen specificities of ANNA-I autoantibodies in paraneoplastic neurological disease. *Journal of autoimmunity.* Dec 1999;13(4):435-443.
7. King PH. Differential expression of the neuroendocrine genes Hel-N1 and HuD in small-cell lung carcinoma: evidence for down-regulation of HuD in the variant phenotype. *International journal of cancer.* Aug 22 1997;74(4):378-382.
8. Dalmau J, Furneaux HM, Cordon-Cardo C, Posner JB. The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues. *The American journal of pathology.* Oct 1992;141(4):881-886.
9. de Graaf MT, de Beukelaar JW, Haasnoot GW, et al. HLA-DQ2+ individuals are susceptible to Hu-Ab associated paraneoplastic neurological syndromes. *J Neuroimmunol.* Sep 14 2010;226(1-2):147-149.
10. Rauer S, Kaiser R. Demonstration of anti-HuD specific oligoclonal bands in the cerebrospinal fluid from patients with paraneoplastic neurological syndromes. Qualitative evidence of anti-HuD specific IgG-synthesis in the central nervous system. *J Neuroimmunol.* Nov 1 2000;111(1-2):241-244.
11. de Graaf M, de Beukelaar J, Bergsma J, et al. B and T cell imbalances in CSF of patients with Hu-antibody associated PNS. *J Neuroimmunol.* Mar 2008;195(1-2):164-170.
12. Voltz R, Dalmau J, Posner JB, Rosenfeld MR. T-cell receptor analysis in anti-Hu associated paraneoplastic encephalomyelitis. *Neurology.* Oct 1998;51(4):1146-1150.
13. Bernal F, Graus F, Pifarre A, Saiz A, Benyahia B, Ribalta T. Immunohistochemical analysis of anti-Hu-associated paraneoplastic encephalomyelitis. *Acta neuropathologica.* May 2002;103(5):509-515.
14. Wanschitz J, Hainfellner JA, Kristoferitsch W, Drlicek M, Budka H. Ganglionitis in paraneoplastic subacute sensory neuronopathy: a morphologic study. *Neurology.* Oct 1997;49(4):1156-1159.
15. Bien CG, Vincent A, Barnett MH, et al. Immunopathology of autoantibody-associated encephalitides: clues for pathogenesis. *Brain.* May 2012;135(Pt 5):1622-1638.
16. Sillevis Smitt PA, Manley GT, Posner JB. Immunization with the paraneoplastic encephalomyelitis antigen HuD does not cause neurologic disease in mice. *Neurology.* Oct 1995;45(10):1873-1878.
17. Roberts WK, Darnell RB. Neuroimmunology of the paraneoplastic neurological degenerations. *Curr Opin Immunol.* Oct 2004;16(5):616-622.

18. Rousseau A, Benyahia B, Dalmau J, et al. T cell response to Hu-D peptides in patients with anti-Hu syndrome. *J Neurooncol.* Feb 2005;71(3):231-236.

19. Benyahia B, Liblau R, Merle-Beral H, Tourani JM, Dalmau J, Delattre JY. Cell-mediated autoimmunity in paraneoplastic neurological syndromes with anti-Hu antibodies. *Ann Neurol.* Feb 1999;45(2):162-167.

20. Tanaka M, Maruyama Y, Sugie M, Motizuki H, Kamakura K, Tanaka K. Cytotoxic T cell activity against peptides of Hu protein in anti-Hu syndrome. *J Neurol Sci.* Sep 15 2002;201(1-2):9-12.

21. Roberts WK, Deluca IJ, Thomas A, et al. Patients with lung cancer and paraneoplastic Hu syndrome harbor HuD-specific type 2 CD8+ T cells. *J Clin Invest.* Jul 2009;119(7):2042-2051.

22. Plonquet A, Garcia-Pons F, Fernandez E, et al. Peptides derived from the onconeural HuD protein can elicit cytotoxic responses in HHD mouse and human. *J Neuroimmunol.* Sep 2003;142(1-2):93-100.

23. DeLuca I, Blachere NE, Santomasso B, Darnell RB. Tolerance to the neuron-specific paraneoplastic HuD antigen. *PLoS One.* 2009;4(6):e5739.

24. de Beukelaar JW, Verjans GM, van Norden Y, et al. No evidence for circulating HuD-specific CD8+ T cells in patients with paraneoplastic neurological syndromes and Hu antibodies. *Cancer Immunol Immunother.* Sep 2007;56(9):1501-1506.

25. de Beukelaar JW, Milikan JC, Verjans GM, et al. No evidence for the presence of HuD-specific T cells in the cerebrospinal fluid of patients with Hu-associated paraneoplastic neurological syndromes. *J Neurol.* Feb 2009;256(2):279-282.

26. Psimaras D, Carpentier AF, Rossi C. Cerebrospinal fluid study in paraneoplastic syndromes. *J Neurol Neurosurg Psychiatry.* Jan 2010;81(1):42-45.

27. Kivisakk P, Mahad DJ, Callahan MK, et al. Expression of CCR7 in multiple sclerosis: implications for CNS immunity. *Ann Neurol.* May 2004;55(5):627-638.

28. Giunti D, Borsellino G, Benelli R, et al. Phenotypic and functional analysis of T cells homing into the CSF of subjects with inflammatory diseases of the CNS. *Journal of leukocyte biology.* May 2003;73(5):584-590.

29. Pashenkov M, Huang YM, Kostulas V, Haglund M, Soderstrom M, Link H. Two subsets of dendritic cells are present in human cerebrospinal fluid. *Brain.* Mar 2001;124(Pt 3):480-492.

30. Hatterer E, Touret M, Belin MF, Honnorat J, Nataf S. Cerebrospinal fluid dendritic cells infiltrate the brain parenchyma and target the cervical lymph nodes under neuroinflammatory conditions. *PLoS One.* 2008;3(10):e3321.

31. Keime-Guibert F, Graus F, Broet P, et al. Clinical outcome of patients with anti-Hu-associated encephalomyelitis after treatment of the tumor. *Neurology.* Nov 10 1999;53(8):1719-1723.

32. Graus F, Dalmou J, Rene R, et al. Anti-Hu antibodies in patients with small-cell lung cancer: association with complete response to therapy and improved survival. *J Clin Oncol.* Aug 1997;15(8):2866-2872.

33. Darnell RB, DeAngelis LM. Regression of small-cell lung carcinoma in patients with paraneoplastic neuronal antibodies. *Lancet.* Jan 2 1993;341(8836):21-22.

Part I

Functional T cell analyses

2

Three sensitive assays do not provide evidence for circulating HuD-specific T cells in the blood of patients with paraneoplastic neurological syndromes with anti-Hu antibodies

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ABSTRACT

Anti-Hu antibody-associated paraneoplastic neurological syndromes (Hu-PNS) are severe and often precede the detection of a malignancy, usually small-cell lung cancer (SCLC). In Hu-PNS, it is hypothesized that neuronal cells are destroyed by T cells targeted against HuD, a protein expressed by small-cell lung cancer cells and neurons. There is only limited evidence for the existence of HuD-specific T cells. To detect these T cells in the blood of Hu-PNS patients we employed 3 highly sensitive assays that included T cell stimulation with dendritic cells (DCs) to specifically expand the number of any HuD-specific T cells. A total of 17 Hu-PNS patients were tested with 1 or more of the following 3 assays: (1) tetramer staining after stimulation of T cells with conventionally generated dendritic cells (cDCs) (n=9), (2) interleukin (IL)-13 enzyme-linked immunosorbent spot (ELISpot; n=3), IL-4 and IL-5 and interferon (IFN)-γ multiplex cytokine bead array (n=2) to assay cytokine production by T cells after stimulation with cDCs, and (3) IFN-γ ELISpot and tetramer staining after T cell stimulation with accelerated co-cultured DCs (acDCs, n=11). No circulating HuD-specific T cells were found. We suggest that either autoaggressive T cells in Hu-PNS are not targeted against HuD, or that their numbers in the blood are too low for detection by highly sensitive techniques.

INTRODUCTION

Anti-Hu antibody (Hu-Ab)-associated paraneoplastic neurological syndromes (Hu-PNS) are severe, have no effective treatment, and often precede the detection of a malignancy, usually small-cell lung cancer (SCLC)[1]. Hu-PNSs are thought to be caused by an immune response against the HuD protein that is normally exclusively expressed by neuronal cells and is aberrantly expressed by SCLC cells.[2] Although patients with Hu-PNS have high titers of autoantibodies against the HuD protein, there is no evidence that these autoantibodies directly cause neuronal damage.[3] The intracellular localization of HuD makes it inaccessible to autoantibodies, and therefore it is likely that neuronal destruction in Hu-PNS is caused by HuD-specific cytotoxic T cells. The hypothesis that these T cells cause neuronal damage is supported by autopsy studies that show the presence of cytotoxic T cells around neurons in the nervous tissue of Hu-PNS patients.[4,5]

Previous studies that aimed to detect circulating HuD-specific T cells showed different, often conflicting results.[6-13] First, it is uncertain whether the HuD protein is immunogenic. One study reported that the HuD protein can elicit a T cell response in healthy subjects and mice[10], whereas another study in mice demonstrated tolerance to the HuD protein.[11] Second, it is unclear which epitopes within the HuD protein are the targets of this hypothetical T cell response. Previously, we[12,13] could not confirm T cell responses to T cell epitopes that were identified by others.[6,10] More recently, Roberts et al.[9] described in 3 patients T cell responses to the HuD-derived T cell epitopes Hu133 and Hu157 that have not been confirmed by others until now. Third, it is unclear which cytokines are produced by HuD-specific T cells. Previously, classical $CD8^+$ T cells producing interferon (IFN)-γ were described,[6] whereas Roberts et al. also reported 'type 2' $CD8^+$ T cells that secreted robust amounts of the type 2 cytokines interleukin (IL)-4, IL-5 and IL-13.[9] The detection and further characterization of any HuD-specific T cells potentially could help develop specific therapies for Hu-PNS.

In this study, we aimed to confirm the presence of HuD-specific T cells in a relatively large group of 17 Hu-PNS patients. We used HuD-peptide loaded tetramer staining to detect $CD8^+$ T cells, a combination of IL-13 enzyme-linked immunosorbent spot (ELISpot) and a flow cytometric multiplex bead array to detect type 2 $CD8^+$ T cells; and IFN-γ ELISpot to test for classical 'type 1' cytotoxic T cells. All procedures included the use of dendritic cells (DCs) to specifically expand the number of any HuD-specific T cells and to gain maximal sensitivity.

MATERIALS AND METHODS

Sample Collection and Storage

Heparinized blood was drawn from 17 Hu-PNS patients who met the following criteria: high-titer anti-Hu-Abs, a definite diagnosis of PNS[14], and the presence of the human leukocyte antigen (HLA)-A*0201 and/or HLA-A*0301 restriction element. As procedural controls, three healthy subjects were tested who were cytomegalovirus (CMV) seropositive and HLA-A*0201 and/or HLA-A*0301 positive. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density-gradient centrifugation and cryopreserved in liquid nitrogen as described.[15] Absolute numbers of lymphocytes in the blood (expressed as 10^9 cells/L) and distribution of T cell subsets (expressed as % of lymphocytes) were determined by flow cytometry using a whole-blood stain, lyse, no-wash method based on counting beads (all patients except no. 7, 10 and 14), or with a hematology analyzer and a flow cytometric method that included washing steps (the remaining 3 patients)[16]. The local ethical review committee approved the study and written informed consent was obtained from all participants.

Proteins and Peptides

Recombinant HuD and Yo proteins were produced in *Escherichia Coli* and purified using metal affinity chromatography, essentially as described before.[3] Endotoxins were removed by Triton-X114 phase separation.[17] A HuD protein-spanning peptide mix (HuDm) that consisted of 93 15-mers, with 11 amino acids overlap and a CMV phosphoprotein-65 (pp65) protein-spanning 15-mers mix (pp65m) were obtained from Jerini Peptide Technologies (Berlin, Germany). The single 9-mers Hu133 (NLYVSGLPK) and Hu157 (RIITSRILV), selected based on the observations of Roberts et al.,[9] and NLVP-MVATV (NLV, a CMV pp65-derived peptide) were obtained from Pepscan (Lelystad, the Netherlands). Tetanus toxoid (TTX) was kindly provided by Dr. R. Rappuoli (Novartis Vaccines, Siena, Italy).

Conventionally Generated DCs (cDCs)

After thawing the PBMC, we isolated $CD14^+$ cells by magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured them in RPMI (Roswell Part Memorial Institute) with GlutaMAX (Invitrogen, Carlsbad, CA), supplemented with 1% L-Glutamine, 10% heat-inactivated human AB serum, 1% penicillin/streptomycin, 100U/ml IL-4 (R&D systems, Minneapolis, MN) and 100U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF; Immunotools, Friesoythe, Germany).[18] To induce DC maturation, 1µg/mL prostaglandin $(PG)E_2$ and 50 ng/mL tumor necrosis factor (TNF)-α were added after six days (R&D systems). After 2 additional days of culture (day

8), these conventionally generated DCs (cDCs) were used for in vitro stimulation of $CD8^+$ T cells.

In Vitro Stimulation of $CD8^+$ T Cells with cDCs

In parallel with the generation of cDCs, the $CD14^-$ T cell fraction was cultured for 8 days prior to stimulation using a feeder system, as described.[13] $CD8^+$ T cell were isolated from the $CD14^-$ fraction by magnetic separation (Miltenyi Biotec). Depending on the number available, cDCs were added to the $CD8^+$ T cells at ratios of 1:10 to 1:30. The $CD8^+$ T cells and cDCs were cultured in complete culture medium (RPMI1640 with 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 1% L-Glutamine, 10% human AB serum and 1% penicillin/streptomycin). Peptides (Hu133, Hu157 or NLV) were added at a final concentration of 10 µg/mL. One day after addition of cDCs and peptides, 10 IU/mL IL-2 (R&D systems) was added to the cultures.

In-vitro Stimulation by Accelerated Co-cultured DCs

Thawed PBMCs were incubated for 24-48 h with peptides or proteins together with DC-activating agents to induce DCs and stimulate T cells as described.[19,20] PBMCs were cultured in Adoptive Immunotherapy Media-Vero (AIM-V) medium (Invitrogen) with 1000U/mL of GM-CSF and 500U/mL of IL-4 (R&D systems). Proteins (Yo, HuD) or peptide mixes (HuDm, pp65m) were added at 10 µg/mL or 2 µg/mL respectively. After 24 h, we added DC maturation stimuli (2000 U/ml TNF-α, 20 ng/ml IL-1ß (R&D systems) and 2 µM PGE_2 (Merck Calbiochem, Darmstadt, Germany)), 1ng/mL IL-7 (R&D systems) and single peptides (Hu133, Hu 157 or NLV) at 10 µg/mL. After 48 h, nonadherent cells were collected, washed and used for IFN-γ ELISpot and tetramer staining.

Tetramer Staining

Up to $2{\cdot}10^6$ cells were stained with phycoerythrin(PE)-conjugated tetramers, anti-CD3 fluorescein isothiocyanate (FITC), anti-CD8 allophycocyanin (APC; Becton Dickinson, San Jose, CA) and 7-amino-actinomycin-D (7AAD; Sigma-Aldrich, St. Louis, MO) as described.[15] The tetramers Hu133 HLA-A*0301 and Hu157 HLA-A*0201that were selected based on the observations of Roberts et al.,[9] and NLV HLA-A*0201, were obtained from Beckman Coulter (Marseille, France). Irrelevant tetramers loaded with glycoprotein (GP)100-derived peptides or HIV-derived peptides were obtained from Beckman Coulter or provided by Dr. W.A.F. Marijt (Leiden University Medical Center, the Netherlands). Listmode data were acquired on a FACSCalibur or FACSCanto flow cytometer (Becton Dickinson). We gated on viable T cells ($7AAD^-$, $CD3^+$ cells with appropriate side and forward scatter properties). A positive response was defined as (1) a distinct population of $CD8^+$ tetramer-positive cells and (2) a higher percentage of $CD8^+$ tetramer-positive cells than irrelevant-tetramer-positive cells.

IFN-γ ELISpot

After stimulation with accelerated co-cultured (ac)DCs, PBMCs were assayed for 6 h as described previously.[19] Spots were counted with a Bioreader 3000 (BioSys, Karben, Germany). A positive response was defined as (1) a weak response (3-4 SD above the mean number of spot-forming cells (SFC) in wells without peptide) that could be reproduced in a second experiment or (2) an intermediate to strong response (>4 SD above the mean number of SFC in wells without peptide). Previously, these cut-off values were shown to yield a high sensitivity (86.4%) and specificity (90.9%) for detecting autoreactive T cells in type 1 diabetes.[21]

Tests for the Detection of Type 2 $CD8^+$ T cells

$CD8^+$ T cells that were stimulated with cDCs for 8 days were plated in triplicates of 100,000 cells/ 100µL/ well in polyvinylidene fluoride (PVDF) plates (Millipore, Billerica, MA) coated with anti-IL-13 Abs (Mabtech, Nacka Strand, Sweden). Subsequently, 25,000 peptide-pulsed T2 cells in 100µL per well were added and incubated for 20 h. Culture supernatants were collected and stored at -80°C. ELISpot plates were processed according to the manufacturer's instructions (Mabtech). Cytokine concentrations of culture supernatants were determined with the Th1/2 cytometric bead array kit from Becton Dickinson, according to the manufacturer's instructions. Since receiver-operator characteristics data are not available for these methods under these specific conditions, we used a more stringent definition of a positive response than for the IFN-γ ELISpot assay: (1) a number of SFC or cytokine level >2× background level in wells with T2 cells but without peptides; and (2) an increase in the number of SFC or cytokine levels after stimulation with peptide-pulsed cDCs.

RESULTS AND DISCUSSION

Seventeen Hu-PNS patients were included who tested positive for the HLA-A*0201 restriction element and/or the HLA-A*0301 restriction element (Table 1). The median age of the patients was 68 years (range, 46-77). In 14 patients an underlying tumor was detected, mostly SCLC (n=10). All but 3 patients (no. 2, 6 and 17) had progressive neurological symptoms in the 4 weeks prior to study entry, indicating ongoing neuronal destruction. Most patients had not received immunomodulatory or cancer treatment prior to blood withdrawal, and showed normal numbers of lymphocytes in the blood. Two patients who had received chemotherapy showed subnormal lymphocyte counts (nos. 3 and 6, Table 1).

Table 1. Patient characteristics

Hu-PNS patient	Age/ gender	Hu-Ab titer	PNS	Tumor	Therapy prior to blood withdrawal	Symptoms- study (months)	HLA-A	Peripheral blood lymphocytes			
								Total (10^9/L)	CD3+ (%)	CD3+, 4+ (%)	CD3+, 8+ (%)
1	76/F	3,200	EM	SCLC	None	5	0201	1,95	82	51	29
2	66/F	3,200	SN	NSCLC	Chemo+RT[A]	33	0201	1,90	73	55	14
3	75/M	3,200	SN	SCLC	Chemo+RT	5	0201, 0301	0,50	81	62	17
4	68/M	6,400	LE, CD	SCLC	Chemo	8	0201	1,12	80	46	27
5	61/F	12,800	EM	SCLC	Chemo	2	0301	1,36	77	64	11
6	46/M	6,400	EM	SCLC	Chemo	3	0301	0,65	62	47	14
7	72/F	12,800	SN	No	None	4	0201, 0301	1,80	54	42	12
8	74/F	800	CD	Lung[B]	None	2	0201	1,22	72	56	14
9	59/M	12,800	CD	No	None	6	0201	1,65	67	56	10
10	68/F	1,600	SN	SCLC	None	2	0201	1,16	48	28	20
11	66/M	25,600	SN	NSCLC	None	3	0301	1,55	73	52	19
12	61/M	6,400	CD	SCLC	None	4	0301	1,85	81	64	16
13	77/M	800	CD	Prostate[C]	None	8	0201	1,24	70	43	22
14	61/F	800	CD	No	None	6	0201	ND	55	35	20
15	73/F	6,400	SN	SCLC	None	2	0201	2,05	78	40	32
16	71/F	12,800	EM	SCLC	Steroids	1	0301	1,26	68	37	27
17	73/M	800	AN	SCLC	Chemo	9	0201	1,26	64	52	11

[A] Patient received chemotherapy 2 years prior to study entry, and was in complete remission.

[B] PET and CT scanning suspect for lung tumor, not pathologically confirmed.

[C] Prostate carcinoma 10 years prior to neurological symptoms.

Abbreviations: PNS: paraneoplastic neurological syndrome, F: female, M: male, EM: encephalomyelitis, SN: sensory neuronopathy, LE: limbic encephalitis, CD: cerebellar degeneration, AN: autonomic neuropathy, SCLC: small cell lung cancer, NSCLC: non-SCLC, No: no tumor found after tumor-workup, Chemo: chemotherapy, RT: radiotherapy, ND: not determined.

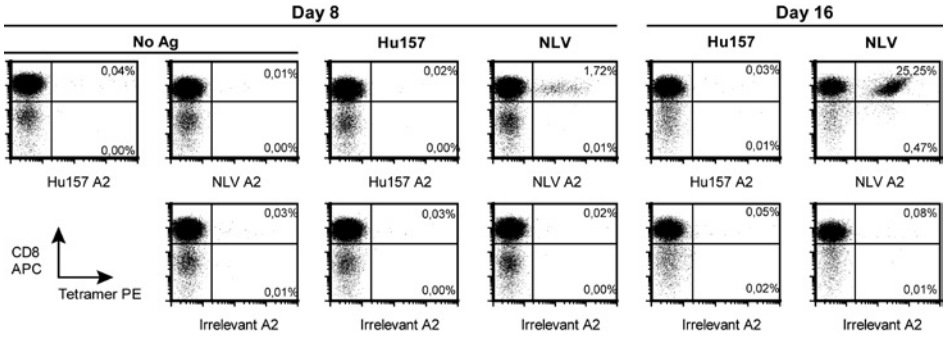

Figure 1. Tetramer staining of T cells after stimulation with cDCs
Tetramer staining of T cells from patient 1 after 1 cycle (day 8) and 2 cycles (day 16) of stimulation with cDCs. CD8-enriched T cells were stimulated with cDCs pulsed with Hu157, the CMV-pp65 derived peptide NLV, or no antigen (No Ag) as indicated in boldface, this indication applies to both rows of panels. Then, the stimulated T cells were stained with HLA-A*0201 tetramers loaded with Hu157, NLV, or irrelevant peptide as indicated by the x-axis labels of each panel. The percentage of $CD8^+$ NLV-tetramer-positive T cells (upper right quadrants) increased from 0.01% of the T cells (No Ag, day 8) to 25% of the T cells (NLV, day 16). The percentage of Hu157-tetramer-positive T cells remained similar to that of the negative control tetramer (Irrelevant A2).
Abbreviations: No Ag: no antigen added to cDCs, Hu157: cDCs pulsed with Hu157 peptide, NLV: cDCs pulsed with the CMV pp65-derived peptide NLV, Hu157 A2: HLA-A*0201 tetramer loaded with Hu157, NLV A2: HLA-A*0201 tetramer loaded with NLV, Irrelevant A2: tetramer loaded with irrelevant peptide.

First, we stimulated $CD8^+$ T cells of 9 Hu-PNS patients (nos. 1-9) with cDCs pulsed with the HuD-derived HLA-A*0301-binding peptide Hu133 or the HLA-A*0201-binding peptide Hu157 and used HLA-peptide tetramers to reproduce the results of Roberts et al.[9] Staining of $CD8^+$ T cells from Hu-PNS patient no. 1 with Hu157-loaded tetramers after 2 cycles of stimulation with peptide-pulsed cDCs did not show any Hu157-specific T cells, whilst stimulation with the CMV pp65-derived positive control peptide NLV resulted in a dramatic increase in NLV-specific T cells from 0.01% to 25% of the T cells (Figure 1). The other 8 patients were stimulated for 1 cycle prior to tetramer staining, and did not show any Hu133- or Hu157-specific T cells (data not shown). Since the median number of acquired $CD8^+$ T cells was 124,000 (range, 12,000-811,000) and a count of 100 tetramer-positive cells is needed for a reliable positive result,[22] we reached a median sensitivity of 0.08% (range, 0.01-0.85%) of stimulated $CD8^+$ T cells. Therefore, the sensitivity of our assay should have been sufficient to detect similar frequencies as reported by Roberts et al (i.e., 0.26-0.79%).

Since Roberts et al. also reported robust secretion of the type 2 cytokines IL-4, IL-5 and IL-13 in stimulated bulk $CD8^+$ T cells, we additionally tested for the presence of these 'type 2' $CD8^+$ T cells in three patients (no. 7-9). Figure 2 shows the results of an IL-13

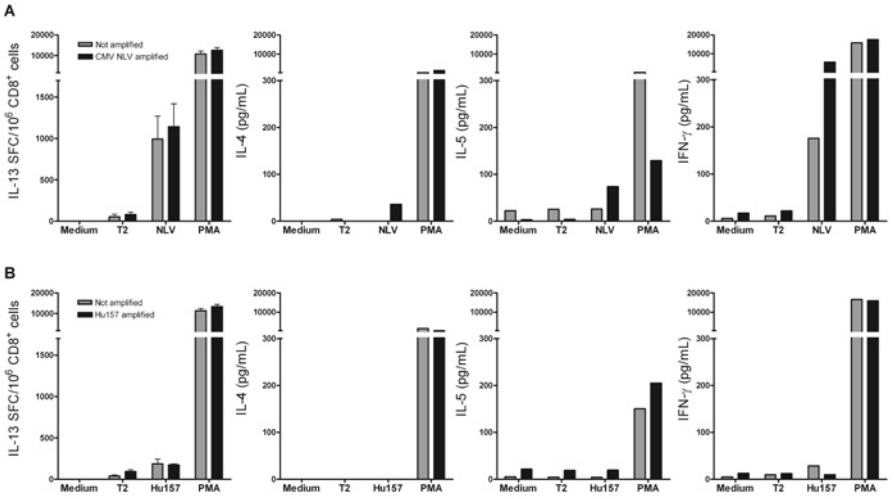

Figure 2. Secretion of type 2 cytokines and IFN-γ by $CD8^+$ T cells after stimulation with cDCs
$CD8^+$ T cells were tested for secretion of type 2 cytokines and IFN-γ in response to peptide-pulsed T2 cells after stimulation with (i) peptide-pulsed cDCs (amplified, black bars) or (ii) cDCs without peptides (not amplified, grey bars). Upper panels (A) show the results of T cells obtained from a CMV seropositive healthy donor, lower panels (B) show the results for Hu-PNS patient no. 7. $CD8^+$ T cells were tested in medium, and against T2 cells (T2), T2 cells pulsed with the CMV pp65-derived peptide NLV, T2 cells pulsed with the HuD-derived peptide Hu157, or T2 cells that were added simultaneously with PMA plus ionomycin (PMA). Panels on the left show the numbers of IL-13 SFC/10^6 $CD8^+$ T cells, the other panels show cytokine concentrations in culture supernatants (pg/mL) of IL-4, IL-5 and IFN-γ. The $CD8^+$ T cells of the CMV seropositive healthy donor (upper panels) secreted both, IL-13 and IFN-γ. $CD8^+$ T cells of Hu-PNS patient no. 7 (lower panels) did neither secrete the type 2 cytokines IL-4, IL-5 and IL-13, nor IFN- γ.

ELISpot and a IL-4, IL-5 and IFN-γ bead array after stimulation with cDCs in Hu-PNS patient no. 7 and a HLA-A*0201-positive CMV seropositive healthy control. $CD8^+$ T cells of patient no. 7 did not show IL-13, IL-4, IL-5 or IFN-γ secretion in response to the Hu157 peptide. $CD8^+$ T cells of the healthy control showed a significant increase in the number of IL-13 spot-forming cells (SFC) and IFN-γ concentration in culture supernatant in response to the NLV peptide. We additionally tested 2 Hu-PNS patients (patient 8 and 9) with IL-13 ELISpot and 1 of these patients (no. 9) also with the flow cytometric bead array and did not detect any type 2 HuD-specific T cells.

Because of these negative results, we decided to test 11 patients (no. 7-17) using an alternative approach based on methods that have been proven successful in detecting low numbers of circulating auto-reactive T cells in type 1 diabetes. This approach involved 48-h stimulation with acDCs and IFN-γ ELISpot with IL-7 co-stimulation. Advantages of these methods are reduction of culture time and number of manipula-

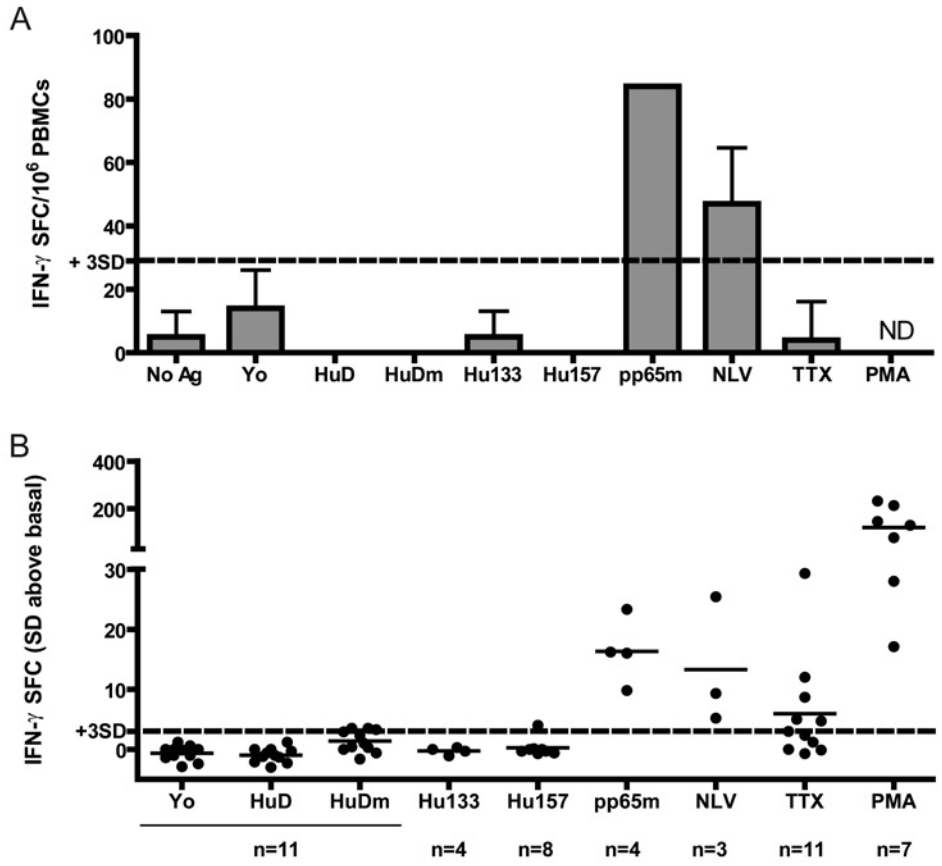

Figure 3. IFN-γ ELISpot of PBMCs after stimulation with acDCs
In panel A the numbers of IFN-γ SFC/10^6 PBMC are presented after stimulation with acDCs in Hu-PNS patient no. 8. Panel B shows the summary for all tested 11 patients as a relative value representing the mean number of SFC in wells with antigen minus the mean number of SFC in wells without antigen divided by the SD of wells without antigen. The cut-off value for a weakly-positive response (+3SD) is indicated by the dotted line. Responses to CMV antigens are shown for CMV seropositive patients only. Responses to peptides that are predicted to bind HLA-A*0201 or HLA-A*0301 molecules are shown for individuals with the corresponding HLA-A phenotype only. Stimulation with positive control peptides (pp65m and NLV) and PMA with ionomycin (PMA) resulted in significantly higher number of IFN- γ spot-forming cells (>4SD) in CMV seropositive patients and other patients. Stimulation with HuD, or HuD-derived peptides (HuDm, Hu133 and Hu157) did result in either no respons (<3 SD), or weak responses (3-4SD) that could not be reproduced.
Abbreviations: No Ag: no antigen, Yo: Yo protein, HuD: HuD protein, HuDm: HuD 15-mers mix, Hu133: Hu133 peptide, Hu157: Hu157 peptide, pp65m: CMV-derived pp65 15-mers mix, NLV: CMV-derived NLV peptide, PMA: phorbol myristate acetate plus ionomycin, TTX: tetanus toxoid, ND: not determined.

tions, which may therefore offer a better representation of the in vivo situation and a high sensitivity due to stimulation with acDCs, reduction of T cell background activity by using serum-free culture media and an increase in the amplitude of the antigen-specific response by IL-7 co-stimulation.[19] In order to test for other HuD-epitopes than Hu133 and Hu157, we also tested against the entire HuD protein and a HuD protein-spanning 15-mers mix (HuDm). Figure 3 shows the results of the IFN-γ ELISpot assays after stimulation with acDCs in 11 Hu-PNS patients. Testing against the HuD protein, HuDm, Hu133 and Hu157 showed either negative results (<3 SD above background) or weak responses (3-4 SD above background) that could not be reproduced and were therefore considered negative. All 4 CMV-seropositive patients tested positive against the CMV-pp65 peptide mix (pp65m). Three of them were HLA-A*0201 and responded to the CMV pp65-derived HLA-A*0201-binding peptide NLV. In addition, we performed Hu133 and Hu157-loaded tetramer staining ex-vivo, and after 12 days of stimulation with acDCs. We acquired a median number of 147,000 (range, 15,000-645,000) T cells ex-vivo and 9,000 (range, 1,000-166,000) T cells after acDC stimulation, corresponding to median sensitivities of 0.07% (range, 0.02-0.67%) and 1.14% (range, 0.06-9.58%) of T cells respectively; no HuD-specific T cells were detected.

There are several possible explanations for our negative results, and the discrepancies between our results and those of Roberts et al. First, methodological differences could have been responsible. We tried to limit these differences by using exactly the same protocols for stimulation of $CD8^+$ T cells with cDCs and using the same reagents for tetramer staining, IL-13 ELISpot and the multiplex bead array. However, Roberts et al. used separate vials for separated cryopreservation of monocytes and T cells, while we used single vials with PBMCs. Hence, we had to keep the T cells in culture for 8 additional days during the generation of cDCs. Theoretically, the numbers of HuD-specific T cells may have decreased during this period. However, we also used stimulation by acDCs as an alternative approach to reduce culture time and the number of manipulations, and this approach did not result in detectable levels of any HuD-specific T cells.

Second, differences in patient characteristics between the two studies may exist. Theoretically, a delay in our patient inclusion could have resulted in more chronically ill patients with stable symptoms in whom certain auto-aggressive T cells may have disappeared. Of note, Roberts et al. could only detect Hu133-specific T cells in a single patient with progressive symptoms for 5 months, and not in two chronically ill patients who had symptoms for more than 10 months. However, since 14 of our 17 patients in this study had progressive neurological symptoms, and 13 of the 17 patients were included not later than 6 months after onset of their neurological symptoms,

we consider it unlikely that a delay in patient inclusion could have accounted for our negative results.

Third, the number of circulating HuD-specific T cells in our study may have been too low for detection with even the most sensitive techniques. HuD-specific T cells may be preferentially found in or around target tissues such as CNS, dorsal roots or SCLC rather than in peripheral blood.

Fourth, our negative results suggest that HuD itself might not be the target of the hypothetical auto-aggressive T cells in Hu-PNS. Although the high expression of HuD in both neurons and SCLC cells makes HuD an attractive target of the auto-aggressive T cells in Hu-PNS, other proteins may be involved. For example, another Hu-protein, HuB is also expressed by neurons and SCLC cells.[23] Even proteins that do not belong to the family of Hu proteins may be involved, despite the presence of high titers of Hu-Ab in our patients. In celiac disease, for example, a B cell response targets the enzyme tissue transglutaminase itself, while T cell responses are target at the products of this enzyme.[24]

Finally, the high titers of immunoglobulin G_1 anti-Hu-Abs in patients with Hu-PNS suggest the help of $CD4^+$ T cells. In this paper, we focused on $CD8^+$ T cells because these cells are believed to directly cause neuronal damage by the release of cytotoxins (classical $CD8^+$ T cells) and because HuD-specific type 2 $CD8^+$ T cells had been reported in the literature.[9] Although we could also have detected $CD4^+$ T cells with the third assay, further research is needed to specifically address the role of $CD4^+$ T cells in Hu-PNS.

In summary, we propose either that auto-aggressive $CD8^+$ T cells in Hu-PNS do not target HuD, but rather other antigens (e.g. HuB), or that these cells are extremely rare in the blood, which makes their detection not amenable to clinical application.

ACKNOWLEDGEMENTS

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REFERENCES

1. de Beukelaar JW, Sillevis Smitt PA. Managing paraneoplastic neurological disorders. *The oncologist.* Mar 2006;11(3):292-305.
2. Roberts WK, Darnell RB. Neuroimmunology of the paraneoplastic neurological degenerations. *Curr Opin Immunol.* Oct 2004;16(5):616-622.
3. Sillevis Smitt PA, Manley GT, Posner JB. Immunization with the paraneoplastic encephalomyelitis antigen HuD does not cause neurologic disease in mice. *Neurology.* Oct 1995;45(10):1873-1878.
4. Bernal F, Graus F, Pifarre A, Saiz A, Benyahia B, Ribalta T. Immunohistochemical analysis of anti-Hu-associated paraneoplastic encephalomyelitis. *Acta neuropathologica.* May 2002;103(5):509-515.
5. Wanschitz J, Hainfellner JA, Kristoferitsch W, Drlicek M, Budka H. Ganglionitis in paraneoplastic subacute sensory neuronopathy: a morphologic study. *Neurology.* Oct 1997;49(4):1156-1159.
6. Rousseau A, Benyahia B, Dalmau J, et al. T cell response to Hu-D peptides in patients with anti-Hu syndrome. *J Neurooncol.* Feb 2005;71(3):231-236.
7. Benyahia B, Liblau R, Merle-Beral H, Tourani JM, Dalmau J, Delattre JY. Cell-mediated autoimmunity in paraneoplastic neurological syndromes with anti-Hu antibodies. *Ann Neurol.* Feb 1999;45(2):162-167.
8. Tanaka M, Maruyama Y, Sugie M, Motizuki H, Kamakura K, Tanaka K. Cytotoxic T cell activity against peptides of Hu protein in anti-Hu syndrome. *J Neurol Sci.* Sep 15 2002;201(1-2):9-12.
9. Roberts WK, Deluca IJ, Thomas A, et al. Patients with lung cancer and paraneoplastic Hu syndrome harbor HuD-specific type 2 CD8+ T cells. *J Clin Invest.* Jul 2009;119(7):2042-2051.
10. Plonquet A, Garcia-Pons F, Fernandez E, et al. Peptides derived from the onconeural HuD protein can elicit cytotoxic responses in HHD mouse and human. *J Neuroimmunol.* Sep 2003;142(1-2):93-100.
11. DeLuca I, Blachere NE, Santomasso B, Darnell RB. Tolerance to the neuron-specific paraneoplastic HuD antigen. *PLoS One.* 2009;4(6):e5739.
12. de Beukelaar JW, Verjans GM, van Norden Y, et al. No evidence for circulating HuD-specific CD8+ T cells in patients with paraneoplastic neurological syndromes and Hu antibodies. *Cancer Immunol Immunother.* Sep 2007;56(9):1501-1506.
13. de Beukelaar JW, Milikan JC, Verjans GM, et al. No evidence for the presence of HuD-specific T cells in the cerebrospinal fluid of patients with Hu-associated paraneoplastic neurological syndromes. *J Neurol.* Feb 2009;256(2):279-282.
14. Graus F, Delattre JY, Antoine JC, et al. Recommended diagnostic criteria for paraneoplastic neurological syndromes. *J Neurol Neurosurg Psychiatry.* Aug 2004;75(8):1135-1140.
15. Gratama JW, van Esser JW, Lamers CH, et al. Tetramer-based quantification of cytomegalovirus (CMV)-specific CD8+ T lymphocytes in T-cell-depleted stem cell grafts and after transplantation may identify patients at risk for progressive CMV infection. *Blood.* Sep 1 2001;98(5):1358-1364.
16. de Beukelaar JW, Sillevis Smitt PA, Hop WC, et al. Imbalances in circulating lymphocyte subsets in Hu antibody associated paraneoplastic neurological syndromes. *Eur J Neurol.* Dec 2007;14(12):1383-1391.

17. Bordier C. Phase separation of integral membrane proteins in Triton X-114 solution. *The Journal of biological chemistry.* Feb 25 1981;256(4):1604-1607.

18. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *The Journal of experimental medicine.* Apr 1 1994;179(4):1109-1118.

19. Martinuzzi E, Afonso G, Gagnerault MC, et al. acDCs enhance human antigen-specific T-cell responses. *Blood.* Aug 25 2011;118(8):2128-2137.

20. Fourlanos S, Perry C, Gellert SA, et al. Evidence that nasal insulin induces immune tolerance to insulin in adults with autoimmune diabetes. *Diabetes.* Apr 2011;60(4):1237-1245.

21. Mallone R, Martinuzzi E, Blancou P, et al. CD8+ T-cell responses identify beta-cell autoimmunity in human type 1 diabetes. *Diabetes.* Mar 2007;56(3):613-621.

22. Gratama JW, Kraan J, Keeney M, Mandy F, Sutherland R, Wood BL. Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline - Second Edition. Vol 27: Clinical and Laboratory Standards Institute; 2007:Appendix D, 62-64.

23. King PH. Differential expression of the neuroendocrine genes Hel-N1 and HuD in small-cell lung carcinoma: evidence for down-regulation of HuD in the variant phenotype. *International journal of cancer.* Aug 22 1997;74(4):378-382.

24. Schuppan D, Junker Y, Barisani D. Celiac disease: from pathogenesis to novel therapies. *Gastroenterology.* Dec 2009;137(6):1912-1933.

Part II

Cellular composition of cerebrospinal fluid

3

Flow cytometric characterization of cerebrospinal fluid cells

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ABSTRACT

Flow cytometry facilitates the detection of a large spectrum of cellular characteristics on a per cell basis, determination of absolute cell numbers and detection of rare events with high sensitivity and specificity. White blood cell (WBC) counts in cerebrospinal fluid (CSF) are important for the diagnosis of many neurological disorders. WBC counting and differential can be performed by microscopy, haematology analyzers or flow cytometry. Flow cytometry of CSF is increasingly being considered as the method of choice in patients suspected of leptomeningeal localization of haematological malignancies. Additionally, in several neuro-inflammatory diseases such as multiple sclerosis (MS) and paraneoplastic neurological syndromes (PNS), flow cytometry is commonly performed to obtain insight into the immunopathogenesis of these diseases. Technically, the low cellularity of CSF samples, combined with the rapidly declining WBC viability, makes CSF flow cytometry challenging. Comparison of flow cytometry with microscopic and molecular techniques shows that each technique has its own advantages and are ideally combined. We expect that increasing the number of flow cytometric parameters that can be simultaneously studied within one sample, will further refine the information on CSF cell subsets in low-cellular CSF samples and enable to define cell populations more accurately.

INTRODUCTION

White blood cell (WBC) counts and their differential into mononuclear (MNC) and polymorphonuclear cells (PMN) in cerebrospinal fluid (CSF) are critical in the diagnosis of many infectious and inflammatory neurological disorders.[1] In acute bacterial meningitis, WBC counts usually range between several hundred to more than 60,000/µl, predominantly PMN (90%-95% of WBC count), although early in the disease WBC counts can be lower than 100 WBC/µL.[2] In viral meningitis, cell counts are usually between 10 - 1,000 WBC/µL, but may exceed 1,000/µL. Here, MNC predominate, but in the very acute stages of disease PMN can account for more than 80% of leukocytes.[2] In multiple sclerosis (MS), two-thirds of patients have a normal CSF cell count and a low level of mononuclear pleocytosis is found in one-third of the cases.[2] The CSF WBC count is usually normal (<5 leukocytes/µL) in patients with the Guillain-Barré syndrome, whereas in case of an increased WBC count other diagnoses should be considered.[3] In paraneoplastic neurological syndromes (PNS), 47% of patients have a lymphocytic pleocytosis before the 3rd month after onset of the neurological symptoms, while after the 3rd month only 28% of patients have elevated cell counts.[4] CSF WBC counts are also routinely determined in patients with suspected leptomeningeal metastases of solid or haematological malignancies and half of these patients have a lymphocytic pleocytosis.[5,6]

MICROSCOPY

Generally, cell counts and differential can be obtained by evaluating cell number and morphology in microscopic slides, by automatic counting based on cellular scatter properties or by flow cytometry in which antigen expression of cells is assessed in combination with light scatter properties. Specifically, microscopic counting of WBC and red blood cells (RBC) is performed using Neubauer or Fuchs-Rosenthal counting chambers, which contain a microscopically visible counting grid and are used with a fixed sample volume. Staining with Samson or Türk reagent may be added to the procedure to facilitate WBC counting and perform differential.[6] However, in low-cellular CSF samples (<5 leukocytes/µL), differential by microscopy is not performed. When the WBC count is higher than the upper reference value, most laboratories perform cytospin centrifugation of the sample, followed by Wright or May-Grunwald-Giemsa staining to enable morphological differential of cells in CSF. This technique permits rapid differential between monocytes, lymphocytes and granulocytes, which is of utmost importance for patients with acute neurological disease.[7] Counting of erythrocytes is important to exclude traumatic bleeding as the cause of an elevated WBC

count.[8] Although microscopic counting and differential has for a long time been used in routine CSF analysis, the clinical laboratory faces several challenges in performing it. First, the analysis is time-consuming and should ideally be performed within 1 hour after lumbar puncture as CSF cell counts decrease rapidly after sampling.[9] Second, counting and differential results have relatively high intra- and interobserver variation[8], as have other manual microscopic techniques. Furthermore, skilled and continuously trained technicians are required for this assay on a 24 hours/7 day basis.[10]

HAEMATOLOGY ANALYZERS

Nowadays an increasing number of clinical laboratories replace the microscopic technique by haematology analyzers (HA) for cell counting and differential in CSF.[8,10,11] HA may provide fast, low-cost and standardized cell counting of CSF and other body fluids such as ascites or pleural fluid. However, special attention is needed regarding background signal, carry-over and interference in view of the low cell concentrations in these fluids. Two dedicated, FDA-approved, applications for CSF counting and differential on HA are available, i.e., the ADVIA® 120/2120 CSF assay (Siemens AG, München, Germany) and the Body Fluid mode on a XE-5000™ analyzer (Sysmex, Kobe, Japan).[8,11] ADVIA® CSF assay uses light scatter and absorbance measurement for counting and differential, after mixing the sample with CSF reagent to sphere and fix the cells. Not only RBC, WBC and PMN/MNC are reported, but also lymphocytes, monocytes and eosinophils. XE-5000™ Body Fluid mode uses sheath flow impedance for counting RBC, while light scatter and fluorescence intensity after DNA/RNA staining is used to analyse WBC. The application reports WBC, PMN/MNC and high-fluorescent cells. For CSF WBC counting, comparison of the Fuchs-Rosenthal counting chamber with both the XE-5000™ analyzer and the ADVIA® 120/2120 CSF assay showed linearity between 1 and 10,000 cells per µL.[8,11,12] Automatic MNC counts also correlated well with manual counts, but PMN counts showed poor agreement being almost two-fold higher using the XE-5000 analyzer.[12] Especially in low WBC counts (<20 cells per µL) high imprecision was observed in both techniques compared to manual counting.[8,11] The detection limit of the XE-5000 is 10 cells per µL. When the WBC count is below that limit, differential into MNC and PMN cannot be made.[12] In these cases, manual counting or evaluation of stained cytospin slides should be performed. In automatic cell counting of low-cellular samples the same problem arises as in flow cytometry (see *Rapid decline of leukocyte counts upon lumbar puncture* section): due to high imprecision, under- or overestimation of CSF WBC counts may lead to erroneous results. In clinical practice, HA are more widely available and have lower material costs than flow cytometers.

CYTOMORPHOLOGY

Morphologic examination of CSF cells is performed on cytospin preparations stained with May-Grunwald-Giemsa.[6] Whilst highly specific (>95%), conventional cytomorphological analysis is associated with only a limited sensitivity with up to 20% to 60% false-negative results.[6,13] Interpretation of cytological findings may be difficult because of paucity of cells in CSF and possible morphological similarities between benign and malignant cells.[14] Cytomorphological examination is used in patients with suspected leptomeningeal dissemination of solid tumours or haematological malignancies. Typically, only 50% of patients have malignant cells identified by cytomorphological examination on the first lumbar puncture.[15] This yield is increased to 80% with a second CSF examination[5], but even three lumbar punctures will still miss tumour cells in approximately 10% of patients.[16] Despite its low sensitivity, CSF cytologic examination has been the gold standard for leptomeningeal metastasis because of its 100% specificity.[16] If clinical suspicion is high, gadolinium-enhanced MRI of the brain and spine can provide definitive evidence of leptomeningeal metastasis, even without a positive CSF cytology.[17,18]

IMMUNOCYTOCHEMISTRY

Immunocytochemistry allows the detection of cell surface antigens on CSF cells by cytospins. For detection of leptomeningeal localization of haematological malignancies a sensitivity of 89% to 95% and a specificity of 89% to 100% was shown by this technique.[19] For CSF samples with low cell counts, immunocytochemistry should be used subsequent to cytomorphology and the selection of the antibodies should be determined by the cytological findings in combination with the patients history.[20,21] Alternatively, it is stated that this technique should only be used when CSF cytomorphology fails in patients with a strong suspicion of leptomeningeal metastases.[22] Compared to flow cytometry, immunocytochemistry gave similar results in detection of high-density surface markers, whereas in analysis of antigens that are expressed at low density immunocytochemistry may be more reliable in some applications.[23] Since flow cytometry is used in the detection of CNS involvement of haematological malignancies besides cytomorphological analysis, as discussed in the *Applications of flow cytometry to study CSF* section, immunocytochemistry has no major role anymore. In contrast, immunohistochemistry is still used in combination with cytology in the detection of leptomeningeal metastases of solid tumours.

POLYMERASE CHAIN REACTION (PCR)

PCR requires the selection of primers specific for tumour cell-derived DNA. In haematological B-cell malignancies, analysis of immunoglobulin heavy chain gene rearrangements in the third complementarity determining region (CDR3) by PCR in blood and bone marrow cells is a state-of-the-art technique for diagnosis, monitoring response to treatment and detection of minimal residual disease.[24,25] Presence of clonally rearranged CDR3 is the molecular signature of malignant B lymphocytes and is present in 80-95% of B-cell lymphomas and leukaemias.[26] Until now this technique has not been generally applied to CSF samples. PCR can also be used for detection of leptomeningeal metastasis in solid malignancies. Although it would be ideal to use primers for DNA sequences common to all metastatic cells, the use of sequences for specific primary cancer histopathologies might provide a more practical option, as many are known already.[13,27-29] Additional molecular tumour markers or oncogenes can be used for other types of cancer and might eliminate the need for biopsy in selected patients.[29]

FLOW CYTOMETRY

In patients with suspected leptomeningeal metastases of haematological tumours, flow cytometry of CSF samples is used in addition to cell counting and cytomorphology. In this review we will focus on the applications and recent developments of CSF flow cytometry. Although this procedure has only a narrow clinical indication, it has significant prognostic and therapeutic implications in individual patients.

Use of polychromatic flow cytometry

The advent of polychromatic flow cytometry, i.e. advanced instrumentation and reagent development[30], allows detection of a large spectrum of cellular characteristics, even in samples with small amounts of cells like CSF. Apart from differentiating between major leukocyte subsets by assessing granularity and volume of cells, a wide range of cell populations can be specified by immunophenotyping using surface membrane, cytoplasmatic and nuclear antigens.[7,14,31] However, intracellular staining should be limited to those cases in which it is essential to reach the immunological conclusion, because its use is associated with relatively pronounced cell loss.[31] The number of characteristics on one single cell that can be determined in a single tube, depends on the number of fluorescent colours available on the flow cytometer used and the number of monoclonal antibodies per tube. The applicability of the assay can even be further enhanced by combining two antigens expressed by non-overlapping

cell subsets on a single fluorochrome (e.g., CD4 present on T lymphocytes and CD19 present on B lymphocytes).[14] In CSF, the simultaneous assessment of 13 parameters (11 colours plus forward and sideward scatter) has been reported in this way.[14] However, problems with spectral overlap and colour compensation increase when more than 6 colours are used, but these problems can be reduced if markers and fluorochromes are combined judiciously.[32]

Absolute cell counts

Frequencies of different WBC populations in CSF are most widely investigated. However, knowledge of absolute numbers of the major cell populations can be of great help to evaluate the sample.[33] Due to possible cell loss during concentration and centrifugation steps[31,34], absolute cell counts may be an underestimation of the real CSF cell number. Addition of counting beads to the monoclonal antibody-stained CSF cell suspension allows accurate enumeration of absolute numbers of cell subsets.[31] By using this technique, we showed that PNS patients stood out by highly increased absolute counts of the major lymphocyte subsets in CSF, but above all, by B-lymphocyte counts that had increased more than 20-fold as compared to controls without neurological disease.[35] In these patients, the frequency of B lymphocytes (expressed as fraction of lymphocytes) had increased only three-fold.[35] When merely frequencies would have been studied, this enormous B-lymphocyte expansion suggesting an important role for these cells in PNS, would have remained unnoticed. This result indicates that assessment of absolute counts besides frequencies is also important in CSF.

Cellular composition of normal CSF

To use CSF flow cytometry in research of neuro-inflammatory diseases, knowledge of the composition of cells in normal CSF is needed. Because CSF of healthy controls is usually not available due to ethical considerations[36], NIND controls are often included instead.[37] However, Svenningsson et al.[38,39] did study normal CSF by assessing the percentages of lymphocyte subsets in CSF of 34 healthy individuals, after informed consent, with 2- or 3-colour flowcytometry. In addition, we studied both absolute numbers and percentages of leukocyte, lymphocyte, T lymphocyte and dendritic cell subsets by 6-colour flow cytometry in 84 individuals without neurological disease undergoing spinal anaesthesia (Table 1).[40] The two published studies[38,39], as well as our data[40], showed that normal CSF is predominantly composed of $CD4^+$ T lymphocytes, mostly with a central memory phenotype, and in addition contains very low frequencies of B lymphocytes, NK cells and NKT lymphocytes (Table 1). Dendritic cells, both myeloid as well as plasmacytoid, were also present in normal CSF, although in very low frequencies (Table 1).[40,41]

Table 1. Reference values of WBC subsets in CSF (adapted from de Graaf et al. [40])

Subset	Absolute number[A]
Leukocytes	1.12 (0.40 – 3.17)
Granulocytes	0.08 (0.02 – 0.43)
Monocytes	0.23 (0.08 – 1.11)
Lymphocytes	0.66 (0.16 – 1.88)
T cells	0.62 (0.15 – 1.83)
$CD4^+$ T cells	0.44 (0.08 – 1.43)
$CD8^+$ T cells	0.13 (0.04 – 0.40)
NKT cells	0.01 (0.00 – 0.06)
B cells	0.00 (0.00 – 0.03)
NK cells	0.01 (0.00 – 0.05)
Dendritic cells	0.04 (0.01 – 0.18)
Myeloid	0.02 (0.00 – 0.13)
Plasmacytoid	0.01 (0.00 – 0.03)

[A]Medians (5^{th}-95^{th} percentiles) of absolute numbers x10^6/L are given.

Sensitivity and specificity

Flow cytometry is a sensitive method capable of detecting abnormal monoclonal B lymphocytes, which comprise as little as 0.01% of total lymphocytes.[42,43] The detection rate of CSF involvement in hematological malignancies is up to 86% higher in flow cytometry than in conventional cytomorphological analysis[6,14,42-48] (Table 2). Although it was previously suggested that this method may not be suitable in the evaluation of samples with low cellularity[23], it has been shown that CSF T lymphocytes, the predominant lymphocyte subset in CSF, can be reliably detected in samples with a cell count lower than 5 leukocytes/µL.[46] Kleine et al.[49] showed that precision of lymphocyte flow cytometry is high (coefficient of variance [CV] ≤10%) provided that a sufficient number of events has been acquired.[50] However, the CV may increase to values up to 30% for the minor subsets in CSF, e.g. NK cells and NKT lymphocytes.[49]

Several studies comparing flow cytometry and conventional cytomorphology to detect CSF involvement in haematological malignancies (Table 2) describe samples in which flow cytometry is positive whilst cytology is negative.[6,14,43,51] Presence of neurological symptoms compatible with leptomeningeal disease is highly suggestive for CNS involvement in such patients, whereas absence of symptoms and lack of recurrence of CNS disease during clinical follow-up indicate a false-positive flow cytometric result.[6,14] Results of clinical follow-up were documented in three studies. Sancho et al.[51] suggest that a flow cytometry-positive/cytology-negative result is associated with a poor clinical outcome in aggressive B-cell lymphomas, as compared to samples with

Table 2. Comparison of flow cytometry and conventional cytomorphology in detection of CSF involvement in haematological malignancies

Malignancies Reference	N	CSF involvement[A]	FC+/ CC+	FC+/ CC-	FC-/ CC+	Detection rate by using CC alone[B]	Detection rate by using FC alone[C]
Lymphoma							
Finn, 1998 [47]	27	10	6	3	1	7/10 (70%)	9/10 (90%)
Hegde, 2005 [43]	60	14	2	12	0	2/14 (14%)	14/14 (100%)
Quijano, 2009 [14]	123	25	7	17	1	8/25 (32%)	24/25 (96%)
Sancho, 2010 [51]	105	22	7	15	0	7/25 (28%)	22/22 (100%)
Acute leukaemia							
Subira, 2001 [46]	168	21	11	7	3	14/21 (67%)	18/21 (86%)
Sayed, 2009 [48]	45	23	8	13	2	10/23 (43%)	21/23 (91%)
Lymphoma, leukaemia							
French, 2000 [42]	35	9	4	3	2	6/9 (67%)	7/9 (78%)
Roma, 2002 [45]	53	21	12	9	0	12/21 (57%)	21/21 (100%)
Nückel, 2006 [44]	45	18	12	3	3	15/18 (83%)	15/18 (83%)
Bromberg, 2007 [6]	219	43	15	24	4	19/43 (44%)	39/43 (91%)

[A]CSF involvement was diagnosed when flow cytometry, conventional cytomorphology or both were positive.
[B]Detection rate of CSF involvement by using conventional cytomorphology alone: ([FC+/CC+] + [FC-/CC+])/CSF involvement.
[C]Detection rate of CSF involvement by using flow cytometry alone: ([FC+/CC+] + [FC+/CC-])/CSF involvement.
FC = flow cytometry; CC = conventional cytomorphology.

absence of neoplastic cells by both methods. In addition, Bromberg et al.[6] described the absence of CNS recurrence in only 1/24 flow cytometry-positive, cytology-negative patients. Hegde et al.[43] found that 3/40 patients at increased risk for central nervous system involvement but with negative cerebrospinal fluid by flow cytometry on initial staging relapsed in the central nervous system and died. These follow-up data indicate that flow cytometric analysis of CSF samples has a low risk of being both false-positive and false-negative in patients with haematological malignancies.

Rare event detection

When leptomeningeal localization of a haematologic malignancy is suspected, the presence of a pathological (monoclonal) population and phenotypic characterization of that population can be assessed by using the proper antibody reagent panel adapted to the number of cells and previous histological and immunophenotypical diagnosis or suspected diagnosis together with appropriate gating strategies.[31] Pathological

cells usually occur at very low percentages in the order of 0.01% in CSF. In addition, CSF samples contain a limited number of cells rendering pathologic cells in CSF very rare. To detect low numbers of rare cells, the background fluorescence of the reagents should be minimal, and a sufficient cell number is required to analyze lymphocyte subsets reproducibly. Therefore, the CSF sample has to be as large as possible and cell loss during work up has to be prevented as discussed in the *Technical pitfalls* section.

APPLICATIONS OF FLOW CYTOMETRY TO STUDY CSF

CNS involvement with lymphoproliferative disorders

Involvement of the central nervous system (CNS) is a relatively uncommon complication of leukaemia and lymphoma[14,46], which is suspected in patients who develop neurological symptoms or signs [14] or in patients at high risk of CNS localization.[6] It has grave prognostic significance and requires important therapeutic decisions including the administration of intrathecal chemotherapy.[6,46] Leptomeningeal localization is diagnosed by conventional cytomorphological analysis through identification of malignant lymphocytes in CSF.[31,52] However, this technique has a relatively high rate of false-negative results in up to 60% of cases.[22,53] Recent reports suggest that multiparameter flow cytometric assessment of CSF samples could improve the efficiency of detection of CNS involvement, due to its high specificity and greater sensitivity.[6,52,54] Table 2 gives an overview of studies which investigated the value of flow cytometry and conventional cytomorphology in detection of CSF involvement in haematological malignancies. These studies showed that the use of flow cytometry alone increased the detection rate of CSF involvement up to 86% compared to the use of cytomorphology alone. Combined use of flow cytometry and cytomorphology increased the detection rate with 17%-86% compared to cytomorphology alone. Therefore, the National Comprehensive Cancer Network (USA) has recommended the routine use of flow cytometry in conjunction with cytomorphological analysis for the diagnosis of CNS lymphoma.[55] CNS involvement is diagnosed if one of these diagnostic procedures is positive. For detection of haematological malignancies, flow cytometry depends on the analysis of light chain restriction (Figure 1) and/or aberrant antigen expression, which should be interpreted within the context of the patient's histological diagnosis.[56] Still, cytomorphological examination of CSF has additional diagnostic and possibly prognostic value and should still be performed in conjunction with flow cytometry.[6]

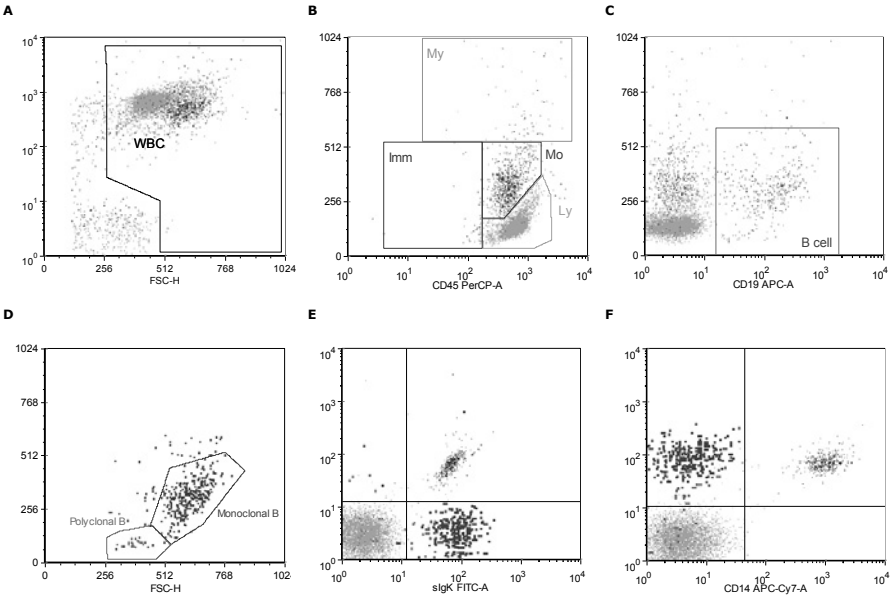

Figure 1. 6-colour flow cytometric CSF analysis
Example of a 6-colour flow cytometric analysis for B-lymphocyte clonality in CSF. Each dot represents a single cell. For analysis, debris and non-leukocyte events were excluded by gating on forward scatter (FSC) and CD45 (gate 1, panel **A**). The leukocyte subsets (My = myeloid lineage; Imm = immature lineage; Mo = monocytes; Ly = lymphocytes) were defined with CD45 expression and side scatter (panel **B**) and show two major subsets: lymphocytes ($CD45^{+}$, SSC^{lo}, FSC^{im}; green dots) and monocytes ($CD45^{+}$, SSC^{im}, FSC^{hi}, $CD4^{dim}$; cyan dots). B lymphocytes were gated using the lineage-specific marker CD19 and side scatter (SSC) (purple dots; gate 2, panel **C**). Next, physical properties of B lymphocytes were confirmed by FSC and SSC (gate 3, panel **D**). Analysis of clonality revealed that the purple dots represent a population of B lymphocytes with monoclonal expression of sIgK but not sIgL light chains (panel **E**), compatible with B cell lymphoma. The cyan dots that bind both sIgK and sIgL (panel E) are monocytes - as revealed by their CD14 reactivity - that have bound cytoplasmic Ig (panel **F**).

Inflammatory neurological diseases

Multiple sclerosis

Flow cytometry of CSF is also used as a research tool in various neuro-inflammatory diseases. The distribution of lymphocyte subpopulations in the CSF may be a consistent indicator of the type of immune response active in these diseases.[57] Several studies have reported on flow cytometric analysis of lymphocytes and their subsets in CSF of patients with MS. The CSF cell populations in MS patients have been shown to consist of approximately 60% $CD4^{+}$ T lymphocytes[58] with a higher frequency of the regulatory phenotype[59,60] and a higher CD4/CD8 ratio[61], while the frequency of NKT lymphocytes is lower[62] than in control patients with non-inflammatory neuro-

logical disorders (NIND). Compared to blood, CSF of MS patients showed a relative depletion of $CD8^+$ effector memory T lymphocytes.[63] In relation to disease activity, patients with active MS had higher percentages of activated $CD4^+$ T lymphocytes[64-67] and lower percentages of activated $CD8^+$ T lymphocytes[64,65,67] in their CSF than inactive MS patients. Moreover, the percentage of naïve $CD45RA^+$, $CD50^+$ (ICAM-3) lymphocytes in CSF is significantly increased[68], while cell surface expression of CD54 (ICAM-1) on T lymphocytes in CSF is significantly decreased[69] in patients with relapses compared to patients in remission. Both are suggested to be used as markers of MS disease activity in CSF as well as blood.[68,69] With regard to B lymphocytes in CSF of MS patients, a significant accumulation of mature B lymphocytes and plasma blasts is observed.[70,71] Most B lymphocytes have a memory phenotype[71-74] and more B lymphocytes express CD80 (costimulatory molecule inducing T lymphocyte activation) than in NIND and healthy controls.[75,76] Furthermore, the number of dendritic cells is elevated in CSF of MS patients.[41] CSF flow cytometry was also used in evaluation of MS treatment with immunosuppressive drugs. Both rituximab (anti-CD20 monoclonal antibody [mAb])[77] and natalizumab (anti-α_4 integrin mAb)[78] reduced the number of B and T lymphocytes, while high-dose methylprednisolone induced changes in the expression of CD25, CD26 and HLA-DR on $CD4^+$ T lymphocytes.[79]

Paraneoplastic neurological syndromes

We and others have reported on CSF lymphocyte subsets in patients with PNS. In PNS associated with anti-Hu antibodies, CSF is characterized by (i) a very substantial (20-fold) B-lymphocyte expansion and (ii) a 3-fold T-cell expansion (including both $CD4^+$ and $CD8^+$ subsets) compared to controls.[35] Children with paraneoplastic opsoclonus-myoclonus syndrome had normal CSF cell counts, but higher percentages of B lymphocytes[80,81], activated T lymphocytes and γδ-T lymphocytes, lower percentages of $CD4^+$ T lymphocytes and a lower CD4/CD8 ratio[80] compared to NIND controls.

TECHNICAL PITFALLS

Low cellularity

The low number of cells in CSF (normal range: <5 leukocytes/μL) hampers the use of flow cytometry.[82] To analyze lymphocyte subsets reproducibly, measuring a sufficient cell number is required. However, the minimal number of CSF cells required is not universally defined. In literature, the minimal CSF cell number varies between 100 gated lymphocytes in lymphocyte subset characterization[49] and 1000 cells in suspected CSF localization of lymphoma.[54] A subpopulation was reliably identified whenever 13 or more clustered events displaying identical features were present,

whereas the presence of fewer than 5 clustered events could not be related to the presence of a specific cell population.[82] In another study, a minimum of 15 events is reported to ascribe them to a specific cell population with a high probability.[83] In leptomeningeal metastasis of haematological malignancies, one study describes the presence of at least 10 clustered events with abnormal patterns of antigen expression for diagnosis[14], while another publication prescribes to classify clusters of more than 25 events as positive, 10-25 events as suspicious and below 10 events as negative.[31] In our laboratory, we agree with the latter publication and consider 25 clustered events as positive. However, this accounts for the use of three or four-colour flow cytometry. When a higher number of colours is used, less clustered events are required to define a cell population.

To obtain a maximal number of cells for analysis, CSF samples have to be concentrated by low-speed centrifugation. No significant cell loss was observed in hypocellular samples (<10 leukocytes/µl) when CSF cells were enriched by centrifuging at 200*g* for 15 minutes at 4°C.[49] CSF samples containing >10 leukocytes/µL can be stained and analyzed without cell enrichment.[36] However, in case of rare event detection, e.g. in CNS involvement of lymphoproliferative disorders, CSF cells should also be concentrated in samples containing >10 leukocytes/µl to increase the sensitivity. Another way to deal with the low cell numbers in CSF, is the use of a two step approach.[31,46] First, one third of the sample is analyzed with a screening tube, which in most cases will answer the clinical question. When this first staining is not conclusive, the process should be repeated with the remaining CSF and the same reagent combination. Combining the list mode data of the first and second staining will increase sensitivity by enabling analysis of a larger number of cells. Second, if a pathological population is identified in the first step, immunophenotyping may be extended.

Rapid decline of leukocyte counts upon lumbar puncture

Another difficulty of CSF studies is the rapid decay of leukocytes after sampling as described in several studies (Table 3). Within 30 minutes after sampling, the CSF cell number decreases significantly.[9,14,34,84-86] Also, differences in survival rate between different leukocyte subsets were observed: monocytes and granulocytes showed a more rapid cell loss than lymphocytes[9,34,85] (Table 3). In flow cytometric analysis, selective cell losses may cause underestimation of cell counts.[23] These errors can affect clinical decisions. E.g., in CSF samples with a pleocytosis, underestimation of the cell number may result in a normal cell count and pathology, such as a neuro-inflammatory disease, may be considered ruled out.

Table 3. Decline of CSF cell yields as a function of storage time

CSF additive Reference	First assessment (hours)	Second assessment (hours)	Temperature	Cell loss relative to first assessment (%)			
				WBC	Lymphocytes	Monocytes	Granulocytes
No							
Chow, 1984 [83]	0[A]	2 and 24	22°C	40 and 60	-	-	-
	0[A]	2 and 24	4°C	15 and 39	-	-	-
Steele, 1986 [84]	0[B]	2 and 4	Ambient	27[G] and 44[G]	12 and 34	20 and 39	50 and 58
Dux, 1994 [34]	0[C]	1.5	On ice	62[G]	35	90	
De Graaf, 2011 [9]	0.5	1	Ambient	26	4	36	34
Saline							
Steele, 1986 [84]	0[B]	4	Ambient	6[G]	0	0	18
Serum-containing buffer							
Veerman, 1985 [85]	0.5[D]	24	Ambient	0	-	-	-
Dux, 1994 [34]	0[C]	1.5	On ice	-	10	-	-
De Graaf, 2011 [114]	0.5[E]	1	Ambient	9	2	18	0
Chemical stabilization							
Quijano, 2009 [14]	0[F]	24 and 48	Unknown	21 and 40	-	-	-

[A]Spiking: homologous blood was added to accellular CSF samples.
[B]Spiking: lymphocytes, monocytes and neutrophils were obtained from peripheral blood and subsequently spiked into CSF samples that had been centrifuged to remove cells or into saline.
[C]Spiking: after CSF withdrawal, cells were pelleted by centrifugation and resuspended in CSF or in PBS containing 5% fetal calf serum. The cell number at resuspension was set at 100%.
[D]CSF in sterile physiologic medium (one part Earle's balanced salt solution and one part 20% human serum albumin) added directly after CSF withdrawal.
[E]CSF in serum-containing medium (RPMI-1640 with HEPES, L-Glutamine, Penicillin/Streptomycin, heat-inactivated fetal bovine serum and heparin) added directly after CSF withdrawal.
[F]CSF directly collected into tubes containing EDTA and 0.2 ml of Transfix™.
[G]WBC loss is calculated by adding up the lymphocyte, monocyte and granulocyte loss.

This cell loss in native CSF can be reduced by addition of medium to CSF directly after sampling. In an earlier study, we showed that addition of serum-containing medium (RPMI-1640 with HEPES, L-Glutamine, Penicillin/Streptomycin, heat-inactivated fetal bovine serum and heparin) preserves CSF cells until at least five hours after sampling.[9] Another study showed that immediate addition of Earle's balanced salt solution with human serum albumin to CSF prevents total WBC loss until at least 24h after sampling.[86] Also, addition of TransFix™ (fixative) has been shown to reduce CSF cell loss.[14] Other previous reports that investigated CSF cell preservation methods, were more laboratory based than clinical. Spiking of CSF cells into 5% fetal calf serum[34] or saline[85] showed no significant cell loss, while spiking into acellular CSF did[34,85] (Table 3). In addition, immediate cooling of the CSF sample[84], a minimum of centrifugation steps[34] and aspiration of the supernatant instead of decanting the sample[31] all ameliorate the loss of cells. Furthermore, due to lack of serum in CSF washing CSF cells before surface immunoglobulin staining is not necessary, which minimizes the wash steps in the protocol.[31] The function of media, in most studies serum-containing, in preserving leukocytes is probably a buffering one. Both increase in pH in CSF after removal from the body (due to diffusion of CO_2 out of the sample) and hypotonicity of CSF (causing movement of water and solutes from the extracellular to the intracellular compartments) have been hypothesized to contribute to cell death[85,87,88], although the effect of both factors has not been confirmed. It remains to be investigated which medium is the most effective in preventing CSF cell loss, being both practical for use in clinical settings and inexpensive. Evidently, CSF samples used for cell counting should be handled carefully by (i) collection in a buffering medium to prevent the rapid cell loss; (ii) a minimum of wash and centrifugation steps; (iii) aspirating instead of decanting; and (iv) data acquisition at least within 24h after sampling[86], although the maximal storage time has not been determined yet.

Nonspecific fluorescence

Nonspecific or "background" fluorescence may constitute a serious problem, especially in rare event detection in CSF samples. Its cause can be categorized into three groups.[89] First, autofluorescence by excitation of naturally occurring cellular components (e.g., granule-associated flavoproteins in granulocytes) other than the antibody bound fluorochrome.[89] This problem may be reduced by the use of a 532 nm laser[90] or specific tools like single laser excitation[91] and cell-by-cell autofluorescence correction.[92] Second, spectral overlap which becomes significant when more than four colours are used and can be minimized by choosing combination of fluorochromes that have no or little overlap with each other.[32] Third, non-specific antibody binding may occur which can be eliminated by optimizing antibody concentration using titration assays.[93]

Blood contamination

Red cells present in CSF reflect either CNS bleeding or a traumatic lumbar puncture in which peripheral blood contaminates the CSF. The possibility of blood contamination of CSF samples can be ruled out by absence of cell populations which are highly represented in blood and most frequently absent in normal CSF, e.g. neutrophils and erythrocytes, particularly if these cells are present in blood at normal or high numbers and not detected in the CSF sample. In flow cytometric absolute cell counting of blood contaminated CSF samples, correction of the number of leukocytes has to be performed. We prefer to use the leukocyte/erythrocyte ratio in peripheral blood for correction[94], because this method accounts for a patient's individual situation. Alternatively, the CSF leukocyte number may be arbitrarily adjusted by correction according to the CSF erythrocyte count, which results in a correction of 1 leukocyte per 500 erythrocytes present in CSF.[95,96] This latter method is widely used in clinical practice, because information on peripheral blood is not needed.

In blood contaminated CSF samples, which are investigated for the presence of CNS involvement of haematological malignancies, detection of a small population of neoplastic cells is only diagnostic of CSF involvement if these cells are not detected in a simultaneously obtained blood sample.[31] In acute leukaemia, lumbar puncture should not be performed in an acute phase of the disease when the frequency of circulating malignant cells is high. In case of a traumatic lumbar puncture, malignant cells from the blood may become detectable in CSF leading to apparently false-positive CSF results. Moreover, iatrogenic contamination of CSF with malignant cells might be caused.[97]

Detection of monoclonal B-cell populations

Detection of a monoclonal B-lymphocyte population in CSF diagnoses CNS localization of a B-cell lymphoma in patients with haematological malignancies.[6,43] B-lymphocyte clonality can be investigated by flow cytometry by assessing surface immunoglobulin light chain expression on $CD19^+$ B-lymphocytes and comparison of the "light chain ratio (LCR)" or "kappa/lambda ratio", which is determined by dividing the percentage of cells with the dominant light chain by the percentage of cells with the minor light chain.[52] Normal ranges for the LCR differ between laboratories. A LCR threshold of 2 was reported to have a specificity of 92.3% and a sensitivity of 73.1% [98], while other studies proposed thresholds ranging from 2 to 6, with highest specificities and sensitivities around 70%.[99-104] This indicates that if e.g., a threshold of 2 is used, approximately 10% of patients with a LCR above 2 are reported to have a monoclonal B-cell population, but do not have a B-cell lymphoma. Therefore, a LCR shift is only suggestive for presence of a monoclonal B-cell population, and further analysis of the

CSF has to be performed. Assessment of additional markers may increase specificity as an abnormal forward scatter/sideward scatter/CD19/ CD20 phenotypic pattern indicates the presence of a malignant cell population[14], although absence of such an abnormal pattern does not rule out malignancy. Usually, assessment of monoclonality and additional markers are combined, e.g. looking for monoclonality in a large forward scatter (FSC) or dim CD20 population, as presented in Figure 1. Furthermore, detection of clonally identical rearranged DNA sequences in malignant B lymphocytes by PCR is suggestive of the presence of B-cell lymphoma[25], but only a minority of clinical laboratories have this technique operational. Another important point of attention is that not every monoclonal B-cell population in CSF indicates symptomatic CNS disease.[42] In patients with indolent haematological disorders, including chronic lymphatic leukaemia (CLL), malignant B cells in the CSF may represent asymptomatic leptomeningeal involvement and may require treatment only when (new) symptoms arise.[52,105,106] At last, clinical follow-up will aid in diagnosing CNS disease.

PERSPECTIVES

We expect that the expansion of the number of colours amenable to flow cytometry will enable the simultaneous study of more parameters within the same sample. Using this approach, more refined information on CSF cell subsets will become available and cell populations can be defined more accurately.[107] An ongoing challenge is the search for new fluorochromes that can be used in conjunction with current ones and yet do not contribute to significant spectral overlap.[108] Even without new fluorochromes or lasers, instruments will improve through advances in software for data processing. However, visualizing these data becomes more and more complex and would require multiple sequential analyses to provide information about each cell subset. Therefore, automated classification systems are being developed.[109-112] Additionally, optimization of storage conditions to preserve CSF cells should result in higher cell yields and thereby increase the detection rate of flow cytometry in CSF samples with low cellularity.

Importantly, flow cytometry can be combined with molecular techniques including PCR to improve sensitivity in detecting CSF involvement of lymphoma.[113,114] Furthermore, broadspectrum tumour cell-specific antigens could be fluorescently labelled and used in flow cytometric detection of CNS malignancy.[13] In future, DNA clonality of the tumour might be identified on biopsy material and then followed by CSF assays along the course of the disease. This allows us to detect whether selection and development of new malignant clones occur in resistant or relapsing disease and will enable us to

appraise the therapeutic and prognostic implications of molecular diagnostic testing of CSF.[26] However, these future techniques have to be internationally validated and standardized to be used in clinical practice.

In summary, these future advances will lead to a higher sensitivity and specificity to detect CNS localization of malignancies, while in neuro-inflammatory diseases (e.g., MS), CSF flow cytometry might become an important tool in the diagnosis, prognosis and follow-up of patients.

REFERENCES

1. Straus SE, Thorpe KE, Holroyd-Leduc J. How do I perform a lumbar puncture and analyze the results to diagnose bacterial meningitis? *Jama.* Oct 25 2006;296(16):2012-2022.
2. Fishman R, editor. *Cerebral fluid in diseases of the nervous system.* 2nd ed. Philadephia, Pennsylvania: Saunders; 1992.
3. Van der Meche FG, Van Doorn PA, Meulstee J, Jennekens FG. Diagnostic and classification criteria for the Guillain-Barre syndrome. *Eur Neurol.* 2001;45(3):133-139.
4. Psimaras D, Carpentier AF, Rossi C. Cerebrospinal fluid study in paraneoplastic syndromes. *J Neurol Neurosurg Psychiatry.* Jan 2009;81(1):42-45.
5. Wasserstrom WR, Glass JP, Posner JB. Diagnosis and treatment of leptomeningeal metastases from solid tumors: experience with 90 patients. *Cancer.* Feb 15 1982;49(4):759-772.
6. Bromberg JE, Breems DA, Kraan J, et al. CSF flow cytometry greatly improves diagnostic accuracy in CNS hematologic malignancies. *Neurology.* May 15 2007;68(20):1674-1679.
7. Hausler M, Sellhaus B, Schweizer K, Ramaekers VT, Opladen T, Kleines M. Flow cytometric cerebrospinal fluid analysis in children. *Pathol Res Pract.* 2003;199(10):667-675.
8. Mahieu S, Vertessen F, Van der Planken M. Evaluation of ADVIA 120 CSF assay (Bayer) vs. chamber counting of cerebrospinal fluid specimens. *Clinical and laboratory haematology.* Jun 2004;26(3):195-199.
9. de Graaf MT, van den Broek PD, Kraan J, et al. Addition of serum-containing medium to cerebrospinal fluid prevents cellular loss over time. *J Neurol.* Aug 2011;258(8):1507-1512.
10. Kleine TO, Nebe CT, Lower C, et al. Modifications of haematology analyzers to improve cell counting and leukocyte differentiating in cerebrospinal fluid controls of the Joint German Society for Clinical Chemistry and Laboratory Medicine. *Cytometry A.* Aug 2009;75(8):688-691.
11. Boer K, Deufel T, Reinhoefer M. Evaluation of the XE-5000 for the automated analysis of blood cells in cerebrospinal fluid. *Clin Biochem.* May 2009;42(7-8):684-691.
12. de Jonge R, Brouwer R, de Graaf MT, et al. Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med.* May 2010;48(5):665-675.
13. Chamberlain MC, Glantz M, Groves MD, Wilson WH. Diagnostic tools for neoplastic meningitis: detecting disease, identifying patient risk, and determining benefit of treatment. *Semin Oncol.* Aug 2009;36(4 Suppl 2):S35-45.
14. Quijano S, Lopez A, Manuel Sancho J, et al. Identification of leptomeningeal disease in aggressive B-cell non-Hodgkin's lymphoma: improved sensitivity of flow cytometry. *J Clin Oncol.* Mar 20 2009;27(9):1462-1469.
15. Glass JP, Melamed M, Chernik NL, Posner JB. Malignant cells in cerebrospinal fluid (CSF): the meaning of a positive CSF cytology. *Neurology.* Oct 1979;29(10):1369-1375.
16. DeAngelis LM, Cairncross JG. A better way to find tumor in the CSF? *Neurology.* Feb 12 2002;58(3):339-340.
17. Straathof CS, de Bruin HG, Dippel DW, Vecht CJ. The diagnostic accuracy of magnetic resonance imaging and cerebrospinal fluid cytology in leptomeningeal metastasis. *J Neurol.* Sep 1999;246(9):810-814.
18. Freilich RJ, Krol G, DeAngelis LM. Neuroimaging and cerebrospinal fluid cytology in the diagnosis of leptomeningeal metastasis. *Ann Neurol.* Jul 1995;38(1):51-57.

19. Zeiser R, Burger JA, Bley TA, Windfuhr-Blum M, Schulte-Monting J, Behringer DM. Clinical follow-up indicates differential accuracy of magnetic resonance imaging and immunocytology of the cerebral spinal fluid for the diagnosis of neoplastic meningitis - a single centre experience. *British journal of haematology.* Mar 2004;124(6):762-768.
20. Oertel J, Huhn D. Immunocytochemical methods in haematology and oncology. *J Cancer Res Clin Oncol.* Aug 2000;126(8):425-440.
21. Tani E, Costa I, Svedmyr E, Skoog L. Diagnosis of lymphoma, leukemia, and metastatic tumor involvement of the cerebrospinal fluid by cytology and immunocytochemistry. *Diagnostic cytopathology.* Feb 1995;12(1):14-22.
22. Hovestadt A, Henzen-Logmans SC, Vecht CJ. Immunohistochemical analysis of the cerebrospinal fluid for carcinomatous and lymphomatous leptomeningitis. *Br J Cancer.* Oct 1990;62(4):653-654.
23. Windhagen A, Maniak S, Heidenreich F. Analysis of cerebrospinal fluid cells by flow cytometry and immunocytochemistry in inflammatory central nervous system diseases: comparison of low- and high-density cell surface antigen expression. *Diagnostic cytopathology.* Nov 1999;21(5):313-318.
24. Jaeger U, Kainz B. Monitoring minimal residual disease in AML: the right time for real time. *Ann Hematol.* Mar 2003;82(3):139-147.
25. Hug A, Storch-Hagenlocher B, Haas J, Vogt-Schaden ME, Goldschmidt H, Wildemann B. Single-cell PCR analysis of the immunoglobulin heavy-chain CDR3 region for the diagnosis of leptomeningeal involvement of B-cell malignancies using standard cerebrospinal fluid cytospins. *J Neurol Sci.* Apr 15 2004;219(1-2):83-88.
26. Ekstein D, Ben-Yehuda D, Slyusarevsky E, Lossos A, Linetsky E, Siegal T. CSF analysis of IgH gene rearrangement in CNS lymphoma: relationship to the disease course. *J Neurol Sci.* Aug 15 2006;247(1):39-46.
27. Rhodes CH, Honsinger C, Sorenson GD. PCR-detection of tumor-derived p53 DNA in cerebrospinal fluid. *American journal of clinical pathology.* Apr 1995;103(4):404-408.
28. Swinkels DW, de Kok JB, Hanselaar A, Lamers K, Boerman RH. Early detection of leptomeningeal metastasis by PCR examination of tumor-derived K-ras DNA in cerebrospinal fluid. *Clin Chem.* Jan 2000;46(1):132-133.
29. Rhodes CH, Honsinger C, Sorenson GD. Detection of tumor-derived DNA in cerebrospinal fluid. *J Neuropathol Exp Neurol.* Jul 1994;53(4):364-368.
30. Lugli E, Roederer M, Cossarizza A. Data analysis in flow cytometry: the future just started. *Cytometry A.* Jul 2010;77(7):705-713.
31. Kraan J, Gratama JW, Haioun C, et al. Flow cytometric immunophenotyping of cerebrospinal fluid. *Current protocols in cytometry / editorial board, J. Paul Robinson, managing editor et al.* Jul 2008;Chapter 6:Unit 6 25.
32. McLaughlin BE, Baumgarth N, Bigos M, et al. Nine-color flow cytometry for accurate measurement of T cell subsets and cytokine responses. Part I: Panel design by an empiric approach. *Cytometry A.* May 2008;73(5):400-410.
33. Brando B, Barnett D, Janossy G, et al. Cytofluorometric methods for assessing absolute numbers of cell subsets in blood. European Working Group on Clinical Cell Analysis. *Cytometry.* Dec 15 2000;42(6):327-346.
34. Dux R, Kindler-Rohrborn A, Annas M, Faustmann P, Lennartz K, Zimmermann CW. A standardized protocol for flow cytometric analysis of cells isolated from cerebrospinal fluid. *J Neurol Sci.* Jan 1994;121(1):74-78.

35. de Graaf M, de Beukelaar J, Bergsma J, et al. B and T cell imbalances in CSF of patients with Hu-antibody associated PNS. *J Neuroimmunol.* Mar 2008;195(1-2):164-170.

36. Heinrich A, Ahrens N, Schmidt S, Khaw AV. Immunophenotypic patterns of T-cell activation in neuroinflammatory diseases. *Acta Neurol Scand.* Apr 2006;113(4):248-255.

37. Maxeiner HG, Rojewski MT, Schmitt A, Tumani H, Bechter K, Schmitt M. Flow cytometric analysis of T cell subsets in paired samples of cerebrospinal fluid and peripheral blood from patients with neurological and psychiatric disorders. *Brain Behav Immun.* Jan 2009;23(1):134-142.

38. Svenningsson A, Hansson GK, Andersen O, Andersson R, Patarroyo M, Stemme S. Adhesion molecule expression on cerebrospinal fluid T lymphocytes: evidence for common recruitment mechanisms in multiple sclerosis, aseptic meningitis, and normal controls. *Ann Neurol.* Aug 1993;34(2):155-161.

39. Svenningsson A, Andersen O, Edsbagge M, Stemme S. Lymphocyte phenotype and subset distribution in normal cerebrospinal fluid. *J Neuroimmunol.* Dec 1995;63(1):39-46.

40. de Graaf MT, Smitt PA, Luitwieler RL, et al. Central memory CD4(+) T cells dominate the normal cerebrospinal fluid. *Cytometry.* Jul 14 2011;80(1):43-50.

41. Pashenkov M, Huang YM, Kostulas V, Haglund M, Soderstrom M, Link H. Two subsets of dendritic cells are present in human cerebrospinal fluid. *Brain.* Mar 2001;124(Pt 3):480-492.

42. French CA, Dorfman DM, Shaheen G, Cibas ES. Diagnosing lymphoproliferative disorders involving the cerebrospinal fluid: increased sensitivity using flow cytometric analysis. *Diagnostic cytopathology.* Dec 2000;23(6):369-374.

43. Hegde U, Filie A, Little RF, et al. High incidence of occult leptomeningeal disease detected by flow cytometry in newly diagnosed aggressive B-cell lymphomas at risk for central nervous system involvement: the role of flow cytometry versus cytology. *Blood.* Jan 15 2005;105(2):496-502.

44. Nuckel H, Novotny JR, Noppeney R, Savidou I, Duhrsen U. Detection of malignant haematopoietic cells in the cerebrospinal fluid by conventional cytology and flow cytometry. *Clinical and laboratory haematology.* Feb 2006;28(1):22-29.

45. Roma AA, Garcia A, Avagnina A, Rescia C, Elsner B. Lymphoid and myeloid neoplasms involving cerebrospinal fluid: comparison of morphologic examination and immunophenotyping by flow cytometry. *Diagnostic cytopathology.* Nov 2002;27(5):271-275.

46. Subira D, Castanon S, Roman A, et al. Flow cytometry and the study of central nervous disease in patients with acute leukaemia. *British journal of haematology.* Feb 2001;112(2):381-384.

47. Finn WG, Peterson LC, James C, Goolsby CL. Enhanced detection of malignant lymphoma in cerebrospinal fluid by multiparameter flow cytometry. *American journal of clinical pathology.* Sep 1998;110(3):341-346.

48. Sayed D, Badrawy H, Ali AM, Shaker S. Immunophenotyping and immunoglobulin heavy chain gene rearrangement analysis in cerebrospinal fluid of pediatric patients with acute lymphoblastic leukemia. *Leukemia research.* May 2009;33(5):655-661.

49. Kleine TO, Albrecht J, Zofel P. Flow cytometry of cerebrospinal fluid (CSF) lymphocytes: alterations of blood/CSF ratios of lymphocyte subsets in inflammation disorders of human central nervous system (CNS). *Clin Chem Lab Med.* Mar 1999;37(3):231-241.

50. Dahmen UM, Boettcher M, Krawczyk M, Broelsch CE. Flow cytometric "rare event analysis": a standardized approach to the analysis of donor cell chimerism. *J Immunol Methods.* Apr 1 2002;262(1-2):53-69.

51. Sancho JM, Orfao A, Quijano S, et al. Clinical significance of occult cerebrospinal fluid involvement assessed by flow cytometry in non-Hodgkin's lymphoma patients at high risk of central nervous system disease in the rituximab era. *Eur J Haematol.* Oct 2010;85(4):321-328.

52. Nowakowski GS, Call TG, Morice WG, Kurtin PJ, Cook RJ, Zent CS. Clinical significance of monoclonal B cells in cerebrospinal fluid. *Cytometry.* Jan 2005;63(1):23-27.

53. Almeida SM, Nanakanishi E, Conto AJ, Souza LP, Antonelli Filho D, Roda CD. Cerebrospinal fluid cytological and biochemical characteristics in the presence of CNS neoplasia. *Arq Neuropsiquiatr.* Sep 2007;65(3B):802-809.

54. Urbanits S, Griesmacher A, Hopfinger G, et al. FACS analysis-a new and accurate tool in the diagnosis of lymphoma in the cerebrospinal fluid. *Clin Chim Acta.* Mar 2002;317(1-2):101-107.

55. Brem S, Black P, Brem H, Chamberlain MC, Chiocca EA. NCCN clinical practice guideline in oncology. Central nervous system cancers. V1.1.2008. 2008.

56. Stetler-Stevenson M, Braylan RC. Flow cytometric analysis of lymphomas and lymphoproliferative disorders. *Semin Hematol.* Apr 2001;38(2):111-123.

57. Moser RP, Robinson JA, Prostko ER. Lymphocyte subpopulations in human cerebrospinal fluid. *Neurology.* Aug 1976;26(8):726-728.

58. Wilson DB, Golding AB, Smith RA, et al. Results of a phase I clinical trial of a T-cell receptor peptide vaccine in patients with multiple sclerosis. I. Analysis of T-cell receptor utilization in CSF cell populations. *J Neuroimmunol.* Jun 1997;76(1-2):15-28.

59. Huang YH, Zozulya AL, Weidenfeller C, et al. Specific central nervous system recruitment of HLA-G(+) regulatory T cells in multiple sclerosis. *Ann Neurol.* Aug 2009;66(2):171-183.

60. Venken K, Hellings N, Thewissen M, et al. Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology.* Jan 2008;123(1):79-89.

61. Oreja-Guevara C, Sindern E, Raulf-Heimsoth M, Malin JP. Analysis of lymphocyte subpopulations in cerebrospinal fluid and peripheral blood in patients with multiple sclerosis and inflammatory diseases of the nervous system. *Acta Neurol Scand.* Nov 1998;98(5):310-313.

62. Svenningsson A, Andersen O, Hansson GK, Stemme S. Reduced frequency of memory CD8+ T lymphocytes in cerebrospinal fluid and blood of patients with multiple sclerosis. *Autoimmunity.* 1995;21(4):231-239.

63. Haegele KF, Stueckle CA, Malin JP, Sindern E. Increase of CD8+ T-effector memory cells in peripheral blood of patients with relapsing-remitting multiple sclerosis compared to healthy controls. *J Neuroimmunol.* Feb 2007;183(1-2):168-174.

64. Okuda Y, Okuda M, Apatoff BR, Posnett DN. The activation of memory CD4(+) T cells and CD8(+) T cells in patients with multiple sclerosis. *J Neurol Sci.* Aug 15 2005;235(1-2):11-17.

65. Matsui M, Araya S, Wang HY, Matsushima K, Saida T. Differences in systemic and central nervous system cellular immunity relevant to relapsing-remitting multiple sclerosis. *J Neurol.* Aug 2005;252(8):908-915.

66. Barrau MA, Montalban X, Saez-Torres I, Brieva L, Barbera N, Martinez-Caceres EM. CD4(+) CD45RO(+)CD49d(high) cells are involved in the pathogenesis of relapsing-remitting multiple sclerosis. *J Neuroimmunol.* Nov 1 2000;111(1-2):215-223.

67. Matsui M, Mori KJ, Saida T, Akiguchi I, Kameyama M. The imbalance in CSF T cell subsets in active multiple sclerosis. *Acta Neurol Scand.* Mar 1988;77(3):202-209.

68. Kraus J, Oschmann P, Engelhardt B, et al. CD45RA+ ICAM-3+ lymphocytes in cerebrospinal fluid and blood as markers of disease activity in patients with multiple sclerosis. *Acta Neurol Scand.* Nov 2000;102(5):326-332.

69. Kraus J, Oschmann P, Engelhardt B, et al. Soluble and cell surface ICAM-1 as markers for disease activity in multiple sclerosis. *Acta Neurol Scand.* Aug 1998;98(2):102-109.

70. Kuenz B, Lutterotti A, Ehling R, et al. Cerebrospinal fluid B cells correlate with early brain inflammation in multiple sclerosis. *PLoS One.* 2008;3(7):e2559.

71. Cepok S, Rosche B, Grummel V, et al. Short-lived plasma blasts are the main B cell effector subset during the course of multiple sclerosis. *Brain.* Jul 2005;128(Pt 7):1667-1676.

72. Harp C, Lee J, Lambracht-Washington D, et al. Cerebrospinal fluid B cells from multiple sclerosis patients are subject to normal germinal center selection. *J Neuroimmunol.* Feb 2007;183(1-2):189-199.

73. Monson NL, Cravens PD, Frohman EM, Hawker K, Racke MK. Effect of rituximab on the peripheral blood and cerebrospinal fluid B cells in patients with primary progressive multiple sclerosis. *Archives of neurology.* Feb 2005;62(2):258-264.

74. Corcione A, Casazza S, Ferretti E, et al. Recapitulation of B cell differentiation in the central nervous system of patients with multiple sclerosis. *Proceedings of the National Academy of Sciences of the United States of America.* Jul 27 2004;101(30):11064-11069.

75. Sellebjerg F, Jensen J, Ryder LP. Costimulatory CD80 (B7-1) and CD86 (B7-2) on cerebrospinal fluid cells in multiple sclerosis. *J Neuroimmunol.* Apr 15 1998;84(2):179-187.

76. Svenningsson A, Dotevall L, Stemme S, Andersen O. Increased expression of B7-1 costimulatory molecule on cerebrospinal fluid cells of patients with multiple sclerosis and infectious central nervous system disease. *J Neuroimmunol.* May 1997;75(1-2):59-68.

77. Cross AH, Stark JL, Lauber J, Ramsbottom MJ, Lyons JA. Rituximab reduces B cells and T cells in cerebrospinal fluid of multiple sclerosis patients. *J Neuroimmunol.* Nov 2006;180(1-2):63-70.

78. Stuve O, Marra CM, Jerome KR, et al. Immune surveillance in multiple sclerosis patients treated with natalizumab. *Ann Neurol.* May 2006;59(5):743-747.

79. Sellebjerg F, Christiansen M, Jensen J, Frederiksen JL. Immunological effects of oral high-dose methylprednisolone in acute optic neuritis and multiple sclerosis. *Eur J Neurol.* May 2000;7(3):281-289.

80. Pranzatelli MR, Travelstead AL, Tate ED, et al. B- and T-cell markers in opsoclonus-myoclonus syndrome: immunophenotyping of CSF lymphocytes. *Neurology.* May 11 2004;62(9):1526-1532.

81. Pranzatelli MR, Travelstead AL, Tate ED, Allison TJ, Verhulst SJ. CSF B-cell expansion in opsoclonus-myoclonus syndrome: a biomarker of disease activity. *Mov Disord.* Jul 2004;19(7):770-777.

82. Subira D, Castanon S, Aceituno E, et al. Flow cytometric analysis of cerebrospinal fluid samples and its usefulness in routine clinical practice. *American journal of clinical pathology.* Jun 2002;117(6):952-958.

83. Gross HJ, Verwer B, Houck D, Hoffman RA, Recktenwald D. Model study detecting breast cancer cells in peripheral blood mononuclear cells at frequencies as low as 10(-7). *Proceedings of the National Academy of Sciences of the United States of America.* Jan 17 1995;92(2):537-541.

84. Chow G, Schmidley JW. Lysis of erythrocytes and leukocytes in traumatic lumbar punctures. *Archives of neurology.* Oct 1984;41(10):1084-1085.

85. Steele RW, Marmer DJ, O'Brien MD, Tyson ST, Steele CR. Leukocyte survival in cerebrospinal fluid. *Journal of clinical microbiology.* May 1986;23(5):965-966.

86. Veerman AJ, Huismans L, van Zantwijk I. Storage of cerebrospinal fluid samples at room temperature. *Acta cytologica.* Mar-Apr 1985;29(2):188-189.

87. Cunniffe JG, Whitby-Strevens S, Wilcox MH. Effect of pH changes in cerebrospinal fluid specimens on bacterial survival and antigen test results. *Journal of clinical pathology.* Mar 1996;49(3):249-253.

88. Fry MM, Vernau W, Kass PH, Vernau KM. Effects of time, initial composition, and stabilizing agents on the results of canine cerebrospinal fluid analysis. *Veterinary clinical pathology / American Society for Veterinary Clinical Pathology.* Mar 2006;35(1):72-77.

89. Hulspas R, O'Gorman MR, Wood BL, Gratama JW, Sutherland DR. Considerations for the control of background fluorescence in clinical flow cytometry. *Cytometry.* Nov 2009;76(6):355-364.

90. Perfetto SP, Roederer M. Increased immunofluorescence sensitivity using 532 nm laser excitation. *Cytometry A.* Feb 2007;71(2):73-79.

91. Alberti S, Parks DR, Herzenberg LA. A single laser method for subtraction of cell autofluorescence in flow cytometry. *Cytometry.* Mar 1987;8(2):114-119.

92. Roederer M, Murphy RF. Cell-by-cell autofluorescence correction for low signal-to-noise systems: application to epidermal growth factor endocytosis by 3T3 fibroblasts. *Cytometry.* Nov 1986;7(6):558-565.

93. Srivastava P, Sladek TL, Goodman MN, Jacobberger JW. Streptavidin-based quantitative staining of intracellular antigens for flow cytometric analysis. *Cytometry.* 1992;13(7):711-721.

94. Mayefsky JH, Roghmann KJ. Determination of leukocytosis in traumatic spinal tap specimens. *Am J Med.* Jun 1987;82(6):1175-1181.

95. Novak RW. Lack of validity of standard corrections for white blood cell counts of blood-contaminated cerebrospinal fluid in infants. *American journal of clinical pathology.* Jul 1984;82(1):95-97.

96. Reske A, Haferkamp G, Hopf HC. Influence of artificial blood contamination of the analysis of cerebrospinal fluid. *J Neurol.* 1981;226(3):187-193.

97. te Loo DM, Kamps WA, van der Does-van den Berg A, van Wering ER, de Graaf SS. Prognostic significance of blasts in the cerebrospinal fluid without pleiocytosis or a traumatic lumbar puncture in children with acute lymphoblastic leukemia: experience of the Dutch Childhood Oncology Group. *J Clin Oncol.* May 20 2006;24(15):2332-2336.

98. Chizuka A, Kanda Y, Nannya Y, et al. The diagnostic value of kappa/lambda ratios determined by flow cytometric analysis of biopsy specimens in B-cell lymphoma. *Clinical and laboratory haematology.* Feb 2002;24(1):33-36.

99. Davidson B, Risberg B, Berner A, Smeland EB, Torlakovic E. Evaluation of lymphoid cell populations in cytology specimens using flow cytometry and polymerase chain reaction. *Diagn Mol Pathol.* Dec 1999;8(4):183-188.

100. Zardawi IM, Jain S, Bennett G. Flow-cytometric algorithm on fine-needle aspirates for the clinical workup of patients with lymphadenopathy. *Diagnostic cytopathology.* Oct 1998;19(4):274-278.

101. Fukushima PI, Nguyen PK, O'Grady P, Stetler-Stevenson M. Flow cytometric analysis of kappa and lambda light chain expression in evaluation of specimens for B-cell neoplasia. *Cytometry.* Dec 15 1996;26(4):243-252.

102. Morse EE, Yamase HT, Greenberg BR, et al. The role of flow cytometry in the diagnosis of lymphoma: a critical analysis. *Ann Clin Lab Sci.* Jan-Feb 1994;24(1):6-11.

103. Geary WA, Frierson HF, Innes DJ, Normansell DE. Quantitative criteria for clonality in the diagnosis of B-cell non-Hodgkin's lymphoma by flow cytometry. *Mod Pathol.* Mar 1993;6(2):155-161.

104. Witzig TE, Banks PM, Stenson MJ, et al. Rapid immunotyping of B-cell non-Hodgkin's lymphomas by flow cytometry. A comparison with the standard frozen-section method. *American journal of clinical pathology.* Sep 1990;94(3):280-286.

105. Hanse MC, Van't Veer MB, van Lom K, van den Bent MJ. Incidence of central nervous system involvement in chronic lymphocytic leukemia and outcome to treatment. *J Neurol.* Jun 2008;255(6):828-830.

106. Cramer SC, Glaspy JA, Efird JT, Louis DN. Chronic lymphocytic leukemia and the central nervous system: a clinical and pathological study. *Neurology.* Jan 1996;46(1):19-25.

107. Pedreira CE, Costa ES, Barrena S, et al. Generation of flow cytometry data files with a potentially infinite number of dimensions. *Cytometry A.* Sep 2008;73(9):834-846.

108. Nomura L, Maino VC, Maecker HT. Standardization and optimization of multiparameter intracellular cytokine staining. *Cytometry A.* Nov 2008;73(11):984-991.

109. Habib LK, Finn WG. Unsupervised immunophenotypic profiling of chronic lymphocytic leukemia. *Cytometry.* May 2006;70(3):124-135.

110. Boddy L, Wilkins MF, Morris CW. Pattern recognition in flow cytometry. *Cytometry.* Jul 1 2001;44(3):195-209.

111. Costa ES, Arroyo ME, Pedreira CE, et al. A new automated flow cytometry data analysis approach for the diagnostic screening of neoplastic B-cell disorders in peripheral blood samples with absolute lymphocytosis. *Leukemia.* Jul 2006;20(7):1221-1230.

112. Costa ES, Pedreira CE, Barrena S, et al. Automated pattern-guided principal component analysis vs expert-based immunophenotypic classification of B-cell chronic lymphoproliferative disorders: a step forward in the standardization of clinical immunophenotyping. *Leukemia.* Nov 2010;24(11):1927-1933.

113. Rhodes CH, Glantz MJ, Glantz L, et al. A comparison of polymerase chain reaction examination of cerebrospinal fluid and conventional cytology in the diagnosis of lymphomatous meningitis. *Cancer.* Feb 1 1996;77(3):543-548.

114. Gleissner B, Siehl J, Korfel A, Reinhardt R, Thiel E. CSF evaluation in primary CNS lymphoma patients by PCR of the CDR III IgH genes. *Neurology.* Feb 12 2002;58(3):390-396.

4

Effector memory and late memory T cells accumulate in the blood of CMV-carrying individuals but not in their cerebrospinal fluid

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ABSTRACT

Cytomegalovirus (CMV)-carrying individuals have significantly higher levels of effector memory and late memory T lymphocytes in their blood than noncarriers. To date, it is well recognized that the central nervous system (CNS) is subjected to active immunosurveillance, as evidenced by the presence of central memory T cells in cerebrospinal fluid (CSF) of healthy individuals. In order to investigate whether levels of effector memory and late memory T cells were also increased in the CSF of CMV-carrying individuals, we characterized $CD4^+$ and $CD8^+$ T-cell subsets in CSF and blood of both groups. Effector memory and late memory T cells were only rarely seen in CSF, which was similar in CMV carriers and noncarriers. In conclusion, there was no demonstrable difference in the numbers of effector memory and late memory T cells between CMV seronegative and CMV seropositive individuals.

INTRODUCTION

CMV-carrying individuals are known to have higher absolute numbers of effector memory and late memory T cells in their blood than non-carriers.[1,2] These T cells express CD57, do not express CD27 or CD28 and are $CD45RA^-$ (effector memory) or $CD45RA^+$ (late memory).[3] In the blood, CMV-specific T cells targeted against a single epitope can make up to 42% of $CD8^+$ T cells, and most CMV-specific T cells are effector memory or late memory T cells.[3] In contrast to earlier paradigms, it has become clear that the central nervous system (CNS) is also subjected to active immunosurveillance.[4] Central memory T cells are found in cerebrospinal fluid (CSF) of healthy individuals, and mice models showed trafficking of these cells between the CNS and cervical lymph lymph nodes.[5-7] Recently, it has been shown that, in contrast to the blood, lymph nodes of CMV-carrying individuals have only low numbers of effector memory and late memory T cells.[3] A possible explanation for this finding is that effector memory and late memory T cells do not express the chemokine receptor CCR7 needed for T cell-homing to the lymph nodes.[8] Other chemokine receptors such as CCR5 andCCR6 are thought to promote T cell homing to the CNS.[9,10] Similar to CCR7, the level of expression of these chemokine receptors is higher in central memory T cells than in effector memory or late memory T cells.[8] Therefore, we hypothesized that the effector memory or late memory T cells in the blood of CMV-carrying individuals do not traffic to the CNS. To test this hypothesis, we studied absolute numbers of naïve, central memory, effector memory and late memory T cells in CSF and blood of CMV-carrying individuals and non-carriers.

METHODS

Twenty-five patients were included who underwent lumbar puncture for spinal anaesthesia (n=15), or for diagnostic purposes (n= 10; Table 1). The study was approved by the local ethical review committee, and written informed consent was obtained. Two to 5 mL CSF was collected in 2 mL serum-containing medium, and analyzed by 6-color flow cytometry as previously described.[5] For enumeration of leukocyte and lymphocyte subsets in CSF, we used a single 6-color mixture of monoclonal antibodies (mAb): CD3 conjugated with fluorescein isothiocyanate (FITC; clone SK7), CD56 conjugated with phycoerythrin (PE; clone C5.9), CD45 conjugated with peridinyl cholorophyllin (PerCP; clone 2D1), CD4 conjugated with PE-Cy7 (clone SK3), CD19 conjugated with allophycocyanin (APC; clone HIB19) and CD8 conjugated with APC-Cy7 (clone SK1). For blood two 4-color mAb mixtures were used: CD3-FITC, CD56-PE, CD45-PerCP and CD19-APC and CD4-FITC (clone HP2/6), CD8-PE (clone SK1), CD45-PerCP and CD3-APC (clone

Table 1. Patient characteristics

	CMV-	CMV+	P[c]
N	12	13	
Gender (M/F)	6/6	4/9	0.43
Age (years)[A]	61 (32-88)	57 (19-76)	0.32
Spinal aneasthesia / diagnostic[B]	9/3	6/7	0.23
CSF leukocyte counts ($\times 10^6$/L)[A]	3 (1-7)	2 (1-5)	0.71
CSF protein concentration (g/L)[A]	0.34 (0.18-0.49)	0.33 (0.20-0.44)	0.37

[A] median, range
[B] Included were patients without neurological disease who underwent lumbar puncture for spinal anaesthesia (usually for orthopaedic surgery) and patients who had a diagnostic lumbar puncture for suspected neurological disease (none of these patients turned out to suffer from a neuro-inflammatory disorder).
[C] P-values were calculated with the Mann-Whitney or Fisher exact test.
Abbreviations: CMV, cytomegalovirus; N, number of patients; M, male; F, female; CNS, central nervous system; CSF, cerebrospinal fluid.

SK7). T cell subsets in both compartments were assessed with a 6-colorpanel: CD45RA-FITC (clone L48), CD127-PE (clone hIL-7R-M21), CD4-PerCP (clone SK3), CD25-PE-Cy7 (clone 2A3), CD27/28-APC (clone L128/CD28.2) and CD3-APCeFluor780 (clone UCHT1). All mAb were obtained from BD Biosciences (San Jose, CA) with the exception of CD56-PE (Dako, Glostrup, Denmark), and CD3-APC-eFluor780 and CD19-APC (eBioscience, San Diego, CA). $CD4^+$ and $CD8^+$ T cells were further classified according to CD45RA expression and CD27 and CD28 expression in naïve T cells ($CD45RA^+$, $27/28^+$), central memory T cells ($CD45RA^-$, $CD27/28^+$), effector memory T cells ($CD45RA^-$, $CD27/28^-$) and late memory T cells ($CD45RA^+$, $CD27/28^-$) as previously described.[5] Samples were acquired on a FACSCanto flow cytometer (BD Biosciences) and analyzed using FCS Express software (De Novo Software, Los Angeles, CA). For absolute numbers of total T lymphocytes in blood, we used a stain, lyse, no-wash method based on counting beads. For further characterization of T cell subsets, we used a lyse, stain and wash technique. CSF cells were concentrated by centrifugation and resuspended in 400 µL PBS. Of this suspension, 100 µL was stained for each panel, washed, and resuspended in 100 µL PBS. For absolute cell counts, 100 µL of counting beads were added [5]. Individuals were excluded from the study if their CSF sample contained less than 25 leukocytes per staining.[11] Since absolute cell numbers in CSF of patients that underwent spinal anesthesia were not significantly different from those of patients that underwent a diagnostic lumbar puncture but were not diagnosed with a neuroinflammatory disease (e.g. tension headache, data not shown), these patient groups were pooled for analysis. The absolute cell numbers of each subset were stratified according to CMV serostatus, and cell numbers in CMV seropositive versus CMV seronegative individuals were compared with the Mann-Whitney test using SPSS version 20 (IBM, Chicago, IL). Figure 2 was made with Prism version 5 (GraphPad, San Diego, CA).

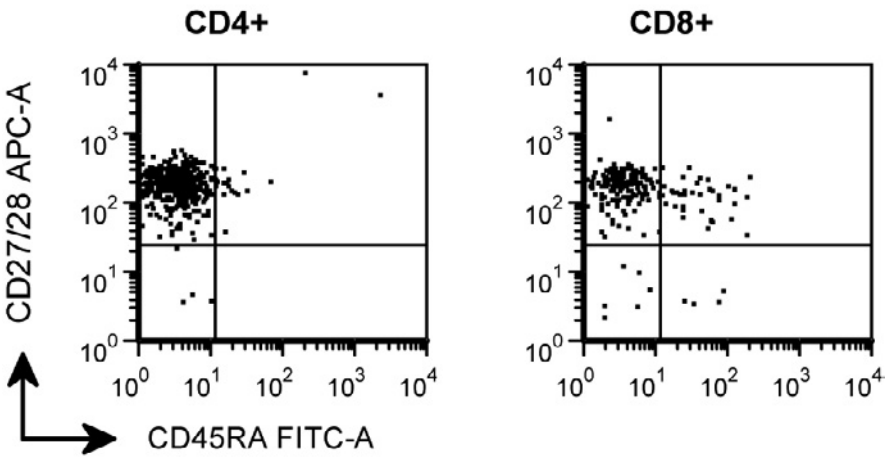

Figure 1.
Representative example of the expression of CD45RA and CD27/28 on $CD4^+$ and $CD8^+$ T cells in CSF of a CMV seropositive patient. The vast majority of $CD4^+$ and $CD8^+$ T cells are $CD45RA^-CD27/28^+$ central memory T cells. $CD45RA^-CD27/28^-$ effector memory T cells and $CD45RA^+CD27/28^-$ late memory T cells were only sporadically detected, similar to CMV seronegative patients (data not shown).

RESULTS

Patient characteristics are shown in table 1. Because all CSF samples contained a statistically sufficient number of leukocytes (we measured a median of 498 leukocytes per panel, while more than 25 leukocytes per panel are required), no patients were excluded from analysis. Both groups (CMV seronegative patients and CMV seropositive patients) did not differ significantly with respect to gender, age and reason for lumbar puncture. Also, total CSF leukocyte counts and CSF protein concentrations were similar in both groups. Figure 2 shows absolute numbers of naïve, central memory, effector memory and late memory $CD4^+$ and $CD8^+$ T cells in CSF (panel A and B) and blood (panel C and D) of CMV seronegative and CMV seropositive individuals. In the blood, a trend was seen towards higher numbers of $CD4^+$ effector memory and $CD4^+$ late memory T cells in CMV seropositive individuals as compared to CMV seronegative individuals, but these differences did not reach statistical significance ($p=0.17$ and $p=0.19$, respectively). The numbers of $CD4^+$ effector memory and $CD4^+$ late memory T cells in CSF were uniformly low in CMV carriers and non-carriers.

The numbers of $CD8^+$ effector memory and late memory T cells in the blood were significantly higher in CMV seropositive individuals. Within the $CD8^+$ T lymphocytes, effector memory and late memory cells were only rarely seen in CSF (median, 0.02;

range, 0.00-0.11 cells/μL and 0.02; 0.00-0.06 cells/μL respectively) and did not differ significantly between CMV seronegative and CMV seropositive individuals.

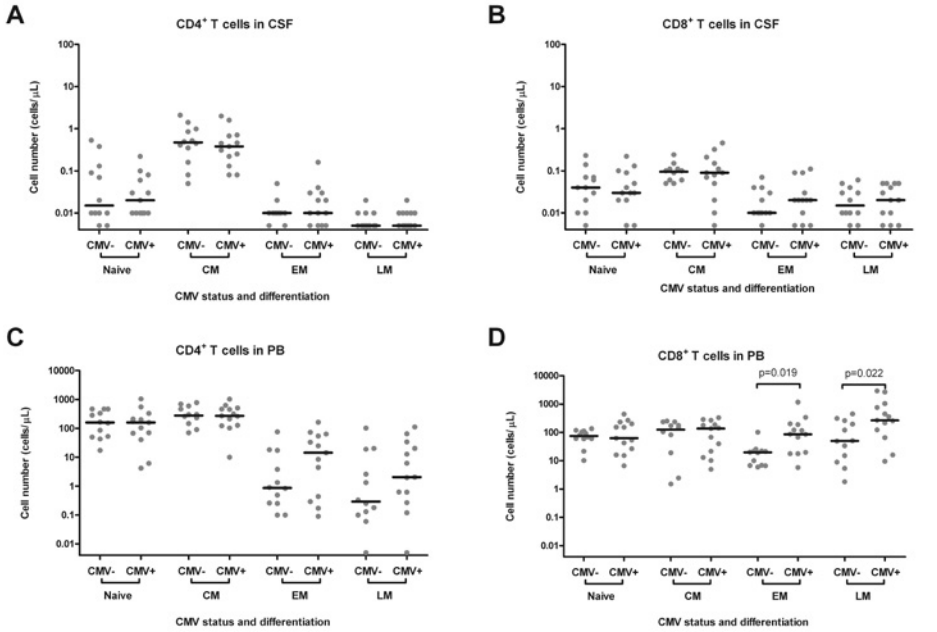

Figure 2.
Absolute numbers of naïve, central memory, effector memory and late memory $CD4^+$ and $CD8^+$ T cells in CSF (panel A and B) and blood (panel C and D) of CMV seronegative and CMV seropositive individuals. Data are shown on logarithmic scales in order to compress the figure. Horizontal lines represent medians. For graphic representation, cell numbers <0.01 cells/μL (corresponding to < 5 measured events) were assigned a value of 0.005 cells/μL. P-values represent the results of the Mann-Whitney test. Abbreviations: CSF, cerebrospinal fluid; PB, peripheral blood; CMV, cytomegalovirus; CMV+, seropositive for CMV; CMV-, seronegative for CMV; CM, central memory; EM, effector memory; LM, late memory.

DISCUSSION

In this study, we found no demonstrable difference between the numbers of effector memory and late memory T cells in CSF of CMV seronegative patients and those of CMV seropositive individuals, despite significant differences in the numbers of these subsets in the blood. These changes in the blood were most prominent and significant within the $CD8^+$ T cells, and to a lesser extend seen in $CD4^+$ T cells (not reaching significance). The numbers of circulating effector memory and late memory T cells in the blood were shown to increase with age, and are strongly correlated with latent

CMV infection.[12] Their presence is thought to at least partly result from antigenic stimulation of T cells by latently CMV-infected endothelial and myeloid cells. CMV-specific T cells are thought to prevent viral reactivation by cytolysis of infected cells.[3,12] Interestingly, the lymph nodes of CMV-carrying individuals contain only few effector memory and late memory T cells, and the few CMV-specific T cells present have a central memory phenotype.[3] Our findings in CSF support the hypothesis that $CD8^+$ effector memory and late memory T cells under normal circumstances do not enter the CNS. This situation may be due to a low level of expression of chemokine receptors such as CCR5 and CCR6 that are needed for T cell homing to the CNS.[8,10] In addition, lack of expression of molecules such as CD162 (selectin P ligand) and CD49D (α 4 subunit of very late antigen 4 receptor) by T lymphocytes or selectin P and very late antigen 4 by endothelial cells may prevent migration of T lymphocytes across vascular endothelium.[4] In this study, we did neither assess the expression of these molecules nor the specificity of the CSF T cells. For this moment, we hypothesize that effector memory and late memory T cells only enter the CNS under exceptional circumstances (for example in case of CMV encephalitis), while under normal conditions, central memory T cells enter and leave the CNS in the context of CNS immunosurveillance.

REFERENCES

1. Kuijpers TW, Vossen MT, Gent MR, et al. Frequencies of circulating cytolytic, CD45RA+CD27-, CD8+ T lymphocytes depend on infection with CMV. *J Immunol.* Apr 15 2003;170(8):4342-4348.
2. Gratama JW, Naipal AM, Oosterveer MA, et al. Effects of herpes virus carrier status on peripheral T lymphocyte subsets. *Blood.* Aug 1987;70(2):516-523.
3. Remmerswaal EB, Havenith SH, Idu MM, et al. Human virus-specific effector-type T cells accumulate in blood but not in lymph nodes. *Blood.* Feb 16 2012;119(7):1702-1712.
4. Ransohoff RM, Engelhardt B. The anatomical and cellular basis of immune surveillance in the central nervous system. *Nature reviews.* Aug 10 2012;12(9):623-635.
5. de Graaf MT, Smitt PA, Luitwieler RL, et al. Central memory CD4(+) T cells dominate the normal cerebrospinal fluid. *Cytometry.* Jul 14 2011;80(1):43-50.
6. Kivisakk P, Mahad DJ, Callahan MK, et al. Expression of CCR7 in multiple sclerosis: implications for CNS immunity. *Ann Neurol.* May 2004;55(5):627-638.
7. Furtado GC, Marcondes MC, Latkowski JA, Tsai J, Wensky A, Lafaille JJ. Swift entry of myelin-specific T lymphocytes into the central nervous system in spontaneous autoimmune encephalomyelitis. *J Immunol.* Oct 1 2008;181(7):4648-4655.
8. Wills MR, Okecha G, Weekes MP, Gandhi MK, Sissons PJ, Carmichael AJ. Identification of naive or antigen-experienced human CD8(+) T cells by expression of costimulation and chemokine receptors: analysis of the human cytomegalovirus-specific CD8(+) T cell response. *J Immunol.* Jun 1 2002;168(11):5455-5464.
9. Yamazaki T, Yang XO, Chung Y, et al. CCR6 regulates the migration of inflammatory and regulatory T cells. *J Immunol.* Dec 15 2008;181(12):8391-8401.
10. Kivisakk P, Trebst C, Liu Z, et al. T-cells in the cerebrospinal fluid express a similar repertoire of inflammatory chemokine receptors in the absence or presence of CNS inflammation: implications for CNS trafficking. *Clinical and experimental immunology.* Sep 2002;129(3):510-518.
11. Kraan J, Gratama JW, Haioun C, et al. Flow cytometric immunophenotyping of cerebrospinal fluid. *Current protocols in cytometry / editorial board, J. Paul Robinson et al.* Jul 2008;Chapter 6:Unit 6 25.
12. Looney RJ, Falsey A, Campbell D, et al. Role of cytomegalovirus in the T cell changes seen in elderly individuals. *Clinical immunology (Orlando, Fla.* Feb 1999;90(2):213-219.

5

Elevated numbers of regulatory T cells, central memory T cells and class-switched B cells in cerebrospinal fluid of patients with anti-Hu antibody associated paraneoplastic neurological syndromes

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ABSTRACT

Multi-parametric flow cytometry was used to study lymphocyte subsets and dendritic cells in paired blood and CSF samples from 11 newly diagnosed patients with progressive anti-Hu antibody associated paraneoplastic neurological syndromes (Hu-PNS), 9 patients with other inflammatory neurologic disorders (IND), and 12 patients with other non-inflammatory neurologic disorders (OND). Hu-PNS patients had elevated numbers of regulatory T cells, central memory T cells, class-switched B cells and dendritic cells in their CSF. These findings support the hypothesis that the immune system is locally activated in Hu-PNS, and suggests common etiological pathways between Hu-PNS and other inflammatory central nervous system disorders.

INTRODUCTION

Anti-Hu antibody associated paraneoplastic neurological syndromes (Hu-PNS) cause severe neurological symptoms and carry a poor prognosis.[1] In Hu-PNS, Hu-antigens expressed by cancer cells are thought to trigger an immune response which cross-reacts with neurons that express the same Hu antigens. Evidence for this immune response is provided by high titers of anti-Hu antibodies (Hu-Ab) in blood and cerebrospinal fluid (CSF) of Hu-PNS patients.[1] Because Hu-proteins are intracellular proteins and because a direct pathogenic role of Hu-Ab could never be proven[2], it has been hypothesized that neuronal destruction in Hu-PNS is mediated by cytotoxic T cells.[3] A pathogenic role for cytotoxic T cells is supported by autopsy studies showing cytotoxic T cells in close proximity to damaged neurons.[4,5] However, studies to identify cytotoxic T cells specific for Hu-proteins have shown conflicting results.[6-11] Therefore, the possible role of the immune system in Hu-PNS remains incompletely understood.

Previously, we have reported that blood of Hu-PNS patients contained higher numbers of regulatory T cells (Tregs) and higher proportions of activated T cells than blood of small-cell lung cancer patients and healthy controls.[12] These findings supported the hypothesis that Hu-PNS is an immune-mediated disease in which $CD4^+$ T cells – including Tregs – and $CD8^+$ T cells are involved. In CSF of Hu-PNS patients, we showed that, next to approximately five times higher numbers of $CD4^+$ T cells and $CD8^+$ T cells, B cell numbers were 20 times higher than in non-cancer controls and cancer patients without PNS.[13] The high B cell numbers in CSF are in line with the intrathecal antibody-production found in these patients.[14]

In other neurological inflammatory diseases, it has been shown that lymphocytes in CSF might search for their specific antigen, and – if they encounter their specific antigen – migrate to the nervous tissue to fulfil their effector functions.[15,16] Additionally, dendritic cells (DC) were shown to be present in CSF and might play a role by transporting antigens from the CNS to deep cervical lymph nodes, but may also stimulate lymphocytes within the CNS compartment.[17,18]

In the present study, we used multi-parametric flow-cytometry to further investigate the CSF in recently diagnosed Hu-PNS patients with progressive neurological disease, focussing on (i) Tregs, (ii) the differentiation stages of T cells and B cells, and (iii) DCs. These findings were compared with paired blood and CSF samples from patients with other inflammatory neurological disorders (IND) of non-paraneoplastic origin and patients with non-inflammatory neurological disorders (OND).

MATERIALS AND METHODS

Specimen collection

All Hu-PNS patients had a definite diagnosis of Hu-PNS[19] and progressive neurological symptoms over the last 4 weeks. Neurological controls included patients with other inflammatory neurological disorders (IND) and patients with other (non-inflammatory) neurological disorders (OND). The IND control group consisted of three patients with meningitis (two patients treated for bacterial meningitis [one of them had an intracranial mycotic aneurysm], and one treated for cryptococcal meningitis), two patients with transverse myelitis, and one each with optic neuritis, cranial neuritis (n. VII caused by VZV reactivation), PML and cerebral vasculitis. The OND patients consisted of three patients with metabolic encephalopathy (toxic in one), two with headache (one with tension type headache and the other with analgesia induced headache), two with hydrocephalus (one with normal pressure hydrocephalus and one with communicating hydrocephalus) and one each with polyneuropathy, steroid myopathy, benign paroxysmal positional vertigo, carpal tunnel syndrome and radiation myelopathy. Patients who received intrathecal or systemic chemotherapy in the last 12 months were excluded. All but three patients had not received any immunomodulatory medication prior to CSF withdrawal. One Hu-PNS patient received intravenous immunoglobulins 3 weeks prior to CSF withdrawal. In one IND patient (with optic neuritis) CSF was drawn immediately after her first infusion with intravenous methylprednisolone, another IND patient (with cranial neuritis) received tacrolimus 2mg/day (after heart transplantation) and prednisone 5 mg/day. Paired blood and CSF samples were drawn. Blood was collected in EDTA tubes. Three to 5 mL CSF was obtained by lumbar puncture with a 20 Gauge atraumatic Sprotte needle, and collected in serum-containing medium [20] to reduce decay of leukocytes. Methods of sample collection and reference values from NNC have been previously published.[20] The study was approved by the local ethical review committee, and written informed consent was obtained.

Staining procedures

We used fluorochrome-conjugated antibody panels for leukocyte subsets, T cell subsets and DC subsets as we described before.[20] The 6-color antibody panel for B cell subsets was: IgD-FITC (clone IA6-2), IgM-PE, CD38-PerCP-Cy5.5 (clone HIT2), CD27 PE-Cy7 (clone O323), CD138-APC (clone MI15) and CD19-APC-Cy7 (clone SJ25C1). All antibodies were obtained from BD Biosciences (San Jose, CA) with exception of CD56-PE (Dako, Glostrup, Denmark), IgD-FITC (Nordic, Taby, Sweden), IgM-PE (Southern Biotech, Birmingham, USA) and CD3-APC-eFluor780, CD19-APC, CD27-PE-Cy7 and CD123-PE-Cy7 (eBioscience, San Diego, CA). Staining procedures have been described before.[20] Briefly, for absolute numbers of leukocyte subsets in blood, we used a stain,

lyse, no-wash method based on counting beads (Flowcount, Beckman Coulter, Miami, FL). For T cell, B cell and DC subsets in blood, we used a lyse, stain and wash technique. CSF cells were concentrated by centrifugation, resuspended in PBS, stained and washed. For absolute cell counts, 100 µL of counting beads were added (Cyto-Cal Count Control, Duke Scientific Corporation, Palo Alto, CA).

Data analysis

List mode data were acquired on a 6-color FACSCanto flow cytometer (BD Biosciences) and analysed with FCS express (De Novo Software, Los Angeles, CA). Definitions of T cell and DC subsets were described before.[20] Immunological definitions of the B cell subsets were adapted from Caraux et al.[21] and included naïve B cells ($CD19^+$, $CD27^-$, $CD38^{-/+}$), IgD/M memory B cells ($CD19^+$, $CD27^+$, $CD38^{-/+}$, IgD/M^+), class-switched memory B cells ($CD19^+$, $CD27^+$, $CD38^{-/+}$, IgD/M^-) and plasma blasts ($CD19^+$, $CD27^+$, $CD38_{high}$). Gating strategies for T cell subsets, B cell subsets and dendritic cells are shown in figure 1. Absolute cell numbers are expressed as medians and 5th-95th percentiles. We used Fisher's test to compare the distribution of gender, and the Mann-Whitney U test to compare age, CSF protein content and cell numbers in Hu-PNS patients with the control groups (as indicated in Table 1 and 2). Prism version 4 (GraphPad, San Diego, CA) and SPSS version 17.0.2 (IBM, Chicago, IL) were used for the statistical analyses. Two-sided p-values <0.05 were considered significant.

RESULTS

Clinical characteristics

Table 1 shows the clinical characteristics of the 11 Hu-PNS patients and the IND and OND control groups. Four of the 11 Hu-PNS patients presented with an encephalomyelitis, three of them also had a sensory neuronopathy; four patients presented with sensory neuronopathy; one patient with subacute cerebellar ataxia; and one patient with limbic encephalitis. The median score on the modified Rankin scale[22] at the time of study entry was 3 (range 2-4). The median duration of neurological symptoms was 5 months (range 1-12) and the median Hu-Ab titer was 1,600 (range 400-12,800). A tumor was found in 10 of the 11 Hu-PNS patients: Five patients had a small-cell lung cancer, three patients had radiographic evidence for a lung tumor, one patient had a myxoid chondrosarcoma, and one patient had a small cell tumor of the parotid gland. Hu-PNS patients were significantly older than IND patients and Hu-PNS patients had a significantly higher CSF protein level than the OND patients (Table 1).

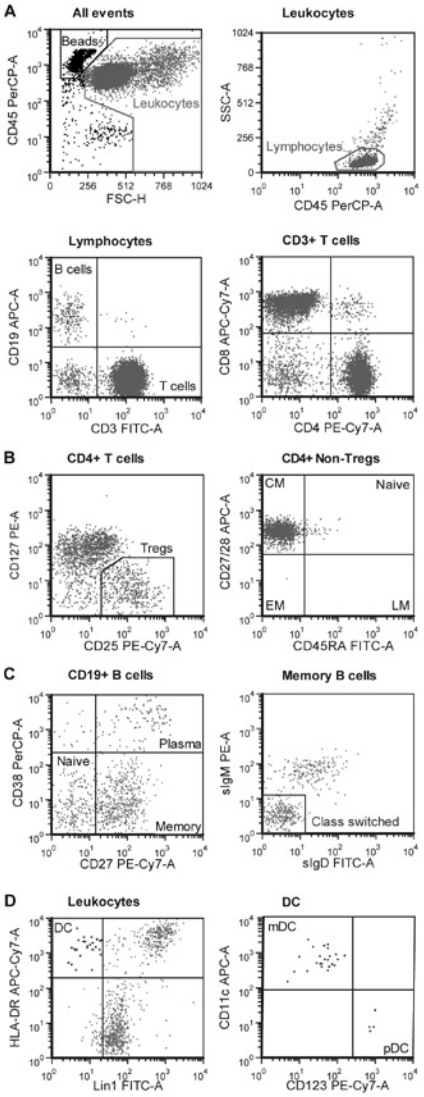

Figure 1.
Gating strategies for the enumeration of major lymphocyte subsets (panel A), CD4+ T cell subsets (panel B), B cell subsets (panel C) and dendritic cells (panel D). CD8+ T cell subsets were gated similarly to the 'CD4+ non-Tregs' in the right plot of panel B. For absolute counting, microspheres were used and defined as FSClow, FLhigh (upper left plot panel A). We used a hierarchical gating strategy, the applied gates are indicated in boldface above each plot and refer to similarly named gates in the previous plots. 'CD4+ Non-Tregs' refers to all (CD3+CD4+) events except those in the 'Tregs' gate (panel B). Dendritic cells were defined as (CD45+HLADR+) events that were negative for expression of CD3, CD14, CD16, CD19, CD20 and CD56 (Lin1-). To confirm that these events represented dendritic cells, we additionally stained for CD11c (marker for myeloid dendritic cells) and CD123 (marker for plasmacytoid dendritic cells; panel D).
Abbreviations: Tregs, regulatory T cells; CM, central memory T cells; EM, effector memory T cells; LM, late memory T cells; Lin1, cocktail composed of CD3, CD14, CD16, CD19, CD20 and CD56 monoclonal antibodies; DC, dendritic cells; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells.

Table 1. Clinical characteristics

	Hu-PNS		IND			OND		
		Range		Range	P[B]		Range	P[C]
n	11		9			12		
Male	2		5		ns	4		ns
Age (years)[A]	65	45-76	44	29-76	0.009	65	32-88	ns
CSF								
Leukocytes (cells/μL)[A]	4.0	1.0-49.0	7.0	0.0-172.0	ns	3.5	1.0-7.0	ns
Protein (g/L)[A]	0.59	0.28-2.47	0.37	0.21-1.77	ns	0.30	0.18-0.67	0.021

[A] median
[B] Hu-PNS vs. IND
[C] Hu-PNS vs. OND
Groups were compared with a Mann-Whitney U test or Fisher's exact test, ns= not significant (p>0.05).

T cell subsets

The CSF of Hu-PNS patients showed higher absolute T cell numbers than CSF of OND patients (Table 2). Both major T cell subsets, $CD4^+$ T cells and $CD8^+$ T cells, were elevated (3.5 and 2.6 fold, respectively). Of the $CD4^+$ T cells, Tregs showed the largest increase in absolute cell numbers (8-fold, Table 2). The proportion of Tregs was also increased: Tregs made up 10.0% of $CD4^+$ T cells (5.7-25.9) in Hu-PNS patients versus 5.5% (0.0-30.3) in OND patients (P=0.014). In Hu-PNS patients, 99.5% (94.6-100.0) of these Tregs did not express CD45RA, indicating that these Tregs were antigen-experienced.

In CSF of Hu-PNS patients, most $CD4^+$ and $CD8^+$ T cells had a central memory phenotype (Table 2 and Figure 2). The absolute number of $CD4^+$ central memory T cells was 2.9 times higher, the number of $CD8^+$ central memory T cells 4.3 times higher than in OND patients (Table 2). The proportions of $CD4^+$ and $CD8^+$ central memory T cells were also increased in Hu-PNS as compared to OND patients (P<0.001 and P=0.014 respectively, Figure 2). The absolute numbers of the further differentiated $CD4^+$ and $CD8^+$ T cell subsets (with effector memory and late memory phenotype) did not differ from OND patients (Table 2).

B cell and DC subsets

The absolute numbers of B cells in CSF of Hu-PNS patients were clearly higher than in CSF of OND patients (Table 2). The largest subsets were class-switched memory B cells, followed by plasma blasts and non-class switched memory B cells. Also the proportion of B cells was elevated in Hu-PNS patients: B cells made up 3.7% (1.0-19.9) of lymphocytes vs. 0.5% (0.0-7.6) in OND patients (P=0.003). The absolute numbers of DCs in CSF of Hu-PNS patients were significantly higher than in OND patients (Table 2). In

Table 2. Absolute numbers of CSF lymphocyte and DC subsets in Hu-PNS patients, IND and OND patients

	Hu-PNS (n=11)			IND (n=9)				OND (n=12)			
Subset	**Median**	**5th percentile**	**95th percentile**	**Median**	**5th percentile**	**95th percentile**	**P[A]**	**Median**	**5th percentile**	**95th percentile**	**P[B]**
T cells (total)	1.21	0.13	22.53	1.11	0.01	64.36	ns	0.50	0.11	4.17	0.016
CD4+ T cells	0.90	0.08	18.37	0.43	0.01	39.49	ns	0.26	0.05	2.98	0.016
Naïve	0.04	0.00	0.28	0.02	0.00	1.70	ns	0.02	0.00	0.53	ns
Central memory	0.69	0.06	13.48	0.49	0.00	35.01	ns	0.24	0.08	2.09	0.023
Effector memory	0.01	0.00	0.04	0.00	0.00	0.04	ns	0.00	0.00	0.04	ns
Late memory	0.00	0.00	0.01	0.00	0.00	0.02	ns	0.00	0.00	0.01	ns
Tregs	0.16	0.01	4.83	0.05	0.00	4.83	ns	0.02	0.00	0.70	0.019
CD8+ T cells	0.50	0.04	8.21	0.55	0.00	23.22	ns	0.19	0.00	1.08	0.049
Naïve	0.34	0.00	1.19	0.05	0.00	2.83	ns	0.03	0.00	0.23	0.019
Central memory	0.26	0.03	6.74	0.18	0.00	6.65	ns	0.06	0.00	0.32	0.010
Effector memory	0.01	0.00	0.23	0.01	0.00	0.43	ns	0.01	0.00	0.09	ns
Late memory	0.02	0.00	0.16	0.02	0.00	0.64	ns	0.00	0.00	0.05	ns
B cells	0.31	0.01	1.94	0.11	0.00	6.53	ns	0.00	0.00	0.03	0.002
Naïve	0.04	0.00	0.44	0.02	0.00	1.37	ns	0.00	0.00	0.02	0.008
Memory IgD/IgM	0.04	0.00	0.49	0.01	0.00	0.88	ns	0.00	0.00	0.01	0.000
Memory class-switched	0.08	0.00	0.70	0.02	0.00	2.50	ns	0.00	0.00	0.01	0.011
Plasma blasts	0.07	0.00	0.27	0.01	0.00	1.50	ns	0.00	0.00	0.01	0.009
Dendritic cells	0.08	0.00	1.54	0.04	0.00	5.50	ns	0.01	0.00	0.43	0.008

Absolute cell numbers are given in cells×10^6/L.

[A] Hu-PNS vs. IND

[B] Hu-PNS vs. OND

Groups were compared with a Mann-Whitney U test, ns= not significant (P>0.05).

Hu-PNS patients, 50.9% (20.4-100.0%) of DCs were CD11c$^+$ myeloid DCs, the remaining DCs were CD123$^+$ plasmacytoid DCs.

Cell numbers in CSF of IND patients

The median absolute cell numbers in IND patients were generally in-between those of Hu-PNS patients and OND patients (Table 2, and data not shown). Compared to Hu-PNS patients, IND patients had lower proportions of CD4$^+$ central memory T cells (P=0.028, Figure 2). Compared to OND patients, IND patients had higher absolute numbers of B cells (P=0.032), especially IgD/M memory B cells (P=0.037, Table 2).

Absolute cell numbers in blood

We additionally compared the absolute cell numbers of the investigated subsets in the blood of Hu-PNS patients with IND and OND patients, and did not find any significant differences between these groups (data not shown).

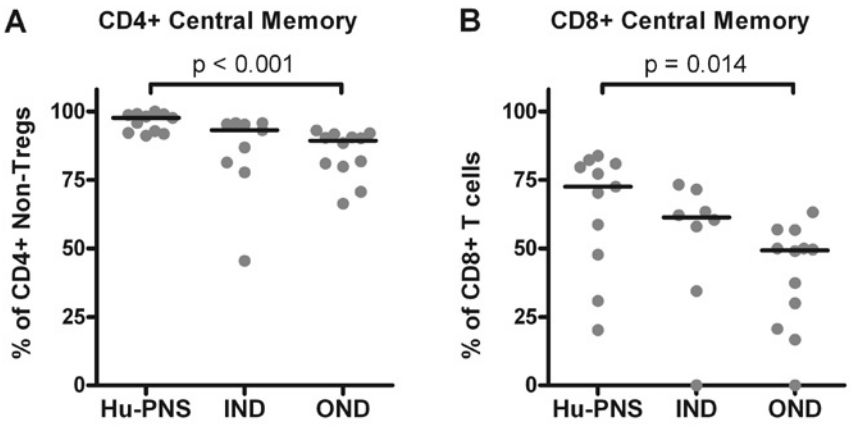

Figure 2.
Percentage of central memory T cells within the non-regulatory CD4+ T cells (panel A) and CD8$^+$ T cells (panel B) in CSF of Hu-PNS patients, IND patients and OND patients. For both subsets (CD4$^+$ and CD8$^+$), central memory T cells were the main differentiation stage, and their percentages were higher in Hu-PNS patients than in OND patients. In Hu-PNS patients, the median percentage of CD4$^+$ central memory cells was 97.7% (5th-95th percentile: 91.2 – 100.0%) of CD4$^+$ T cells, versus 89.4% (66.4-93.1%) in OND patients (P<0.001). The median percentage of CD8$^+$ T cells was 72.6% (20.22-83.8%) in Hu-PNS patients versus 49.3 (0.0-63.3%) in OND patients (p=0.014). One IND had no detectable CD8$^+$ T cells and is therefore not shown in panel B of this figure. The median percentages of central memory T cells in IND controls were generally in-between those of Hu-PNS patients and OND controls (93.2% [45.5-95.8%] of CD4$^+$ T cells, and 61.3% [0.0-73.3%] of CD8$^+$ T cells). Abbreviations: Hu-PNS, patients with anti-Hu antibody associated paraneoplastic neurological syndromes, IND, inflammatory neurological disorders; OND, other (non-inflammatory) neurological disorders.

DISCUSSION

In this study, we confirmed our previous observations that CSF of Hu-PNS patients contained higher numbers of $CD4^+$ T cells, $CD8^+$ T cells and B cells than CSF of patients with non-inflammatory neurological disorders.[13] In addition, we characterized these cells further and found higher numbers of Tregs, $CD4^+$ and $CD8^+$ central memory T cells and class-switched memory B cells in CSF of Hu-PNS patients than in OND patients. Finally we also found higher numbers of DCs in Hu-PNS patients than in OND patients. Median cell numbers in IND patients were generally in-between those of Hu-PNS patients and OND patients, while the cell numbers of OND patients were generally similar to previously published non-neurological patients.[20]

The pronounced expansion of antigen-experienced Tregs in CSF of Hu-PNS patients in the absence of similar changes in the blood suggests that these cells are not just a result of the underlying cancer,[23] but are part of the local immune response in the CNS. A similar increase of Tregs has been described in CSF of multiple sclerosis (MS) patients.[24] Antigen-experienced Tregs are known to suppress immune responses against self-antigens by secretion of cytokines such as IL-10.[25] The progressive disease in our Hu-PNS patients, however, questions the effectiveness of these Tregs in Hu-PNS. This is in line with previous studies that suggested that Treg function is compromised in two ways in immune-mediated diseases of the CNS. First, intrinsic factors in Tregs, such as reduced expression or function of the forkhead/winged helix transcription factor (FoxP3), may compromise suppressive function, as supported by the impaired suppressive function of Tregs in peripheral blood of Hu-PNS patients[26] and MS patients.[27] Second, the local inflammation in the CNS might results in a cytokine milieu (high levels of IL-6 and TNF) that might interfere with effective suppression of effector T cells by Tregs, as shown in experimental autoimmune encephalitis.[28]

The expansion of $CD4^+$ and $CD8^+$ central memory T cells in CSF of Hu-PNS patients supports the hypothesized role of T helper cells and cytotoxic T cells in neuronal destruction in Hu-PNS. Central memory T cells have encountered their antigen in the lymph nodes, and are able to cross the blood-CSF barrier to search for their antigen.[15] Especially in the initiation phase of immune-mediated CNS disease, central memory T cells in CSF are thought to play a key role in disease pathogenesis.[15,16,29] Expansion of $CD4^+$ and $CD8^+$ central memory T cells is also found in CSF of patients with MS.[15,30] Upon a second antigen encounter, central memory T cells quickly differentiate into effector cells that invade the CNS tissue, as supported by autopsy studies showing T cell infiltrates in the CNS of Hu-PNS patients.[4,5]

As we previously described, a pronounced expansion of B cells in CSF of Hu-PNS patients, and to a lesser extend IND patients, was found. Since antibodies were not shown pathogenic in animal models[2] and Hu-proteins are intracellular antigens, the role of B cells in Hu-PNS is long considered of minor importance.[3] B cells could, however, contribute to Hu-PNS in many ways.[29,31] First, like DCs, memory B cells are potent antigen-presenting cells that could contribute to differentiation of central memory T cells in CSF to the effector cells that invade CNS tissue.[29] Interesting is the presence of class-switched B cells. Class-switched $CD27^+$ memory B cells have received help of $CD4^+$ T cells[32] and are more efficient antigen-presenting cells than non-class switched B cells.[33] Second, B cells might contribute to CNS inflammation by the production of pro-inflammatory cytokines.[29,31] Finally, the plasma blasts in CSF may, together with plasma cells in CNS tissue, be responsible for the intrathecal Hu-Ab production found in Hu-PNS patients.[14]

The median cell number in patients with IND were generally in-between those of Hu-PNS patients and patients with OND. These findings are consistent with other studies on inflammatory CNS disease.[16,34] The overlap in cell numbers between Hu-PNS and IND supports the hypothesis that Hu-PNS is an immune-mediated disease. In addition, our results are reminiscent of findings reported in multiple sclerosis. Hence, we speculate that newly developed therapies for MS that interfere with lymphocyte trafficking from the lymph nodes to the CNS, such as fingolimod or natalizumab, may also be effective in Hu-PNS.[35,36]

In the current study, Tregs were defined as ($CD3^+$, $CD4^+$, $CD25_{high}$, $CD127_{dim}$) because it has been shown these cells represents the large majority of $FoxP3^+$ Tregs.[24,37,38] We choose this definition instead of the 'gold standard' ($CD3^+$, $CD4^+$, $CD25_{high}$, $FoxP3^+$), because the fixation and the many washing steps needed for this staining were anticipated to result in too much cell loss in the already paucicellular CSF samples. Indeed, other studies also used immunological definitions limited to surface antigens to overcome this problem.[24,27]

To conclude, we found higher numbers of Tregs, central memory T cells, class-switched memory B cells and DCs in CSF of Hu-PNS patients than in OND patients. The absence of similar changes in the blood of Hu-PNS patients supports the hypothesis that their immune system is locally activated. Besides supporting a role for cytotoxic T cells, T helper cells and B cells, the results of this study suggest that Tregs and DCs are also involved in the pathogenesis of Hu-PNS. Our findings are similar to those reported in MS, and indicate that future studies are needed to study these neuro-inflammatory

diseases side-by-side. We speculate that these studies may identify common targets for therapies.

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REFERENCES

1. de Beukelaar JW, Sillevis Smitt PA. Managing paraneoplastic neurological disorders. *The oncologist.* Mar 2006;11(3):292-305.
2. Sillevis Smitt PA, Manley GT, Posner JB. Immunization with the paraneoplastic encephalomyelitis antigen HuD does not cause neurologic disease in mice. *Neurology.* Oct 1995;45(10):1873-1878.
3. Roberts WK, Darnell RB. Neuroimmunology of the paraneoplastic neurological degenerations. *Curr Opin Immunol.* Oct 2004;16(5):616-622.
4. Bernal F, Graus F, Pifarre A, Saiz A, Benyahia B, Ribalta T. Immunohistochemical analysis of anti-Hu-associated paraneoplastic encephalomyelitis. *Acta neuropathologica.* May 2002;103(5):509-515.
5. Jean WC, Dalmau J, Ho A, Posner JB. Analysis of the IgG subclass distribution and inflammatory infiltrates in patients with anti-Hu-associated paraneoplastic encephalomyelitis. *Neurology.* Jan 1994;44(1):140-147.
6. Tanaka M, Maruyama Y, Sugie M, Motizuki H, Kamakura K, Tanaka K. Cytotoxic T cell activity against peptides of Hu protein in anti-Hu syndrome. *J Neurol Sci.* Sep 15 2002;201(1-2):9-12.
7. de Beukelaar JW, Verjans GM, van Norden Y, et al. No evidence for circulating HuD-specific CD8+ T cells in patients with paraneoplastic neurological syndromes and Hu antibodies. *Cancer Immunol Immunother.* Sep 2007;56(9):1501-1506.
8. de Beukelaar JW, Milikan JC, Verjans GM, et al. No evidence for the presence of HuD-specific T cells in the cerebrospinal fluid of patients with Hu-associated paraneoplastic neurological syndromes. *J Neurol.* Feb 2009;256(2):279-282.
9. Roberts WK, Deluca IJ, Thomas A, et al. Patients with lung cancer and paraneoplastic Hu syndrome harbor HuD-specific type 2 CD8+ T cells. *J Clin Invest.* Jul 2009;119(7):2042-2051.
10. Rousseau A, Benyahia B, Dalmau J, et al. T cell response to Hu-D peptides in patients with anti-Hu syndrome. *J Neurooncol.* Feb 2005;71(3):231-236.
11. de Jongste AH, de Graaf MT, Martinuzzi E, et al. Three sensitive assays do not provide evidence for circulating HuD-specific T cells in the blood of patients with paraneoplastic neurological syndromes with anti-Hu antibodies. *Neuro Oncol.* July 2012 2012;14(7):841-848.
12. de Beukelaar JW, Sillevis Smitt PA, Hop WC, et al. Imbalances in circulating lymphocyte subsets in Hu antibody associated paraneoplastic neurological syndromes. *Eur J Neurol.* Dec 2007;14(12):1383-1391.
13. de Graaf M, de Beukelaar J, Bergsma J, et al. B and T cell imbalances in CSF of patients with Hu-antibody associated PNS. *J Neuroimmunol.* Mar 2008;195(1-2):164-170.
14. Rauer S, Kaiser R. Demonstration of anti-HuD specific oligoclonal bands in the cerebrospinal fluid from patients with paraneoplastic neurological syndromes. Qualitative evidence of anti-HuD specific IgG-synthesis in the central nervous system. *J Neuroimmunol.* Nov 1 2000;111(1-2):241-244.
15. Kivisakk P, Mahad DJ, Callahan MK, et al. Expression of CCR7 in multiple sclerosis: implications for CNS immunity. *Ann Neurol.* May 2004;55(5):627-638.

16. Giunti D, Borsellino G, Benelli R, et al. Phenotypic and functional analysis of T cells homing into the CSF of subjects with inflammatory diseases of the CNS. *Journal of leukocyte biology.* May 2003;73(5):584-590.
17. Pashenkov M, Huang YM, Kostulas V, Haglund M, Soderstrom M, Link H. Two subsets of dendritic cells are present in human cerebrospinal fluid. *Brain.* Mar 2001;124(Pt 3):480-492.
18. Hatterer E, Touret M, Belin MF, Honnorat J, Nataf S. Cerebrospinal fluid dendritic cells infiltrate the brain parenchyma and target the cervical lymph nodes under neuroinflammatory conditions. *PLoS One.* 2008;3(10):e3321.
19. Graus F, Delattre JY, Antoine JC, et al. Recommended diagnostic criteria for paraneoplastic neurological syndromes. *J Neurol Neurosurg Psychiatry.* Aug 2004;75(8):1135-1140.
20. de Graaf MT, Smitt PA, Luitwieler RL, et al. Central memory CD4(+) T cells dominate the normal cerebrospinal fluid. *Cytometry.* Jul 14 2011;80(1):43-50.
21. Caraux A, Klein B, Paiva B, et al. Circulating human B and plasma cells. Age-associated changes in counts and detailed characterization of circulating normal CD138- and CD138+ plasma cells. *Haematologica.* Jun 2010;95(6):1016-1020.
22. Graus F, Vega F, Delattre JY, et al. Plasmapheresis and antineoplastic treatment in CNS paraneoplastic syndromes with antineuronal autoantibodies. *Neurology.* Mar 1992;42(3 Pt 1):536-540.
23. Karagoz B, Bilgi O, Gumus M, et al. CD8+CD28- cells and CD4+CD25+ regulatory T cells in the peripheral blood of advanced stage lung cancer patients. *Medical oncology (Northwood, London, England).* Mar 2010;27(1):29-33.
24. Fritzsching B, Haas J, Konig F, et al. Intracerebral human regulatory T cells: analysis of CD4+ CD25+ FOXP3+ T cells in brain lesions and cerebrospinal fluid of multiple sclerosis patients. *PLoS One.* 2011;6(3):e17988.
25. Miyara M, Sakaguchi S. Human FoxP3(+)CD4(+) regulatory T cells: their knowns and unknowns. *Immunology and cell biology.* Feb 8 2011.
26. Tani T, Tanaka K, Idezuka J, Nishizawa M. Regulatory T cells in paraneoplastic neurological syndromes. *J Neuroimmunol.* May 30 2008;196(1-2):166-169.
27. Venken K, Hellings N, Thewissen M, et al. Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology.* Jan 2008;123(1):79-89.
28. Korn T, Reddy J, Gao W, et al. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med.* Apr 2007;13(4):423-431.
29. Goverman J. Autoimmune T cell responses in the central nervous system. *Nature reviews.* Jun 2009;9(6):393-407.
30. Jacobsen M, Cepok S, Quak E, et al. Oligoclonal expansion of memory CD8+ T cells in cerebrospinal fluid from multiple sclerosis patients. *Brain.* Mar 2002;125(Pt 3):538-550.
31. Dalakas MC. B cells as therapeutic targets in autoimmune neurological disorders. *Nature clinical practice.* Oct 2008;4(10):557-567.
32. Berkowska MA, Driessen GJ, Bikos V, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood.* Aug 25;118(8):2150-2158.

33. Good KL, Avery DT, Tangye SG. Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naive B cells. *J Immunol.* Jan 15 2009;182(2):890-901.

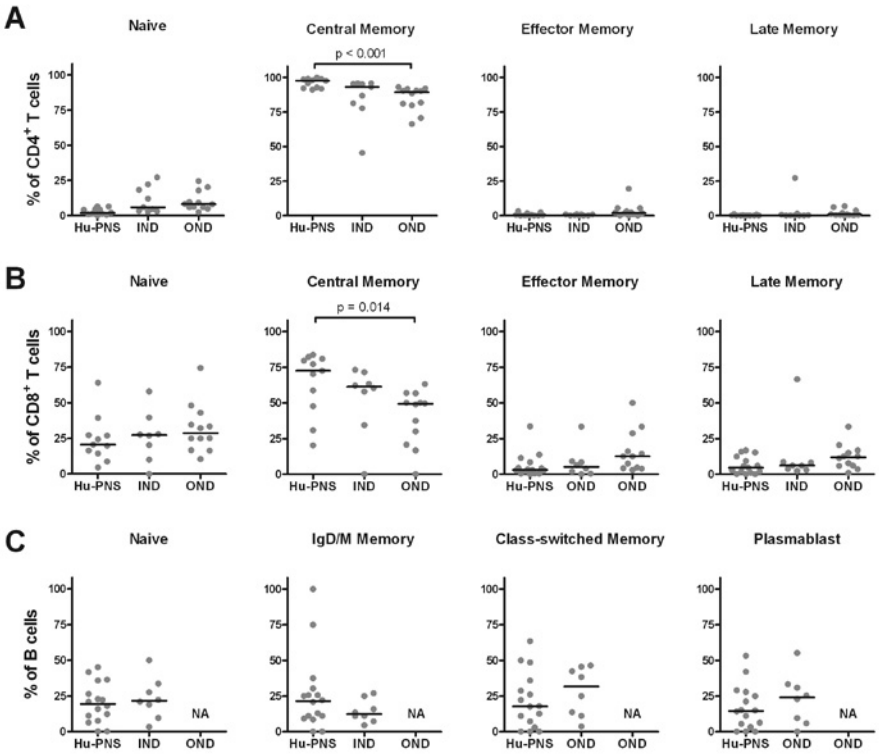
34. Cepok S, Rosche B, Grummel V, et al. Short-lived plasma blasts are the main B cell effector subset during the course of multiple sclerosis. *Brain.* Jul 2005;128(Pt 7):1667-1676.

35. Pelletier D, Hafler DA. Fingolimod for multiple sclerosis. *The New England journal of medicine.* Jan 26 2012;366(4):339-347.

36. Pucci E, Giuliani G, Solari A, et al. Natalizumab for relapsing remitting multiple sclerosis. *Cochrane database of systematic reviews (Online).* 2011(10):CD007621.

37. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *The Journal of experimental medicine.* Jul 10 2006;203(7):1701-1711.

38. Seddiki N, Santner-Nanan B, Martinson J, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *The Journal of experimental medicine.* Jul 10 2006;203(7):1693-1700.



Supplementary Figure 1.
Percentages of T cell subsets within the non-regulatory T cells (panel A), percentages of $CD8^+$ T cell subsets within the $CD8^+$ T cells (panel B), and percentages of B cell subsets within the B cells (panel C) in CSF of Hu-PNS patients, IND patients and OND patients. One IND had no detectable $CD8^+$ T cells and is therefore not shown in panel B of this figure. The vast majority of $CD4^+$ and $CD8^+$ T cells were central memory T cells. The percentages of effector and late memory $CD4^+$ and $CD8^+$ T cells were very low. B cells were virtually absent in CSF of other (non-inflammatory) neurological disorders, and therefore no percentages could be calculated for this patient group (indicated with not applicable, NA). Abbreviations: NA, not applicable; Hu-PNS, patients with anti-Hu antibody associated paraneoplastic neurological syndromes, IND, inflammatory neurological disorders; OND, other (non-inflammatory) neurological disorders.

6

Use of TransFix™ cerebrospinal fluid storage tubes prevents cellular loss and enhances flow cytometric detection of malignant hematological cells after 18 hours of storage

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ABSTRACT

Flow cytometry is a sensitive method for detection of leptomeningeal localizations of hematological malignancies (LHM) in cerebrospinal fluid (CSF). Rapid processing of CSF is needed, as leukocyte numbers appear to decline quickly after lumbar puncture. The cell-stabilizing agent TransFix™ may enhance the detection of LHM in CSF by preventing cellular loss. To study the effects of TransFix™ on leukocyte numbers and the detection of LHM, we prospectively collected 99 CSF samples from patients with suspected or proven LHM in tubes with (i) TransFix™; (ii) serum-containing medium; and (iii) no cell-stabilizing agents (native CSF). Presence of LHM and absolute leukocyte numbers were determined by flow cytometry after 30 minutes and 18 hours of storage. Leukocyte numbers in TransFix™- stabilized CSF were higher than in the corresponding native samples at both time points (1.4× and 2.3× respectively, $P<0.0001$ on each occasion). After 18 hours of storage, TransFix™ enhanced the detection of LHM in CSF. In all discordant paired observations (13/99, $P=0.005$), the level of suspicion (classified as positive, suspicious or negative) in CSF with TransFix™ was higher than in native CSF. We conclude that the use of TransFix™-containing CSF storage tubes prevents cellular loss, and enhances flow cytometric detection of LHM after 18 hours of storage.

INTRODUCTION

The use of flow cytometric analysis of cerebrospinal fluid (CSF) in addition to morphologic CSF examination greatly increases the detection rate of leptomeningeal localizations of hematological malignancies (LHM), as compared to the use of morphology alone.[1-9] Therefore, flow cytometric CSF analysis is recommended in all patients with suspected primary central nervous system lymphomas (PCNSL), patients with other hematological malignancies complicated by neurological signs and symptoms suggestive of LHM, and patients with aggressive non-Hodgkin lymphomas (NHL) who by themselves have an increased risk of developing LHM.[10,11]

Since cell numbers in CSF are low (normally <5 cells/µL) and appear to decrease rapidly ex-vivo,[12,13] CSF samples for flow cytometric analysis should be processed immediately after withdrawal.[14,15] Thus, in order to obtain optimal results, immediate availability of this technique is needed, as any delay in sample transportation or processing will lead to decreased CSF cell numbers and will likely reduce the sensitivity of analysis.

To address this problem, various cell stabilizing methods have been employed to reduce cellular loss in CSF samples prior to flow cytometric analysis, as recently reviewed.[14] However, only two of these methods have been used in large clinical studies aimed at the detection of LHM: (i) CSF withdrawal in tubes with 2 mL serum-containing medium;[1] and (ii) CSF withdrawal in tubes with 0.2 mL of the commercially available fixative agent TransFix™.[4]

Addition of serum-containing medium to CSF prevents cellular loss up to at least 5 hours of storage.[12] However, CSF storage tubes with serum-containing medium are not commercially available and have a limited shelf life of around 3 months. The use of TransFix™ CSF storage tubes may be an attractive alternative for cell stabilization in CSF because of their commercial availability and longer shelf life, i.e. one year.

TransFix™ is a cellular stabilization reagent that contains a buffer, an aliphatic aldehyde, and heavy metal salts.[16] It was originally designed to stabilize whole blood samples for flow cytometric counting of lymphocytes and their subsets such as $CD4^+$ and $CD8^+$ T cells, and was first used by the United Kingdom National External Quality Assessment Service (UK NEQAS) for leukocyte immunophenotyping.[17] Later, TransFix™ became commercially available, and was shown to stabilize whole blood samples for up to 10 days.[18] TransFix™ in combination with EDTA has also been used to stabilize CSF samples to enable overnight shipping to a central flow cytometry facility.[4]

Although the above studies suggest that cell stabilization with serum-containing medium or TransFix™ may enhance the flow cytometric detection of LHM in CSF,[1,4] no studies have been published that directly compare CSF stabilization with TransFix™ with the use of serum-containing medium or native CSF. Therefore, the clinical significance of the use of TransFix™ or serum-containing medium for flow cytometric CSF analysis is unknown.

In this study, we prospectively investigated the effects of TransFix™ on the detection of LHM in CSF and CSF cell numbers by flow cytometry. CSF samples were processed immediately (i.e. 30 minutes after withdrawal), and after overnight storage (i.e. after 18 hours). Results were compared with those of simultaneously collected CSF samples that were stabilized with serum-containing medium, and simultaneously collected CSF samples without cell stabilizing agents (native CSF).

MATERIALS AND METHODS

Sample collection and storage

From January 2011 onwards, 99 diagnostic and follow-up CSF samples were obtained at the Erasmus University Medical Center/ Daniel den Hoed Cancer Center from 43 patients who (i) underwent lumbar puncture for flow cytometric CSF analysis to test for LHM; and (ii) provided written informed consent. This study was performed according to the Declaration of Helsinki, and its procedures were approved by the local ethics committee. In addition to the CSF drawn for conventional tests and procedures, a total of approximately 3 mL extra CSF was directly collected in three polypropylene tubes: ~1 mL in a tube with 0.2 mL TransFix™/EDTA (Caltag Medsystems, Towcester, UK); ~ 1 mL in a tube with 2 mL serum-containing medium (RPMI-1640 with 35mM HEPES, 1 mM L-Glutamine, 2% penicillin/streptomycin, 5% heat-inactivated fetal bovine serum and 2,500 IU heparin); and ~1 mL in a tube without cell-stabilizing agent (native CSF). CSF was drawn in these three tubes in alternating order to avoid a possible influence of the order in which the tubes were filled. Each of the three tubes was equally split into an additional 2 sterile polypropylene tubes: one of these tubes was processed 30 minutes after withdrawal; the other was stored at 4°C overnight (18 hours). Before and after splitting of the samples, the weight of the tubes was recorded to verify CSF volumes.

Flow cytometry

Detailed procedures for CSF processing,[15] determination of absolute cell numbers,[12,19] and to test for LHM[1,14] have been published elsewhere. Briefly, CSF cells were con-

centrated by centrifugation (8 minutes, 450 *g*), and resuspended in 100 µL phosphate buffered saline (PBS). In cases where surface immunoglobulins (sIg) had to be detected, cells were washed with PBS. Six-color antibody panels were chosen based on clinical information, and –if available– results of previous histological or flow cytometric analyses. All panels included anti-CD45 peridinin chlorophyll protein (PerCP; clone 2D1, Dako, Glostrup, Denmark) to distinguish leukocytes from debris and other cells. In addition, we used anti-CD4 fluorescein (FITC; clones SK3+SK4), anti-CD8 FITC (clone SK1), anti-CD5 R-phycoerythrin (PE; clone L17F12), anti-CD13 PE (clone L138), anti-CD34 PE (clone 8G12), anti-CD10 PE (clone HI10a), anti-CD19 PE (clone 4G7), anti-CD4 PE-Cy5 (clone SK3), anti-CD19 allophycocyanin (APC; clone SJ25C1), anti-CD14 APC-H7 (clone MΦP9), anti-CD19 APC-Cy7 (clone SJ5C1), and anti-CD20 APC-Cy7 (clone L27; all from BD Biosciences); anti-sIg κ PE (clone R0436; Dako), anti-sIgκ FITC (clone HP6156; Lucracon, Stuttgart, Germany); anti-sIgλ FITC (clones HP6062/6054; both from Invitrogen, Carlsbad, CA); anti-sIgλ PE (cat. 2072-09) and anti-sIgM PE (cat. 2020-09; both from SouthernBiotech, Birmingham, AL); anti-CD5 PE-Cy7 (clone BL1a) and anti-CD10 PE-Cy7 (clone ALB1; both from Beckman Coulter, Fullerton, CA); and anti-CD7 APC (clone 124-1D1) and anti-CD3 APC-eFluor780 (clone UCHT1; both from eBioscience, San Diego, CA). Fluorochrome-conjugated antibodies were added to 100 µL of the cell suspension, incubated for 15 minutes in the dark, washed, and resuspended in 100 µL PBS. To determine absolute cell numbers, 100 µL of Cyto-Cal Count Control counting beads (Duke Scientific Corporation, Palo Alto, CA) were added. Listmode data were acquired on a FACSCanto flow cytometer (BD Biosciences).

Data analysis

An example of our gating strategy to detect LHM and determine absolute numbers of leukocytes and their subsets is shown in figure 1. To detect LHM, listmode data were analyzed with FACSDiva software (BD Biosciences). Clusters of >25 events fulfilling criteria for malignant populations were classified as positive, clusters of 10 to 25 events as suspicious, and below 10 events as negative, as described before.[1,15] Absolute cell numbers and fluorescence intensities were determined with FCS express software (De Novo Software, Los Angeles, CA). Relative cell numbers were calculated by dividing the absolute cell number of a particular subset, time point and storage condition by the absolute cell number of the same subset in CSF with serum-containing medium at 30 minutes. Discordances in the detection of LHM in CSF with TransFix™ versus the simultaneously collected control CSF samples were compared with the McNemar test using SPSS version 17.0.2 (IBM, Chicago, IL). Cell numbers as well as fluorescence intensities of TransFix™-treated CSF cells versus CSF cells from the paired control samples were compared with the Wilcoxon signed rank test using Prism version 5 (GraphPad, San Diego, CA). The effects of TransFix™ or serum-containing medium on

the detection of LHM, cell numbers, and fluorescence intensities in follow-up samples were assumed to be independent of those in the initially obtained samples of the same patients. Results of continuous variables were expressed as median and range, unless otherwise specified. Two-sided P-values <0.05 were considered significant.

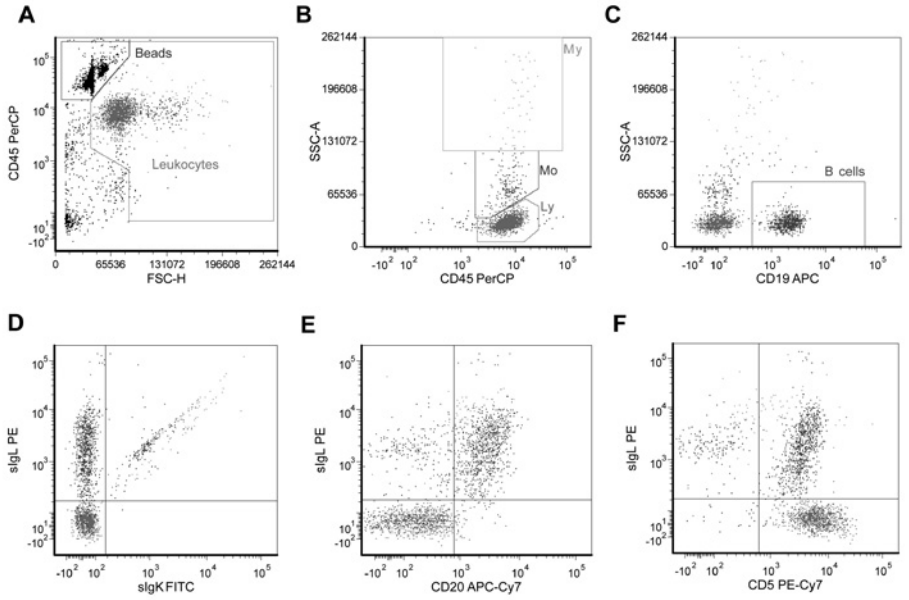

Figure 1.
Gating strategy to detect B lymphocyte clonality and enumerate leukocyte subsets in CSF by 6-color flow cytometry. Leukocytes were distinguished from non-leukocyte events and debris by gating on forward scatter (FSC) and CD45; microspheres were used for absolute counting and defined as FSClow, FLhigh (panel A). Panels B-F show further analysis of the events selected in the leukocyte gate in panel A. Leukocyte subsets were defined with high CD45 expression and side scatter (panel B) and show three subsets: lymphocytes (Ly; CD45+, SSClow; green dots), monocytes (Mo; CD45+, SSCintermediate; cyan dots) and granulocytes (My [myeloid cells]; CD45+, SSChigh; orange dots). B cells were gated using the lineage-specific marker CD19 and side scatter (SSC) (purple dots, panel C). The B cells in this analysis were monoclonal sIgL+ (panel D) and expressed CD20 (panel E) and CD5 (panel F). Data are shown of an 18-hours old CSF sample in TransFix™ from a patient with a B cell non-Hodgkin lymphoma (small lymphocytic lymphoma).

RESULTS

Samples and patients

A total of 99 CSF samples were obtained, 43 diagnostic samples and 56 follow-up samples from 43 patients (24 male patients, 19 female patients), all tested for the oc-

currence of LHM. The median age of these patients was 60 years (range 22-77 years). Of the 43 patients, 26 patients had a systemic localization of a hematological malignancy while 17 patients had a PCNSL. In most cases (30/43, 70%), a B cell NHL was present, usually a diffuse large B cell lymphoma (23/30, 77%). Other cases included patients with acute myeloid leukemia (n=5), T lymphoblastic leukemia/lymphoma (n=3), T cell NHL (n=2), plasma cell myeloma (n=1) and B lymphoblastic leukemia (n=2). The median volumes of CSF drawn were: 1.14 mL (0.48-2.45) CSF in TransFix™/EDTA; 1.28 mL (0.47-2.66) CSF in serum-containing medium; and 1.25 mL (0.45-2.33) of native CSF. The median leukocyte count of the CSF samples was 5 cells/μL (range <1-552 cells/μL); the median protein content was 0.38 g/L (range 0.15-2.42 g/L).

Qualitative detection of hematological malignancies

Thirty minutes from CSF withdrawal, we compared the flow-cytometric detection of LHM in TransFix™-treated CSF samples with CSF samples with serum-containing medium (Table 1), and native CSF samples (Table 2). Compared to CSF with serum-containing medium and native CSF, CSF with TransFix™ showed concordant results in 86/99 (87%) and 83/99 (84%) of cases, respectively. Discordant results included paired observations in which the level of suspicion was higher (i.e. positive vs. suspicious, positive vs. negative and suspicious vs. negative) in CSF with TransFix™, as well as paired observations in which the level of suspicion was higher in CSF with serum-containing medium or native CSF. Hence, 30 minutes after withdrawal, use of TransFix™ had neither a significant beneficial nor significant unfavorable effect on the detection of LHM, as compared to serum-containing medium or native CSF. The flow-cytometric detection of LHM after 18 hours in CSF samples with TransFix™ versus CSF samples with serum-containing medium and native CSF samples is shown in tables 3 and 4. Compared to CSF with serum-containing medium and native CSF, CSF with TransFix™ showed concordant results in 92/99 (93%) and 86/99 (87%) of cases, respectively. In all discordant pairs of observations, the level of suspicion was higher in CSF with TransFix™ than in CSF with serum-containing medium (7/99 [7%], P=0.07) or native CSF (13/99 [13%], P=0.005). To exclude that the asymmetrical distribution of the discordances seen between TransFix™-treated CSF and native CSF was due to a higher volume of TransFix™-stabilized CSF than that of native CSF, we analyzed the CSF volumes of the discordant pairs of samples separately, and found no significant difference (0.56 mL [0.31-0.81] vs. 0.56 mL [0.23-0.92] respectively, P=0.80). All samples that scored positive in CSF with TransFix™ were derived from patients that were also diagnosed with LHM by our conventional diagnostic procedure involving flow cytometric analysis of >2 mL CSF with serum-containing medium.[1]

Table 1. Detection of LHM after 30 minutes: TransFix vs. medium

t= 30 min		Medium			
		positive	suspicious	negative	Total
TransFix™	positive	25	3	0	28
	suspicious	3	1	2	6
	negative	1	4	60	65
Total		29	8	62	99

P=0.64
(McNemar test)

Table 2. Detection of LHM after 30 minutes: TransFix vs. native CSF

t= 30 min		Native			
		positive	suspicious	negative	Total
TransFix™	positive	21	3	4	28
	suspicious	2	0	4	6
	negative	0	3	62	65
Total		23	6	70	99

P=0.23
(McNemar test)

Table 3. Detection of LHM after 18 hours: TransFix vs. medium

t= 18 hours		Medium			
		positive	suspicious	negative	Total
TransFix™	positive	28	3	3	34
	suspicious	0	1	1	2
	negative	0	0	63	63
Total		28	4	67	99

P=0.07
(McNemar test)

Table 4. Detection of LHM after 18 hours: TransFix vs. native CSF

t= 18 hours		Native			
		positive	suspicious	negative	Total
TransFix™	positive	23	6	5	34
	suspicious	0	0	2	2
	negative	0	0	63	63
Total		23	6	70	99

P=0.005
(McNemar test)

Cell numbers

Thirty minutes after withdrawal in CSF with medium, lymphocytes showed the highest absolute cell numbers (0.50 cells/µL [0.01-110.3]), while numbers of monocytes and granulocytes were generally very low (0.27 cells/µL [0.01-9.65] and 0.27 cells/µL [0.04-8.95], respectively). Figure 2 shows the cell numbers of leukocytes, lymphocytes, monocytes and granulocytes after 30 minutes and 18 hours of storage, relative to their cell numbers in CSF with serum-containing medium after 30 minutes. Thirty minutes from withdrawal, the median number of leukocytes in CSF with TransFix™ was similar to those in CSF with serum-containing medium, and 1.4 times higher than in native CSF. After 18 hours of storage, the median leukocyte number in CSF with TransFix™ was 1.8 times higher than in CSF with serum-containing medium, and 2.3 times higher than in native CSF (Figure 2A). These higher total leukocyte numbers in CSF with TransFix™ as compared to medium (after 18 hours) and native CSF (both time points) were mainly due to higher lymphocyte numbers (Figure 2B). Monocytes numbers in CSF with TransFix™ were similar to those in serum-containing medium,

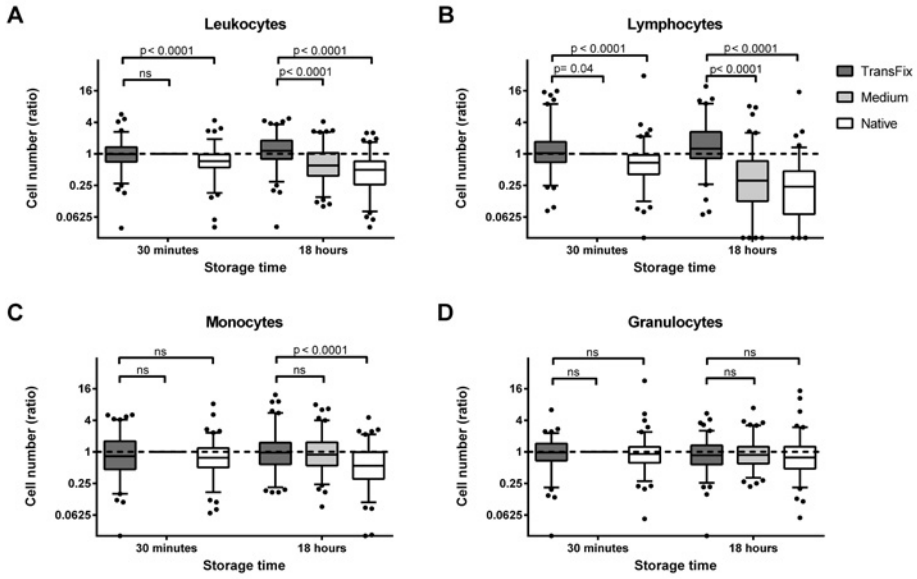

Figure 2.
Relative numbers of leukocytes (A), and their subsets (B, C, D) after 30 minutes and 18 hours of storage in CSF with TransFix™, CSF with serum-containing medium and native CSF. Relative numbers were calculated by dividing the absolute numbers (in cells/µL) by those in CSF with serum-containing medium at 30 minutes. A reference line is drawn at a relative cell number of 1, to indicate the cell number in serum-containing medium at 30 minutes. Boxes represent medians and quartiles, whiskers 5th-and 95-th percentiles. P-values were calculated with the Wilcoxon signed rank test. ns, not significant.

and higher than those in native CSF after 18 hours of storage (Figure 2C), while no significant differences in granulocyte numbers were found between CSF with TransFix™, CSF with serum-containing medium, and native CSF (Figure 2D).

Surface antigen labeling and light scatter

Figure 3 shows the measured fluorescence intensities after staining with fluorochrome-conjugated antibodies against the leukocyte marker CD45, B cell markers (CD19 and CD20), and surface immunoglobulins (sIgκ and sIgM). Thirty minutes after withdrawal, a small but significant decrease in fluorescence intensity is seen for CD45-PerCP in CSF treated with TransFix™ (panel A). At both time points (30 minutes and 18 hours), the fluorescence intensities of B cells labeled with anti-CD19 APC and anti-CD20 APC-Cy7 were significantly lower in TransFix™-treated CSF samples than in CSF samples with serum-containing medium (panels B and C). Similarly, lower fluorescence intensities were seen in CSF with TransFix™ after staining for surface immunoglobulin kappa (panel D). We did not observe any significant increase in background fluorescence in

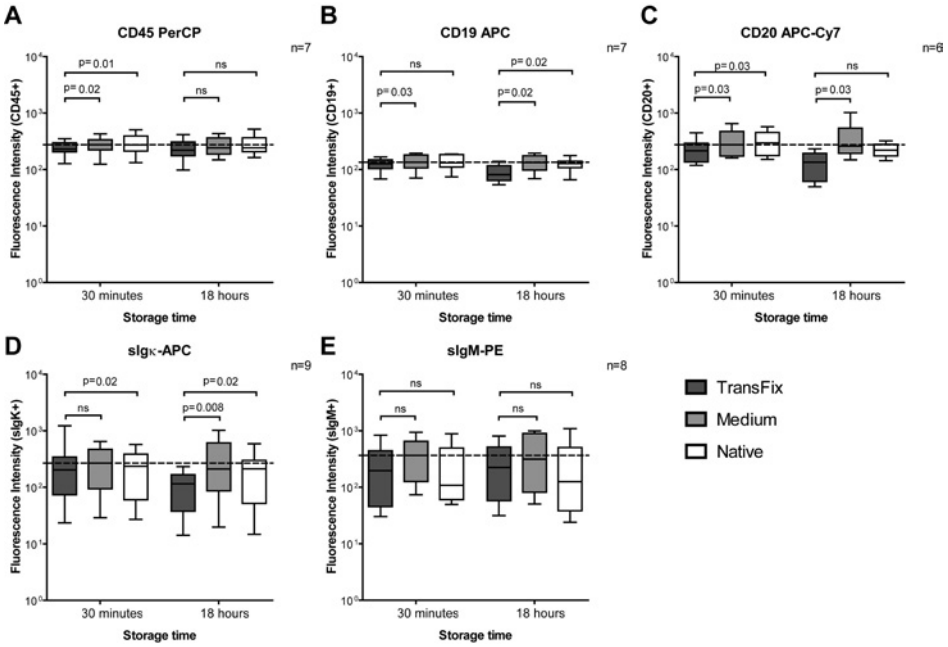

Figure 3.
Fluorescence intensities after staining for the leukocyte marker CD45 (A), the B cell markers CD19 and CD20 (B, C), and the surface immunoglobulins kappa and M (D, E) in CSF with TransFix™, CSF with serum-containing medium, and native CSF after 30 minutes and 18 hours of storage. A reference line is drawn to indicate the median fluorescence intensity in serum-containing medium at 30 minutes. Boxes represent medians and quartiles, whiskers 5th-and 95-th percentiles. P-values were calculated with the Wilcoxon signed rank test. ns, not significant.

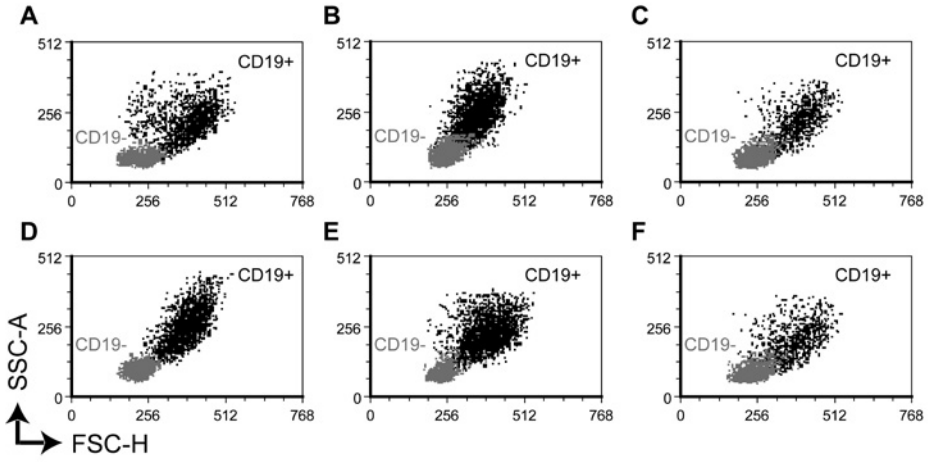

Figure 4.
Light scatter properties of CD19$^+$ diffuse large B cell lymphoma cells (black dots) and CD19$^-$ non-malignant lymphocytes (grey dots) derived from CSF with TransFix™ (A, D), CSF with serum-containing medium (B, E) and native CSF (C, F) after 30 minutes (A, B, C) and 18 hours (D, E, F) of storage. All CD19$^+$ cells (black dots) also expressed CD10 and were monoclonal sIgK$^+$, consistent with the diagnosis diffuse large B cell lymphoma. In all six conditions, diffuse large B cell lymphoma cells could be discriminated from other lymphocytes based on their higher forward light scatter. SSC-A, sideward scatter (area); FSC-H, forward scatter (height).

CSF with TransFix or serum-containing medium (data not shown). Figure 4 shows the light scatter properties of diffuse large B cell lymphoma cells and other lymphocytes in CSF with TransFix™ (panels A en D), CSF with serum-containing medium (panels B and E) and native CSF (panels C and F) in a representative sample. Allthough forward and sideward scatter signals of the depicted cells tended to be lower after stabilisation with TransFix™, diffuse large B cell lymphoma cells could still be discriminated from other lymphocytes based on their higher forward light scatter signal.

DISCUSSION

In this study, we compared the detection of LHM by flow cytometry using TransFix™-stabilized CSF with the detection of LHM using CSF stabilized with serum-containing medium and native CSF. We found that, after 18 hours of storage, use of TransFix™ significantly enhanced the detection of LHM as compared to native CSF and CSF with serum-containing medium, while 30 minutes after withdrawal, detection rates under the 3 conditions were similar.

Currently, it is recommended to immediately process CSF samples for flow cytometric detection of LHM, preferably within 60 minutes after withdrawal.[15,20] This implies immediate availability of a staffed flow-cytometry facility, which is not within the reach of many institutions. A method that enables storage of CSF for later analysis with diagnostic accuracy similar to immediately processed native CSF is therefore highly desirable. Cell-stabilizing agents such as TransFix™ and serum-containing medium have been tested by some laboratories.[1,4] In this study, we tested the use of TransFix™, serum-containing medium and native CSF side-by-side, in order to enable direct comparison of these methods.

Quijano et al.[4] used TransFix™ to stabilize CSF samples of patients with aggressive B cell NHL for overnight shipment to a central flow cytometry facility, and detected LHM in 22% of cases, the same percentage as found in a previous study on fresh native CSF.[3] However, they did not directly compare their results with those of immediately processed native CSF, and hence, a possible negative influence of CSF storage in TransFix™ on the detection of LHM can not be excluded. Our study shows that the diagnostic accuracy of the flow cytometric detection of LHM in 18-hours old TransFix™-stabilized CSF is similar to that of immediately processed CSF.

TransFix™ may stabilize leukocytes in different ways: a buffer may prevent cell death due to an increase in pH,[21] while an aliphatic aldehyde would fixate the cells by cross-linking of amino-acid residues.[16] TransFix™ also contains heavy metal salts that, according to the inventors, further stabilize leukocytes, and reduce excessive autofluorescence caused by aliphatic aldehydes.[22] TransFix™ was originally designed for the stabilization of whole blood samples. In blood, TransFix™ has shown to reduce cellular loss of lymphocytes up to 10 days of storage.[18] In addition, light scatter properties of lymphocytes were well maintained, autofluorescence levels were low, and fixation did not interfere with surface antigen labeling for CD45, CD3 and other markers, enabling reliable enumeration of major lymphocyte subsets.[17,18] However, TransFix™ was less effective in preventing granulocyte and monocyte loss over time. In these leukocyte subsets, it caused a decrease in forward and sideward scatter signals, and negatively affected surface antigen labeling.[18] The effects of TransFix™ on leukocytes in CSF and malignant hematological cells have not been studied in detail so far. Here, we have shown that, similar to blood, TransFix™ potently reduced cellular loss of lymphocytes. In addition, the effects of TransFix™ on light scatter signals seemed not to interfere with the identification of diffuse large B cell lymphoma cells based on their higher forward scatter signal than other lymphocytes. Also the slightly negative effect on surface antigen labeling did not interfere with proper identification of cellular subsets or malignant cells.

The acquired CSF volumes are potential limitations of this study. First, the average volume of CSF studied per condition and time point was ~ 0.5 mL, since it was considered unethical to collect more than 3 mL of extra CSF in addition to the CSF needed for conventional tests and procedures. Normally, it is recommended to analyze a minimum of 2 mL of CSF, in order to obtain sufficient sensitivity.[15] Theoretically, the discrepancies in test results found in this study might not apply to higher CSF volumes. However, we believe that it is reasonable to assume that CSF-stabilizing reagents that enable more sensitive detection of LHM in 0.5 mL of CSF will also have a beneficial effect on larger CSF volumes.

Second, we chose to directly collect CSF in TransFix™ CSF storage tubes, since previous data suggested that significant cellular loss may occur directly after withdrawal.[12] As a consequence, some variation in the acquired CSF volumes was seen between samples with TransFix™, and paired samples with serum-containing medium or native CSF. However, we do not believe that the higher detection rates of LHM in CSF with TransFix™ were caused by a larger test volume, because median CSF volumes in TransFix™ were not higher than in the paired control samples, and in particular in discordant pairs of CSF samples, CSF volumes did not differ significantly.

To conclude, we showed that TransFix™ enables flow-cytometric detection in 18-hours old CSF samples with a similar or higher detection rate than in rapidly processed CSF with serum-containing medium or native CSF. We propose that the use of TransFix™ may facilitate flow cytometric analysis of CSF samples that were collected outside office hours, and enable the use of external flow cytometry facilities in institutions without a flow cytometry facility of their own. In addition, TransFix™ may save costs, since it allows storage and batch-processing of CSF samples instead of immediate processing that requires continuous availability of a flow cytometry facility.

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REFERENCES

1. Bromberg JE, Breems DA, Kraan J, et al. CSF flow cytometry greatly improves diagnostic accuracy in CNS hematologic malignancies. *Neurology.* May 15 2007;68(20):1674-1679.
2. French CA, Dorfman DM, Shaheen G, Cibas ES. Diagnosing lymphoproliferative disorders involving the cerebrospinal fluid: increased sensitivity using flow cytometric analysis. *Diagnostic cytopathology.* Dec 2000;23(6):369-374.
3. Hegde U, Filie A, Little RF, et al. High incidence of occult leptomeningeal disease detected by flow cytometry in newly diagnosed aggressive B-cell lymphomas at risk for central nervous system involvement: the role of flow cytometry versus cytology. *Blood.* Jan 15 2005;105(2):496-502.
4. Quijano S, Lopez A, Manuel Sancho J, et al. Identification of leptomeningeal disease in aggressive B-cell non-Hodgkin's lymphoma: improved sensitivity of flow cytometry. *J Clin Oncol.* Mar 20 2009;27(9):1462-1469.
5. Finn WG, Peterson LC, James C, Goolsby CL. Enhanced detection of malignant lymphoma in cerebrospinal fluid by multiparameter flow cytometry. *American journal of clinical pathology.* Sep 1998;110(3):341-346.
6. Nuckel H, Novotny JR, Noppeney R, Savidou I, Duhrsen U. Detection of malignant haematopoietic cells in the cerebrospinal fluid by conventional cytology and flow cytometry. *Clinical and laboratory haematology.* Feb 2006;28(1):22-29.
7. Roma AA, Garcia A, Avagnina A, Rescia C, Elsner B. Lymphoid and myeloid neoplasms involving cerebrospinal fluid: comparison of morphologic examination and immunophenotyping by flow cytometry. *Diagnostic cytopathology.* Nov 2002;27(5):271-275.
8. Sayed D, Badrawy H, Ali AM, Shaker S. Immunophenotyping and immunoglobulin heavy chain gene rearrangement analysis in cerebrospinal fluid of pediatric patients with acute lymphoblastic leukemia. *Leukemia research.* May 2009;33(5):655-661.
9. Subira D, Castanon S, Roman A, et al. Flow cytometry and the study of central nervous disease in patients with acute leukaemia. *British journal of haematology.* Feb 2001;112(2):381-384.
10. Brem SS, Bierman PJ, Black P, et al. Central nervous system cancers: Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw.* Sep 2005;3(5):644-690.
11. Zelenetz AD, Advani RH, Buadi F, et al. Non-Hodgkin's lymphoma. Clinical practice guidelines in oncology. *J Natl Compr Canc Netw.* Mar 2006;4(3):258-310.
12. de Graaf MT, van den Broek PD, Kraan J, et al. Addition of serum-containing medium to cerebrospinal fluid prevents cellular loss over time. *J Neurol.* Aug 2011;258(8):1507-1512.
13. Dux R, Kindler-Rohrborn A, Annas M, Faustmann P, Lennartz K, Zimmermann CW. A standardized protocol for flow cytometric analysis of cells isolated from cerebrospinal fluid. *J Neurol Sci.* Jan 1994;121(1):74-78.
14. de Graaf MT, de Jongste AH, Kraan J, Boonstra JG, Smitt PA, Gratama JW. Flow cytometric characterization of cerebrospinal fluid cells. *Cytometry.* May 12 2011(80B):271-281.
15. Kraan J, Gratama JW, Haioun C, et al. Flow cytometric immunophenotyping of cerebrospinal fluid. *Current protocols in cytometry / editorial board, J. Paul Robinson, managing editor et al.* Jul 2008;Chapter 6:Unit 6 25.
16. Barnett D, Granger V, Mayr P, Reilly JT, Inventors. Preparation and stabilisation of cells. US patent WO 95/017961995.

17. Barnett D, Granger V, Mayr P, Storie I, Wilson GA, Reilly JT. Evaluation of a novel stable whole blood quality control material for lymphocyte subset analysis: results from the UK NEQAS immune monitoring scheme. *Cytometry.* Sep 15 1996;26(3):216-222.

18. Canonico B, Zamai L, Burattini S, et al. Evaluation of leukocyte stabilisation in TransFix-treated blood samples by flow cytometry and transmission electron microscopy. *J Immunol Methods.* Dec 2004;295(1-2):67-78.

19. de Graaf MT, Smitt PA, Luitwieler RL, et al. Central memory CD4(+) T cells dominate the normal cerebrospinal fluid. *Cytometry.* Jul 14 2011;80(1):43-50.

20. Ahluwalia MS, Wallace PK, Peereboom DM. Flow cytometry as a diagnostic tool in lymphomatous or leukemic meningitis: ready for prime time? *Cancer.* Apr 1 2012;118(7):1747-1753.

21. Cunniffe JG, Whitby-Strevens S, Wilcox MH. Effect of pH changes in cerebrospinal fluid specimens on bacterial survival and antigen test results. *Journal of clinical pathology.* Mar 1996;49(3):249-253.

22. Granger V, Barnett D, Reilly JT, Mayr PSM, Fay SP, Inventors. Preparation and stabilisation of cells using aged transition metals. US patent US 6,197,540. Mar. 6, 2001, 2001.

Part III

Clinical studies

7

Current and future approaches for treatment of paraneoplastic neurological syndromes with well characterized onconeural antibodies

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ABSTRACT

Introduction

Paraneoplastic neurological syndromes (PNS) are severely disabling conditions that are associated with cancer. Well characterized onconeural antibodies (Abs) are, by definition, virtually exclusively present in patients with cancer and include anti-Hu, Yo, CV2, Ri, Ma2, amphiphysin and DNER (anti-Tr). More recently a second group of antineuronal antibodies has been described that occur both in patients with and without cancer.

Areas covered

This review is focused on putative T cell mediated immunopathogenetic mechanisms and treatment of PNS associated with well characterized onconeural Abs. As of December 2013, only uncontrolled open-label clinical trials, retrospective case series and case reports were detected in the literature. Six clinical studies in Hu-PNS reported the effect of immunotherapy on functional outcome.

Expert opinion

When taken together, these 6 studies showed an improvement of one point or more on the modified Rankin scale (mRS) in 11% (7/61) of patients. In Yo-PNS, 8% (2/26) of patients improved on the mRS. PNS with other well characterized onconeural Abs also responded poorly to immunotherapies. Potential new immunotherapies include natalizumab, fingolimod, alemtuzumab and combination of rituximab with cyclophosphamide.

INTRODUCTION

Paraneoplastic neurological syndromes (PNS) are severely disabling conditions that are associated with cancer, but not caused by invasion of the tumor or its metastases.[1] In patients with PNS, various antibodies (Abs) have been found directed at neuronal antigens that are also expressed by the associated tumors in most cases (Table 1). These Abs can be directed at intracellular or cell surface proteins.[2,3]

In 2004, the term 'well characterized onconeural Abs' was defined based on (1) Abs for which there are recognizable reactivity patterns on routine immunohistochemistry, confirmed by immunoblotting on recombinant proteins; (2) the number of cases reported associated with tumors; (3) the description of well characterized neurological syndromes associated with the antibodies; (4) the unambiguous identification of the Abs among independent studies, and (5) the (virtual) absence of these Abs in patients without cancer.[4] These 'well characterized onconeural Abs' are by definition almost exclusively found in patients with cancer and include anti-Hu, Yo, CV2, Ri, Ma2 and amphiphysin.[4] Since the recent identification of the transmembrane protein Delta/Notch-like epidermal growth factor-related receptor (DNER) as the Tr-antigen, anti-Tr (anti-DNER) can also be considered a well characterized onconeural Ab due to its close association with Hodgkin lymphoma.[5]

Patients with any neurological syndrome with unknown cause who harbor these well characterized onconeural Abs fulfill the diagnostic criteria of a 'definite PNS' and should be screened for the presence of a tumor.[4,6] In 2006, a European task force evaluated the management of PNS and concluded that immune therapies such as steroids, plasma exchange or intravenous immunglobulins had generally no or only modest effects on PNS associated with well characterized onconeural Abs.[7] The main recommendations of the task force included treatment of the underlying tumor, if histologically proven, and symptomatic therapies. In 2012, a Cochrane review concluded that there is only class IV evidence of the effect of immunomodulation on paraneoplastic neuropathy.[8]

The prognosis of PNS patients with well characterized onconeural Abs is poor: improvement of the neurological disability is rare, and median survival is 12 to 22 months.[9-11] Apart from anti-DNER, all well characterized onconeural antibodies are directed against intracellular proteins. Since intracellular antigens are not directly accessible to Abs, the majority of these well characterized onconeural Abs are probably not pathogenic but are considered disease markers of a concurrent T cell-mediated immune response targeted against the same proteins.[12-15]

A second group of antineuronal Abs is found in patients with PNS that can also be detected in non-paraneoplastic neurological autoimmune syndromes (Table 1).[16] These Abs can therefore not be categorized as 'well characterized onconeural Abs' according to the criteria of Graus et al.[4] Examples include anti-N-methyl-D-aspartate (NMDA) receptor Abs and anti-voltage gated calcium channels (VGCC) Abs.[16-18] Most of these Abs are potentially pathogenic as they are directed against easily accessible neuronal surface antigens.[16] The associated PNS often respond much better to tumor treatment and immunotherapies than PNS with well characterized onconeural Abs.[3,16,17]

In this review, we provide an update on the available evidence on the immunopathogenesis and immunotherapy of PNS associated with well characterized onconeural Abs.

METHODS

The PubMed (MEDLINE; 1946 - December 2013) and Cochrane Libraries (*The Cochrane Library*; December 2013) were searched for published articles on the pathogenesis and treatment of paraneoplastic neurological syndromes. Search terms included the medical subject headings "paraneoplastic syndromes, nervous system" or "paraneoplastic neurological syndromes", in combination with either (i) "etiology.fs." or "pathogenesis", or (ii) "drug therapy.fs" or "treatment". In addition, we searched for specific PNS using the following search terms: (1) "Hu" or "ANNA-1" or "anti-neuronal nuclear antibody 1" or "HuD" or "HuC" or "Hel-N1"; (2) "Yo" or "PCA-1" or "type 1 Purkinje cell cytoplasmic autoantibodies" or "CDR62"; (3) "CV2" or "CRMP5" or "collapsin response mediator protein 5"; (4) "Ri" or "ANNA-2" or "anti-neuronal nuclear antibody 2" or "Nova-1" or "Nova-2"; (5) "Ma2" or "Ta"; (6) "amphiphysin"; (7) "recoverin", and (8) "Tr" or "DNER"; any of these 8 in combination with "paraneoplastic" or "treatment".

To systematically evaluate the effectiveness of immunotherapy in patients with PNS associated with antibodies against intracellular proteins, we selected prospective clinical trials, and case series that (i) included patients with PNS associated with well characterized onconeural Abs, (ii) were aimed at evaluating the effects of immunotherapy and (iii) reported functional outcome using a well-defined clinical scale such as the modified Rankin scale (mRS).[19] Patients with mRS ≤3 are ambulatory patients, while patients with mRS ≥4 are bedridden or wheelchair-bound. Functional improvement or deterioration were defined as a change of at least 1 point on the mRS. Treatment was considered successful when a patient with mRS ≤3 improved or stabilized and when a patient with mRS ≥4 (bed- or wheelchair-bound) improved one

Table 1. Paraneoplastic antibodies and their associations with neurological syndromes and tumors

Antibody	Neurological syndromes	Tumors (prevalence)
Well characterized onconeural antibodies		
Anti-Hu (ANNA-1)	EM, LE, SSN, PCD, AN	SCLC, neuroblastoma, prostate (84-88%) [A] [9,11,84,85]
Anti-Yo (PCA-1)	PCD	Ovary, breast (79-95%)[10,52,86]
Anti-CV2 (CRMP5)	SSN, AN, PCD, EM, LE, ON, Chorea	SCLC, thymoma (86-91%)[64,87]
Anti-Ri (ANNA-2)	OMS, BE, PCD	SCLC, breast (86-100%)[65,88]
Anti-Ma2 (Ta)	EM, LE, PCD, BE	Testicle, lung (63%-89%)[66,67]
Anti-Amphiphysin	SPS, EM, SSN, SMN	SCLC, breast (79-100%)[69,70,89]
Anti-DNER (Tr)	PCD	Hodgkin's lymphoma (89%)[5,51]
Partially characterized onconeural antibodies		
Anti-ANNA-3	SMN, PCD, AN, LE, BE	SCLC (82%)[90]
Anti-Recoverin	Cancer-associated retinopathy	SCLC (100%)[91,92]
Antibodies associated with paraneoplastic and non-paraneoplastic (i.e. autoimmune) neurological syndromes		
Anti-GAD	SPS, PCD, LE	Lung, neuroendocrine tumors (1-9%)[69,93]
Anti-VGCC	LEMS, PCD	SCLC (57%)[18]
Anti-AchR	Myasthenia gravis	Thymoma (19%)[94]
Anti-gAchR	AN	SCLC, thymoma (11%)[95]
Anti-NMDAR	Anti-NMDAR-associated encephalitis	Ovarian teratoma (38%)[96]
Anti-LGI1	LE	Rarely paraneoplastic (8%)[97-100]
Anti-Caspr2	Morvan syndrome or neuromyotonia	Thymoma (22%)[98,101-103]
Anti-AMPAR	LE	Lung, breast, thymus (66%)[104,105]
Anti-$GABA_BR$	LE	SCLC (60%)[106-109]
Anti-mGluR1	PCD	Hodgkin lymphoma, prostate carcinoma (60%)[110-113]
Anti-mGluR5	Ophelia syndrome	Hodgkin lymphoma (100%)[112,114]
Anti-GlyR	SPS, PERM	Hodgkin lymphoma, thymoma, breast, lung (19%)[115,116]

[A] In children younger than 18 years, anti-Hu was associated with neuroblastoma (or any other tumor) in only 2 of 8 (25%) patients.[117]

Abbreviations: prop, proportion (patients with detectable tumor/total) used to calculate prevalence; ANNA, anti-neuronal nuclear antibody; EM, encephalomyelitis; LE, limbic encephalitis; SSN, subacute sensory neuronopathy; PCD, paraneoplastic cerebellar degeneration; AN, autonomic neuropathy; SCLC, small-cell lung cancer; PCA, Purkinje cell cytoplasmatic autoantibodies; CRMP, collapsin response mediator protein; ON, optic neuritis; BE, brainstem encephalitis; SPS, stiff person syndrome; SMN, sensory motor neuropathy; DNER, delta/notch-like epidermal growth factor-related receptor; GAD, glutamic acid decarboxylase; VGCC, voltage-gated calcium channel; LEMS, Lambert-Eaton myasthenic syndrome; AchR, acetylcholine receptor; g, ganglionic; NMDAR, N-methyl-D-aspartate receptor; yrs, age in years; VGKC, voltage gated potassium channel; Ag, antigen; AMPAR, α-amino-3-hydroxy-5-methyl-4-osixazolepropionic acid receptor; GABABR, gamma-aminobutyric acid B receptor; mGluR, metabotropic glutamate receptor.

point or more.[20] For PNS with onconeural antibodies for which no prospective clinical trials or case series were available, we reviewed the available case reports. To identify possible predictors for functional outcome after immunotherapy in patients with PNS with anti-Hu Abs (Hu-PNS), we pooled data from the available therapeutic studies. Prism version 5 (GraphPad, San Diego, CA) was used for univariate statistical analyses (Fisher's exact test or Mann-Whitney test), SPSS version 20 (IBM, Chicago, IL) was used for multivariate analysis (logistic regression; using successful functional outcome as dependent variable and age, CNS involvement, initial mRS, detectable tumor and anti-tumor therapy as independent variables; no correction for 'study'). Two-sided P-values <0.05 were considered significant.

IMMUNOPATHOGENESIS

Anti-Hu is the most frequent well characterized onconeural Ab and is usually associated with paraneoplastic encephalomyelitis and sensory neuronopathy.[9,21] Anti-Hu Abs are directed at a family of RNA-binding proteins (HuD, HuC, Hel-N1 and HuR).[22] Three of these proteins (HuD, HuC and Hel-N1) are neuronal specific while HuR is ubiquitously expressed. In an ELISA assay, anti-Hu Ab titers against HuR were much lower than against HuD, HuC and Hel-N1 indicating that the immunoreactivity against HuR represents cross-reactive antibody specificities directed against conserved sequences in this family.[23] In addition, only HuD and Hel-N1 are expressed by SCLC and SCLC-derived tumor cell lines.[22,24]

The presence of high titers of anti-Hu Abs in the blood and cerebrospinal fluid of Hu-PNS patients led to the hypothesis that Hu-PNS are caused by an immune response triggered by Hu expression in tumor cells that also reacts with Hu-expressing neurons.[25] Supporting evidence for a putative immunopathogenetic mechanism is found in HLA-associations,[26] intrathecal antibody production,[27] cerebrospinal fluid (CSF) pleocytosis,[28,29] and infiltrates of oligoclonal cytotoxic T cells around damaged neurons at autopsy.[30-33] Since immunization of animals against HuD did result in high-titered anti-Hu Abs but no disease, it was hypothesized that the anti-Hu Abs themselves were not pathogenic.[14,34] In addition, these studies showed that anti-Hu Abs did not enter neurons and bind the intracellular Hu-proteins, suggesting that the observed intraneuronal IgG accumulation in autopsy studies in humans may have resulted from a postmortem artefact.[14] Finally, autopsy studies showed only weak complement reactivity and absence of natural killer (NK) cells, suggesting that complement-mediated and antibody-dependent cell cytotoxicity were not pathogenic.[35] In view of this lack of support for Ab-mediated pathogenesis of PNS, cytotoxic $CD8^+$ T cells were proposed

to be responsible for neuronal destruction in Hu-PNS, as supported by the $CD8^+$ T cell infiltrates around damaged neurons found at autopsy.[15,31] Based on this hypothesized $CD8^+$ T cell response, several studies have been performed to detect HuD-specific cytotoxic $CD8^+$ T cells, but initial positive results[36-38] could not be reproduced.[39,40] More recently, $CD8^+$ HuD-specific T cells were described with an abnormal 'type 2' phenotype[41], but these data could not be reproduced either.[42] In summary, cumulative evidence suggests that neuronal destruction in Hu-PNS is caused by a T cell-mediated immune response, but the specificity, phenotype and function of these autoreactive T cells remain largely unknown.

Similar to Hu-PNS, also PNS with Abs against other intracellular antigens are probably T cell-mediated. Animal models failed to demonstrate a pathogenic effect of anti-Yo Abs.[12,13] Postmortem studies of patients with paraneoplastic cerebellar degeneration and anti-Yo Abs (Yo-PNS) showed $CD8^+$ T cell infiltration in the cerebellum and loss of Purkinje cells.[43,44] Yo-specific T cells have been described, but these results could not be reproduced.[45,46] Immunopathological studies in PNS with anti-CV2, anti-Ma2 and anti-Ri Abs also showed cytotoxic T cells infiltrates and neuronal loss supporting the hypothesis that these PNS may also be T cell-mediated.[33,47-49]

An exception is PNS with anti-amphiphysin Abs, as autoantibodies against intracellular amphiphysin are probably pathogenic. Amphiphysin is an intracellular neuronal protein involved in synaptic vesicle endocytosis. Intrathecal passive transfer of affinity purified anti-amphiphysin IgG into rats caused stiffness and muscle spasms resembling stiff-person syndrome (SPS).[50] Electrophysiological studies identified reduced presynaptic GABA-ergic inhibition as an underlying mechanism while immunofluorescence studies provided evidence that anti-amphiphysin IgGs can enter the synapses of neurons.[50] How the Abs get taken up into the nerve terminals remains unclear although one may speculate that epitopes of amphiphysin are exposed during GABA release when synaptic vesicles transiently fuse with the neuronal membrane.

Contrary to anti-amphiphysin Abs that are directed at an intracellular antigen and may still be pathogenic, anti-DNER Abs are directed at the extracellular domain of DNER, but may not be pathogenic. Anti-DNER Abs did not change the morphology of neurons in vitro, suggesting that the Abs did not directly interfere with DNER function.[5] Anti-Tr/DNER PNS patients have a poor prognosis and an autopsy study showed loss of Purkinje cells.[51,52] These findings may indicate that complement-mediated toxicity or antibody-dependent cytotoxicity play a role, although anti-DNER PNS may also be caused by a T cell-mediated immune response despite the accessability of the target antigen to the Abs.[53]

IMMUNOTHERAPY

Rationale for the use of immunotherapy

In patients with PNS and well characterized onconeural antibodies, detection and treatment of underlying tumors offers the best chance to stabilize their PNS.[54] However, even after tumor treatment, only 3-6% (5/164; 3/51) of Hu-PNS patients improve, while 30% (15/51) deteriorate.[9,54] Median survival for Hu-PNS patients is 12 months, and most patients die from progression of their PNS (60%, 76/126), not their tumor.[9] In PNS patients with anti-Yo Abs, 73% (14/19) further deteriorate after diagnosis of their PNS, 78% (15/17) of patients become bedridden, 36% (8/22) die from their PNS, and median survival is 13-22 months.[10,52] Hence, there is a clear need for immunotherapies that can effectively suppress the harmful auto-immune response in PNS and prevent further deterioration, morbidity and death.

A possible negative effect of immunotherapy might be the suppression of a concurrent beneficial immune response against the associated tumor. There is conflicting evidence that SCLC patients with low titers of anti-Hu Abs without PNS may have a better tumor prognosis,[55,56] and spontaneous tumor regressions have been reported in Hu-PNS patients indicating that the immune response against Hu may control tumor growth.[57] However, in a series of 51 Hu-PNS patients, no negative effect was seen of immunotherapy (i.e., intravenous IgG, plasma exchange or cytoreductive therapy and immunosuppression) on the outcome of anti-tumor therapy,[54] and complete cancer remissions after chemotherapy were frequently reported in studies on immunotherapies in PNS.[58-60] In conclusion, the severity of PNS and the poor functional outcome after tumor treatment alone indicate that there is a need for effective immunotherapy in PNS. There is no substantial evidence that such a treatment would negatively affect tumor outcome.

Functional outcome and survival after immunotherapy in Hu-PNS

Most studies on immunotherapy in PNS involve patients with Hu-PNS, reflecting the relatively high frequency of anti-Hu Abs.[21] Six studies evaluated functional outcome after immunotherapy using the mRS (Table 2).[20,58-62] A seventh study on the effect of tacrolimus + prednisone included 6 Hu-PNS patients but did not formally asses functional outcome.[63] All studies were uncontrolled open-label studies, the included numbers of Hu-PNS patients were small (range 7-18 patients), and only three studies reported a prospective design.[58-60] Taken together, these studies reported functional improvement, defined as an improvement of one point or more on the mRS, in 11% (7/61) of patients, which is slightly, and not significantly higher than in the largest published case series on Hu-PNS thus far (i.e. 5% (10/200), Fisher's exact test: p= 0.08).[9]

Table 2. Clinical outcome after immunotherapy in Hu-PNS

Study				Age	Gender		Response to treatment (n, %) [A]				Survival
(author, year)	Design	Treatment	N	(years) [B]	(M/F)	mRS [B]	Improved	Stable	Worse	Succesful	(months) [C]
Graus 1992[61]	R	PE+MP (+CTX) [D]	7	58 (49-64)	7/0	4 (4-5)	0 (0%)	5 (71%)	2 (29%)	0 (0%)	3 (1-7)
Uchuya 1996[62] [E]	NR	ivIg	17	60 (35-74)	12/5	3 (1-5)	1 (6%)	8 (47%)	8 (47%)	6 (35%)	10.5 (2-48+)
Keime-Guibert 2000[20] [E]	NR	ivIg+CTX+MP	9	62 (50-72)	6/3	3 (1-4)	0 (0%)	5 (56%)	4 (44%)	2 (20%)	7 (3-35+)
Vernino 2004[60]	P	PE (+CTX) [F]	5	63 (57-78)	3/2	3 (2-4)	2 (40%)	1 (20%)	2 (40%)	3 (60%)	>20 (1-41+)
Shams'ili 2006[58]	P	Rituximab	8	58,5 (52-80)	4/4	4 (3-5)	2 (25%)	3 (38%)	3 (38%)	4 (50%)	8 (1-13+)
v. Broekhoven 2010[59]	P	hCG	15	66 (51-75)	7/8	3 (2-4)	2 (13%)	6 (40%)	7 (47%)	7 (47%)	18 (0-42+)
Pooled	NA	NA	61	61 (35-80)	39/22	3 (1-5)	7 (11%)	28 (46%)	26 (43%)	22 (36%)	10 (0-48+)

[A] Improved/worse indicates a decrease/increase of ≥ 1 on the mRS; successful treatment is defined as a stable mRS ≤3, or a mRS ≥ 4 and an decrease of ≥ 1 on the mRS.
[B] Median (range)
[C] Median survival (range) from the start of immunotherapy, ">" indicates that the survival was >50% at the longest follow-up time.
[D] 4 patients additionally received CTX
[E] These 2 studies each included 1 patient who was 'not evaluable' after treatment and hence not included in this table on treatment outcome[20,62]
[F] 2 patients additionally received CTX
Abbreviations: N, number of patients; M, male; F, female; mRS, modified Rankin scale; R, retrospective; P, prospective; NR, not reported; PE, plasma exchange; ivIg, intravenous immunoglobulins; CTX, cyclophosphamide; MP, methylprednisolone; hCG, human chorionic gonadotropin; NA, not applicable; +, patient with longest survival still alive at last follow-up.

Table 3. Clinical outcome after immunotherapy in Yo-PNS

Study				Age	Gender		Response to treatment (n, %) [A]				Survival
(author, year)	Design	Treatment	N	(years) [B]	(M/F)	Rankin [B]	Improved	Stable	Worse	Successful	Median (month) [C]
Graus 1992[61]	R	PE	4	71 (41-78)	0/4	3.5 (3-4)	0 (0%)	3 (75%)	1 (25%)	2 (50%)	30 (6-53+)
Uchuya 1996[62]	NR	ivIg	4	69 (43-78)	0/4	4 (3-5)	0 (0%)	2 (50%)	2 (50%)	0 (0%)	6 (3-8)
Keime-Guibert 2000[20]	NR	ivIg+CTX+MP	7	67 (52-70)	0/7	4 (3-4)	0 (0%)	3 (43%)	4 (57%)	1 (14%)	5 (2-38+)
Vernino 2004[60]	P	PE(+CTX) [D]	11	56 (39-74)	0/11	4 (3-4)	2 (18%)	4 (36%)	5 (45%)	3 (27%)	>30 (2-38+)
Pooled	NA	NA	26	65 (39-78)	0/26	4 (3-5)	2 (8%)	12 (46%)	12 (46%)	6 (23%)	18 (2-53+)

[A] Improved/worse indicates a decrease/increase of ≥ 1 on the mRS; succesful treatment is defined as a stable mRS ≤3, or a mRS ≥ 4 and an decrease of ≥ 1 on the mRS.
[B] Median (range)
[C] Median survival from the start of immunotherapy, ">" indicates that the survival was > 50% at the longest follow-up time
[D] 4 patients additionally received CTX
Abbreviations: N, number of patients; M, male; F, female; R, retrospective; P, prospective; NR, not reported; PE, plasma exchange; ivIg, intravenous immunoglobulins; CTX, cyclophosphamide; MP, methylprednisolone; NA, not applicable, +, patient with longest survival still alive at last follow-up.

In this setting, a therapeutic benefit can be considered as either at least stabilization in patients who are still ambulatory (mRS≤3) or improvement (decrease of ≥1 on the mRS) in bedridden patients (RS≥4).[20] According to these criteria, immunotherapy was considered successful in 36% of cases, which is within the range of previous clinical series (14-53%) (Table 2).[11] In the study investigating tacrolimus + prednisone, subjective improvement was reported in 3/6 Hu-PNS patients. Median survival from the start of immunotherapy was 10 months (range 0 to >48) in the studies shown in Table 2. In the study with tacrolimus + prednisone, the median survival from the start of immunotherapy was 57 months (range, 2 - 61; n=6).[63]

Functional outcome and survival after immunotherapy in Yo-PNS

Four of the 6 clinical studies described in Table 2 also included four or more Yo-PNS patients (Table 3).[20,60-62] Taken together, these studies reported an improvement of one point or more on the mRS in 8% (2/26) of patients. Immunotherapy was considered successful in 6 patients (23%). Survival from the start of immunotherapy was 18 months (range, 2 to >53), similar to larger clinical series that also included patients who did not receive immunotherapy (13-22 months).[10,52] In another study that investigated the effect of tacrolimus + prednisone in 19 Yo-PNS patients who all had ovarian cancer and received tumor treatment, subjective improvement occurred in 11 patients (58%) and a median survival from start of immunotherapy of 48 months was reported.[63]

Functional outcome and survival after immunotherapy in other subgroups of PNS

Patients with PNS with anti-CV2/CRMP5 Abs are on average less disabled and survive longer than Hu-PNS patients (mean mRS 2.5 vs. 3.1; median survival times 47.7 vs. 11.5 months, respectively).[64] The longer survival in CV2/CRMP5-PNS could partly be explained by the fact that CV2/CRMP5-PNS are not only associated with SCLC but also with thymoma. Tumor type is, however, not the only factor that causes a better prognosis in CV2/CRMP5-PNS, since CV2/CRMP5-PNS patients with SCLC seem to have a better prognosis than Hu-PNS patient with SCLC as well.[64] Only two cases of CV2/CRMP5-PNS have been described in studies aimed at evaluating the effects of immunotherapy. One female patient with ataxia and optic neuritis and no tumor remained ambulatory (mRS 3) after treatment with plasma exchange and cyclophosphamide and survived for at least 31 months.[60] Another female encephalopathic patient with a small cell carcinoma was severely disabled (mRS 5), did not respond to tacrolimus and prednisone, and died one month after treatment.[63]

Pittock et al. reported clinical outcome of 17 patients with PNS with anti-Ri Abs after tumor and/or immunotherapy, 14 of them had a tumor.[65] In this series, subjective

clinical improvement was reported by the treating physician in 5 out of 7 patients who received only anti-tumor therapy (radiation, chemotherapy and/or surgery); in 4 out of 6 patients who received only immunotherapy (intravenous immunoglobulins [ivIg], or plasma exchange, and/or methylprednisolone or dexamethasone); and in 3 out of 4 patients that received both therapies. Interestingly, the 2 patients that did not improve upon immunotherapy both had no detectable cancer. Shams'ili et al. reported 6 patients with anti-Ri antibodies and paraneoplastic cerebellar degeneration, 5 of them had a tumor.[52] In this series, the mRS was used to assess functional status and outcome after therapy. Four out of these 6 patients were ambulatory (mRS ≤ 3); 1 patient received anti-tumor treatment but progressed; 2 out of 3 patients who received immunotherapy remained stable or improved; and 2 out of 2 patients that received both therapies remained stable or improved. The median survival of these 6 patients was >69 months.[52]

Dalmau et al. presented clinical outcome of 33 patients with PNS with anti-Ma2 Abs, 30 of them had a tumor.[66] Eight patients received only anti-cancer treatment, 4 of them improved, 3 stabilized and 1 progressed. Ten patients received only immunotherapy (steroids, ivIg, and/or plasma exchange), 3 of them improved, 1 of them stabilized and 6 progressed. Nine patients received anti-tumor therapy and immunotherapy (steroids, ivIg, plasma exchange, IgG adsorption and/or cyclophosphamide), 4 of them improved, 3 stabilized and 2 progressed. All 6 patients who did not receive anti-tumor or immunotherapy progressed. Interestingly, the three patients who improved upon immunotherapy alone did very well, two of them even completely recovered.[66] The chance of neurological stabilization or improvement in patients with anti-Ma2 Abs (reacting solely to Ma2; also called anti-Ta) seems better than that of patients with anti-Ma Abs (reacting to Ma1 and Ma2): 69% vs. 24%, respectively.[67,68]

Clinical improvements have been reported in patients with PNS with anti-amphiphysin Abs after treatment of the tumor and following immunomodulation with steroids, ivIg or plasma exchange.[69-71] In a series of 63 amphiphysin-PNS patients, neurological improvement was reported in 4 patients who received methylprednisolone, while at least one patient did not respond to methylprednisolone.[70] In the same study, two out of four patients who received ivIg improved neurologically. Four patients who received plasma exchange showed no neurological improvement. In this study, neurological improvement was not quantified. Survival did not differ significantly between patients with cancer who received immunotherapy and/or anti-tumor therapy and those who did not receive therapy.[70] In another study on 11 patients with stiff-person syndrome associated with anti-amphiphysin Abs, all patients were female; 10 of them had breast cancer. Nine patients were described as benzodiazepine responsive and

three were described as dramatically better following tumor excision and chemotherapy. Four patients responded to methylprednisolone, while ivIg efficacy was not reported.[69] Efficacy of plasma exchange in patients with paraneoplastic SPS is limited to case reports.[71]

Paraneoplastic cerebellar degeneration (PCD) with anti-DNER Abs and Hodgkin lymphoma responds poorly to therapy, similar to PCD with other well characterized onconeural Abs.[51,52] In a series of 7 anti-DNER PNS patients, 6 patients had a Hodgkin lymphoma.[52] One of these patients, who received anti-tumor therapy resulting in complete remission, showed objective functional improvement (≥ 1 point on the mRS). In a larger series of 28 PCD patients with anti-DNER Abs, 4 patients (14%) showed complete (3) or partial (1) remission of ataxia, while the majority of patients remained with a bad functional status.[51] In comparison to patients with PCD with anti-Hu and anti-Yo Abs, patients with PCD with anti-Tr/DNER Abs survived longer (median >113 months).[52]

Choice of immunotherapy

Based on the evidence for a T cell-mediated immunopathogenesis in Hu-PNS, it has been hypothesized that therapies aimed at depletion of B cells (rituximab) or removal of circulating antibodies (plasma exchange) would be less effective than therapies that include suppression of T cell-mediated immune responses such as methylprednisolone, cyclophosphamide and tacrolimus.[1] However, a small prospective study on the effect of rituximab also showed functional improvement in 2 out of 8 Hu-PNS patients (25%).[52] A possible explanation of the functional improvement after rituximab treatment could be that B cells might contribute to the pathogenesis of Hu-PNS by antigen-presentation to T cells and production of pro-inflammatory cytokines.[72] Similar to rituximab, also ivIg may theoretically be effective in PNS. The main effects of administration of ivIg are thought to be modulation of pathogenic antibodies, interaction with Fc receptors on phagocytic cells and inhibition of complement activation.[73] However, ivIg may also suppress T cell responses by downregulation of cytokines, suppression of T cell functions and interference with antigen recognition. Hence, ivIg have been suggested as second-line immunotherapy in PNS with antibodies against intracellular proteins when corticosteroids are not effective or contra-indicated.[73]

Patient characteristics, tumor treatment and functional outcome

Previously, several factors have been suggested to be associated with better functional outcome including younger age,[60] absence of CNS involvement,[20,62] moderate disability (e.g. mRS ≤3),[9,20,60,62] and tumor treatment.[9] Analysis of pooled data of the studies

shown in Table 2 showed that only functional disability was significantly associated with 'succesful functional outcome' after immunotherapy (Table 4).

Table 4. Possible predictors for functional outcome after immunotherapy for Hu-PNS

Therapeutic studies on Hu-PNS [20,58-62]	Functional outcome					
	Succesful	Not successful	OR [A]	95% CI (OR)	P [A]	P [B]
N of patients	22	39				
Age (years; median, range)	63 (41-78)	61 (35-80)	1.04	0.97-1.11	0.32	0.54
CNS involvement (n, %)	7 (32%)	18 (46%)	0.35	0.09-1.37	0.13	0.30
Initial mRS (median, range)	3 (1-5)	4 (1-5)	0.53	0.30-0.94	0.03	0.003
Detectable tumor [C] (n, %)	18 (82%)	37 (95%)	0.16	0.02-1.37	0.10	0.10
Anti-tumor therapy [D] (n, %)	12 (55%)	15 (39%)	NT	NT	NT	0.09

[A] Odds ratios and P-values derived from multivariate analysis (Logistic regression). Anti-tumor therapy was only considered relevant within patients with detectable tumor, and hence not included in multivariate analysis.
[B] P-values derived from univariate analyses (Fisher exact test or Mann-Whitney test).
[C] At time of first tumor screening.
[D] Chemotherapy and/or radiotherapy and/or surgery *simultaneously with* immunotherapy. Percentages calculated within patients with detectable tumors.
Abbreviations: OR, odds ratio; CI, confidence interval; N, number of patients; n, number of patients positive for predictor; CNS, central nervous system; mRS, modified Rankin score; NT, not tested.

Among the 22 patients with 'successful functional outcome', 7 patients improved in functional status. Among the patients with detectable tumors (n=55), those who received tumor treatment improved more frequently than patients with an untreated tumor (5/27 vs. 0/28; P=0.02 [Fisher exact test]). Four of these 5 patients who showed functional improvement reached complete tumor remission, while in the fifth tumor response was not reported. These trends between improvement in functional status, anti-tumor treatment and complete tumor remission underline the importance of tumor screening and anti-tumor treatment in patients with Hu-PNS. In 2 out of the 7 patients who improved, no tumor was detected when immunotherapy was administered. One patient improved during immunotherapy from a mRS of 3 to a mRS of 2, while it took 20 months before SCLC was detected and successfully treated.[59] The other patient improved from a mRS of 2 to a mRS of 1, while NSCLC was detected and successfully treated after 26 months.[60] This patient survived for more than 41 months. These cases indicate that immunotherapy can be helpful in patients without detectable tumor and does not seem to accelerate tumor growth.

Adverse events

Treatment with ivIg was generally well tolerated. Uchuya et al. reported adverse events in 3 out of 22 PNS patients, two of them could continue treatment (one with

transient headache and one with transient fever, malaise and cyanosis), the third had severe hemolytic anemia.[62] Also treatment with rituximab was well tolerated. Shams'ili et al. treated 9 PNS patients with rituximab, and 3 of them additionally received chemotherapy.[58] Complete B cell-depletion was achieved in 8 of the 9 PNS patients, and none of them had significant adverse events, notably no infections.[58] Treatment with plasma exchange and chemotherapy was tolerated by 16 out of 20 patients. Of the 4 patients with (severe) adverse events, one died from progressive PNS, one developed a central line infection and two had significant hypotension without long-term complications.[60] Adverse events may be more common in patients treated with cyclophosphamide. Keime-Guibert et al. treated 17 PNS patients with 600 mg/m^2 cyclophosphamide i.v. every three weeks in combination with ivIg and methylprednisolone and reported no severe side-effects; only one patient experienced transient feeling of discomfort (shivering and agitation).[20] However, in 9 other PNS patients treated with cyclophosphamide (oral dose of 2 mg/kg/day for 3 months, combined with plasma exchange) all developed grade 1 or 2 anemia (according to the Common Toxicity Criteria)[74], 3 developed grade 3 lymphopenia, 3 developed severe neutropenia (grade 3 or 4) and 1 developed mild hemorrhagic cystitis.[60] In 5 out of 9 patients, treatment with cyclophosphamide had to be discontinued because of hematologic toxicity (n=4) or nausea and vomiting that persisted after dose reduction (n=1).[60] Adverse events were also common after combined treatment with tacrolimus and prednisone.[63] In a series of 26 PNS patients treated with this combination, 23 patients (88%) experienced one or more adverse events.[63] Three of these 23 adverse events were serious and required transfer to an acute care hospital: progressive ataxia, pneumonia, and a progressive encephalopathy in a patient with pneumonia and metastasized small-cell lung cancer.[63]

Quality of evidence

All evidence on immunotherapies for PNS with well characterized onconeural Abs is based on observational studies and uncontrolled open-label trials. Several factors other than immunotherapy may have contributed to the reported clinical outcomes. Patient selection bias may play a role since in the retrospective case series, it is unclear why some patients received immunotherapy and others did not,[9,52] while in some studies on immunotherapy, inclusion criteria (other than PNS) were not defined,[20,61,62] or patients with stabilized PNS and/or severe disabilities were excluded.[58-60] Furthermore, the reviewed studies were hampered by the heterogeneity of included PNS patients and low patient numbers. Hence, according to the criteria of the European Federation of Neurological Societies, the quality of all the evidence presented here should be considered 'very low'.[75]

CONCLUSIONS

Neurological syndromes of unknown cause are 'definite' paraneoplastic neurological syndromes (PNS) when associated with a well characterized onconeural Ab.[4] These 'well characterized onconeural Abs' are by definition almost exclusively found in patients with cancer and include anti-Hu, Yo, CV2, Ri, Ma2, amphiphysin, and DNER (formerly anti-Tr). Apart from anti-DNER, all well-characterized onconeural antibodies are directed against intracellular proteins. Since intracellular antigens are usually not directly accessible to Abs, the majority of these well characterized onconeural Abs are probably not pathogenic but are considered disease markers of a concurrent T cell-mediated immune response targeted against the same proteins.[12-15]

Anti-Hu is the most frequent onconeural Ab and six studies evaluated the functional outcome after various forms of immunotherapy using the mRS. Overall, improvement defined as decrease of one point or more on the mRS occurred in 11% of the patients. When a successful outcome of immunotherapy was defined as improvement by one point or more in bedridden patients and stabilization or improvement in ambulatory patients, immunotherapy was overall successful in 36% of the patients. Four of these six studies also included anti-Yo patients and improvement of one point or more was noted in 8% of the patients with Yo-PNS.

Pooled analysis also showed that in these studies only functional status was associated with 'successful functional outcome'. Trends were observed between improvement in functional status, antitumor treatment and complete tumor remission.

Treatment with ivIg, rituximab (also in combination with chemotherapy), plasma exchange and steroids was generally well tolerated. Adverse events were more common following treatment with cyclophosphamide and combination treatment with tacrolimus and prednisone.

Case series of patients with anti-CV2/CRMP5, Ma, Ri, amphiphysin and DNER are summarized.

EXPERT OPINION

Paraneoplastic neurological syndromes with well characterized onconeural Abs are immune-mediated and, by definition, tightly associated with cancer. When the term 'well characterized onconeural Ab' was introduced, all such Abs were directed at

intracellular neuronal antigens that were also expressed by the associated tumors.[4] Based on the intracellular localization of these neuronal antigens, postmortem studies and animal experiments, it was hypothesized that these PNS were caused by a T cell-mediated immune response against onconeural antigens expressed by neurons and tumor cells.[15]

In the last decade, however, evidence has become available that challenges the hypothesis that intracellular onconeural antigens are targeted by a T cell-mediated immune response, and extracellular onconeural antigens by an antibody-mediated immune response. First, PNS with Abs against the intracellular neuronal antigen amphiphysin may be antibody-mediated, since animal models and in-vitro models showed that anti-amphiphysin immunoglobulin G can enter the nerve terminals, reduce presynaptic GABA-ergic inhibition and cause disease resembling stiff person syndrome.[50] Second, the target of anti-Tr was recently identified as the Delta/Notch-like epidermal growth factor-related receptor (DNER).[5] Given the tight association between PNS with anti-DNER Abs and Hodgkin lymphoma,[51] anti-DNER should now be considered a well characterized onconeural Ab. Anti-DNER Abs are targeted against the extracellular domain of DNER,[5] and hence these Abs could, in theory, directly access their target in the brain and interfere with DNER function. However, anti-DNER Abs did not change neuron morphology in cell cultures.[5] Furthermore, a single autopsy case showed severe loss of Purkinje cells, and DNER-PNS patients respond poorly to therapy.[51] Based on these observations, we suggest that DNER-PNS is most likely either caused by complement dependent toxicity or antibody dependent cellular toxicity; also, a T cell-mediated disease, despite the extracellular target of anti-DNER Abs, remains possible.

The tight association between well characterized onconeural Abs and associated cancers does not necessarily implicate that the targets of these onconeural Abs are expressed by the associated cancers. Immunohistochemical studies using anti-Tr could hardly detect expression of the target antigen in Hodgkin lymphoma tissues of DNER-PNS patients, and normal CRMP proteins could not be detected in thymoma tissue of a CV2-PNS patient (while CRMP5 is highly expressed in SCLC).[51,76] Therefore, we suggest that well characterized onconeural Abs may be better defined as 'tightly associated with the presence of an underlying malignancy' than as 'targeted against neural antigens expressed by the tumor'.[4]

To improve the functional outcome in PNS with well characterized onconeural Abs, various immunotherapies have been used including plasma exchange, ivIg, rituximab, steroids and cyclophosphamide.[20,58-62] There is some evidence that these immunotherapies might improve functional outcome in a few patients. However, within PNS

patients with the most frequent well characterized onconeural (Hu-PNS), only 11% showed objective functional improvement after immunotherapy. The quality of the evidence is classified as 'very low' due to the uncontrolled design of the studies, the low number of patients and the heterogeneity of neurological syndromes and underlying tumor types.

To determine whether some patients respond better to immunotherapy than others, we investigated whether patient characteristics were associated with functional outcome after immunotherapy in Hu-PNS patients. In contrast to previously suggested associations,[9,20,60,62] we could only find a significant relation between prior disability and successful functional outcome (Table 4). Another possible factor may be the disease stage (progressive or stable). We did not include disease stage in this analysis, since data on disease stage were not reported on an individual basis in three of the 6 studies.[20,61,62] However, it must be noted that in these three studies, only one patient improved, and this patient had progressive PNS at the time of treatment.[62] The other studies exclusively included patients with progressive PNS.[58-60] Hence, progressive patients may respond better to immunotherapy than patients who already reached a plateau phase.

In addition, we found that in patients with detectable tumor, concurrent tumor treatment resulted significantly more often in functional improvement. This finding underlines the importance of tumor screening and anti-tumor treatment in patients with Hu-PNS. In these patients, we cannot determine the differential effects of the immunotherapy and anti-tumor therapy. However, also two patients improved after immunotherapy alone while a tumor could only be detected after 20-26 months.[59,60] Thus, tumor treatment seems important, but not obligatory, for functional improvement in Hu-PNS patients who receive immunotherapy.

More effective immunotherapies are clearly needed. Based on the hypothesized immunopathogenesis (Figure 1), several newly available immunotherapies should be investigated.[15,77,78] Examples are fingolimod, a drug that prevents egress of T cells from the lymph nodes by down regulation of the sphingosine 1-phosphate receptor,[79] and natalizumab, a drug that prevents adhesion of T cells and other leukocytes to endothelial cells by binding to very late activation antigen 4 (VLA-4) thereby preventing their extravasation and migration to the central nervous system.[80] Alternatively, one could argue that combined suppression of T and B lymphocyte-mediated immune responses might be more effective than suppression of T lymphocyte-mediated immune responses alone. An example of a single agent that suppresses both, T lymphocytes and B lymphocytes, is alemtuzumab (anti-CD52). Alemtuzumab leads to reduced numbers of

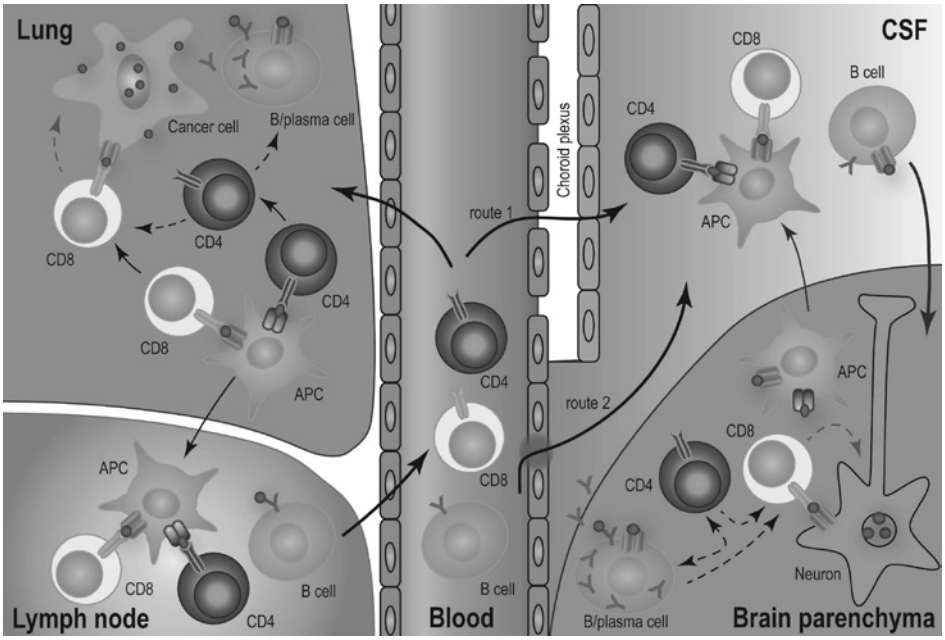

Figure 1.
Schematic representation of the hypothetical immunopathogenesis of Hu-PNS. In the lung, expression of Hu-proteins (red dots) by cancer cells triggers an immune response against Hu proteins. Antigen presenting cells (APCs) present the Hu-proteins to naïve $CD4^+$ and $CD8^+$ T lymphocytes in the lymph nodes, while naïve B lymphocytes undergo an antigen-driven germinal center reaction. These antigen-experienced 'memory' T and B lymphocytes enter the circulation, and travel to the lung. In the lung, the T lymphocytes are re-stimulated by APCs and produce pro-inflammatory cytokines that stimulate $CD8^+$ T cells and B cells ($CD4^+$ T cells, dashed arrows), or kill cancer cells by release of cytotoxic granules ($CD8^+$ T cells, dashed red arrows). Additionally, some of these T and B lymphocytes enter the CSF via the choroid plexus (route 1, at disease initiation), or by adhesion to inflamed endothelium (route 2). The T cells in CSF are re-stimulated by APCs, migrate to the brain parenchyma and kill neurons that express the same Hu proteins. Simultaneously, B cells may contribute to the immunopathogenesis of Hu-PNS by antigen presentation (not shown) and production of pro-inflammatory cytokines (dashed arrows). After stimulation by $CD4^+$ T cells (dashed arrows), B cells can differentiate into plasma cells that produce anti-Hu antibodies. Not shown are the spleen (common place for the B cell germinal center reaction), and bone marrow (common place for antibody-producing plasma cells). Mechanisms involving natural killer (NK) cells are not depicted as these cells were absent in autopsy studies.[35]

circulating T lymphocytes and B lymphocytes and is effective in multiple sclerosis.[81,82] Another strategy to suppress both B and T lymphocytes would be the combination of T cell-targeted therapy and CD20 monoclonal Abs such as rituximab or ofatumumab.[58,83]

To conclude, there is still insufficient evidence to recommend immunotherapy for all patients with PNS associated with well characterized onconeural Abs. In patients with

an identified tumor, immediate antitumor therapy is probably the most import factor contributing to stabilization or improvement of functional outcome. Immunotherapy may be considered in individual patients, especially patients with progressive PNS despite tumor treatment and patients without detectable tumor. In those patients, steroids and ivIg are well tolerated while cyclophosphamide or rituximab could be used as a second-line therapy.[58,73] Available evidence underlines the need for more effective immunotherapies for PNS and the need for larger, controlled clinical trials. This will be a formidable challenge given the rarity and heterogeneity of PNS.

LIST OF ABBREVIATIONS

(g)AchR	acetylcholine receptor; g, ganglionic;
AMPAR	α-amino-3-hydroxy-5-methyl-4-osixazolepropionic acid receptor
AN	autonomic neuropathy
ANNA	anti-neuronal nuclear antibody
APC	antigen presenting cells
BE	brainstem encephalitis
CNS	central nervous system
CRMP	collapsin response mediator protein
CSF	cerebrospinal fluid
CTX	cyclophosphamide
DNER	delta/notch-like epidermal growth factor-related receptor
EM	encephalomyelitis
GABABR	gamma-aminobutyric acid B receptor
GAD	glutamic acid decarboxylase
hCG	human chorionic gonadotropin
ivIg	intravenous immunoglobulins
LE	limbic encephalitis
LEMS	Lambert-Eaton myasthenic syndrome
mGluR	metabotropic glutamate receptor.
MP	methylprednisolone
mRS	modified Rankin scale
NK cells	natural killer cells
NMDAR	N-methyl-D-aspartate receptor
ON	ptic neuritis
PCA	Purkinje cell cytoplasmatic autoantibodies
PCD	paraneoplastic cerebellar degeneration
PE	plasma exchange
PNS	paraneoplastic neurological syndromes
SCLC	small-cell lung cancer
SMN	sensory motor neuropathy
SPS	stiff-person syndrome
SSN	subacute sensory neuronopathy
VGCC	voltage-gated calcium channel
VGKC	voltage gated potassium channel
VLA-4	very late activation antigen 4

REFERENCES

1. de Beukelaar JW, Sillevis Smitt PA. Managing paraneoplastic neurological disorders. *The oncologist.* Mar 2006;11(3):292-305.
2. Lancaster E, Dalmau J. Neuronal autoantigens—pathogenesis, associated disorders and antibody testing. *Nat Rev Neurol.* Jul 2012;8(7):380-390.
3. Rosenfeld MR, Dalmau J. Diagnosis and management of paraneoplastic neurologic disorders. *Curr Treat Options Oncol.* Dec 2013;14(4):528-538.
4. Graus F, Delattre JY, Antoine JC, et al. Recommended diagnostic criteria for paraneoplastic neurological syndromes. *J Neurol Neurosurg Psychiatry.* Aug 2004;75(8):1135-1140.
5. de Graaff E, Maat P, Hulsenboom E, et al. Identification of delta/notch-like epidermal growth factor-related receptor as the Tr antigen in paraneoplastic cerebellar degeneration. *Ann Neurol.* Jun 2012;71(6):815-824.
6. Titulaer MJ, Soffietti R, Dalmau J, et al. Screening for tumours in paraneoplastic syndromes: report of an EFNS task force. *Eur J Neurol.* Jan 2011;18(1):19-e13.
7. Vedeler CA, Antoine JC, Giometto B, et al. Management of paraneoplastic neurological syndromes: report of an EFNS Task Force. *Eur J Neurol.* Jul 2006;13(7):682-690.
8. Giometto B, Vitaliani R, Lindeck-Pozza E, Grisold W, Vedeler C. Treatment for paraneoplastic neuropathies. *Cochrane database of systematic reviews (Online).* 2012;12:CD007625.
9. Graus F, Keime-Guibert F, Rene R, et al. Anti-Hu-associated paraneoplastic encephalomyelitis: analysis of 200 patients. *Brain.* Jun 2001;124(Pt 6):1138-1148.
10. Rojas I, Graus F, Keime-Guibert F, et al. Long-term clinical outcome of paraneoplastic cerebellar degeneration and anti-Yo antibodies. *Neurology.* Sep 12 2000;55(5):713-715.
11. Sillevis Smitt P, Grefkens J, de Leeuw B, et al. Survival and outcome in 73 anti-Hu positive patients with paraneoplastic encephalomyelitis/sensory neuronopathy. *J Neurol.* Jun 2002;249(6):745-753.
12. Graus F, Illa I, Agusti M, Ribalta T, Cruz-Sanchez F, Juarez C. Effect of intraventricular injection of an anti-Purkinje cell antibody (anti-Yo) in a guinea pig model. *J Neurol Sci.* Nov 1991;106(1):82-87.
13. Tanaka K, Tanaka M, Igarashi S, Onodera O, Miyatake T, Tsuji S. Trial to establish an animal model of paraneoplastic cerebellar degeneration with anti-Yo antibody. 2. Passive transfer of murine mononuclear cells activated with recombinant Yo protein to paraneoplastic cerebellar degeneration lymphocytes in severe combined immunodeficiency mice. *Clinical neurology and neurosurgery.* Feb 1995;97(1):101-105.
14. Sillevis Smitt PA, Manley GT, Posner JB. Immunization with the paraneoplastic encephalomyelitis antigen HuD does not cause neurologic disease in mice. *Neurology.* Oct 1995;45(10):1873-1878.
15. Roberts WK, Darnell RB. Neuroimmunology of the paraneoplastic neurological degenerations. *Curr Opin Immunol.* Oct 2004;16(5):616-622.
16. Zuliani L, Graus F, Giometto B, Bien C, Vincent A. Central nervous system neuronal surface antibody associated syndromes: review and guidelines for recognition. *J Neurol Neurosurg Psychiatry.* Jun 2012;83(6):638-645.
17. Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld MR, Balice-Gordon R. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. *Lancet neurology.* Jan 2011;10(1):63-74.

18. Titulaer MJ, Maddison P, Sont JK, et al. Clinical Dutch-English Lambert-Eaton Myasthenic syndrome (LEMS) tumor association prediction score accurately predicts small-cell lung cancer in the LEMS. *J Clin Oncol.* Mar 1 2011;29(7):902-908.

19. van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJ, van Gijn J. Interobserver agreement for the assessment of handicap in stroke patients. *Stroke; a journal of cerebral circulation.* May 1988;19(5):604-607.

20. Keime-Guibert F, Graus F, Fleury A, et al. Treatment of paraneoplastic neurological syndromes with antineuronal antibodies (Anti-Hu, anti-Yo) with a combination of immunoglobulins, cyclophosphamide, and methylprednisolone. *J Neurol Neurosurg Psychiatry.* Apr 2000;68(4):479-482.

21. Giometto B, Grisold W, Vitaliani R, Graus F, Honnorat J, Bertolini G. Paraneoplastic neurologic syndrome in the PNS Euronetwork database: a European study from 20 centers. *Archives of neurology.* Mar 2010;67(3):330-335.

22. Manley GT, Smitt PS, Dalmau J, Posner JB. Hu antigens: reactivity with Hu antibodies, tumor expression, and major immunogenic sites. *Ann Neurol.* Jul 1995;38(1):102-110.

23. King PH, Redden D, Palmgren JS, Nabors LB, Lennon VA. Hu antigen specificities of ANNA-I autoantibodies in paraneoplastic neurological disease. *Journal of autoimmunity.* Dec 1999;13(4):435-443.

24. King PH. Differential expression of the neuroendocrine genes Hel-N1 and HuD in small-cell lung carcinoma: evidence for down-regulation of HuD in the variant phenotype. *International journal of cancer.* Aug 22 1997;74(4):378-382.

25. Dalmau J, Furneaux HM, Cordon-Cardo C, Posner JB. The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues. *The American journal of pathology.* Oct 1992;141(4):881-886.

26. de Graaf MT, de Beukelaar JW, Haasnoot GW, et al. HLA-DQ2+ individuals are susceptible to Hu-Ab associated paraneoplastic neurological syndromes. *J Neuroimmunol.* Sep 14 2010;226(1-2):147-149.

27. Rauer S, Kaiser R. Demonstration of anti-HuD specific oligoclonal bands in the cerebrospinal fluid from patients with paraneoplastic neurological syndromes. Qualitative evidence of anti-HuD specific IgG-synthesis in the central nervous system. *J Neuroimmunol.* Nov 1 2000;111(1-2):241-244.

28. de Graaf M, de Beukelaar J, Bergsma J, et al. B and T cell imbalances in CSF of patients with Hu-antibody associated PNS. *J Neuroimmunol.* Mar 2008;195(1-2):164-170.

29. de Jongste AH, de Graaf MT, van den Broek PD, Kraan J, Smitt PA, Gratama JW. Elevated numbers of regulatory T cells, central memory T cells and class-switched B cells in cerebrospinal fluid of patients with anti-Hu antibody associated paraneoplastic neurological syndromes. *J Neuroimmunol.* May 15 2013;258(1-2):85-90.

30. Voltz R, Dalmau J, Posner JB, Rosenfeld MR. T-cell receptor analysis in anti-Hu associated paraneoplastic encephalomyelitis. *Neurology.* Oct 1998;51(4):1146-1150.

31. Bernal F, Graus F, Pifarre A, Saiz A, Benyahia B, Ribalta T. Immunohistochemical analysis of anti-Hu-associated paraneoplastic encephalomyelitis. *Acta neuropathologica.* May 2002;103(5):509-515.

32. Wanschitz J, Hainfellner JA, Kristoferitsch W, Drlicek M, Budka H. Ganglionitis in paraneoplastic subacute sensory neuronopathy: a morphologic study. *Neurology.* Oct 1997;49(4):1156-1159.

33. Bien CG, Vincent A, Barnett MH, et al. Immunopathology of autoantibody-associated encephalitides: clues for pathogenesis. *Brain.* May 2012;135(Pt 5):1622-1638.

34. Carpentier AF, Rosenfeld MR, Delattre JY, Whalen RG, Posner JB, Dalmau J. DNA vaccination with HuD inhibits growth of a neuroblastoma in mice. *Clin Cancer Res.* Nov 1998;4(11):2819-2824.

35. Jean WC, Dalmau J, Ho A, Posner JB. Analysis of the IgG subclass distribution and inflammatory infiltrates in patients with anti-Hu-associated paraneoplastic encephalomyelitis. *Neurology.* Jan 1994;44(1):140-147.

36. Plonquet A, Garcia-Pons F, Fernandez E, et al. Peptides derived from the onconeural HuD protein can elicit cytotoxic responses in HHD mouse and human. *J Neuroimmunol.* Sep 2003;142(1-2):93-100.

37. Rousseau A, Benyahia B, Dalmau J, et al. T cell response to Hu-D peptides in patients with anti-Hu syndrome. *J Neurooncol.* Feb 2005;71(3):231-236.

38. Tanaka M, Maruyama Y, Sugie M, Motizuki H, Kamakura K, Tanaka K. Cytotoxic T cell activity against peptides of Hu protein in anti-Hu syndrome. *J Neurol Sci.* Sep 15 2002;201(1-2):9-12.

39. de Beukelaar JW, Milikan JC, Verjans GM, et al. No evidence for the presence of HuD-specific T cells in the cerebrospinal fluid of patients with Hu-associated paraneoplastic neurological syndromes. *J Neurol.* Feb 2009;256(2):279-282.

40. de Beukelaar JW, Verjans GM, van Norden Y, et al. No evidence for circulating HuD-specific CD8+ T cells in patients with paraneoplastic neurological syndromes and Hu antibodies. *Cancer Immunol Immunother.* Sep 2007;56(9):1501-1506.

41. Roberts WK, Deluca IJ, Thomas A, et al. Patients with lung cancer and paraneoplastic Hu syndrome harbor HuD-specific type 2 CD8+ T cells. *J Clin Invest.* Jul 2009;119(7):2042-2051.

42. de Jongste AH, de Graaf MT, Martinuzzi E, et al. Three sensitive assays do not provide evidence for circulating HuD-specific T cells in the blood of patients with paraneoplastic neurological syndromes with anti-Hu antibodies. *Neuro Oncol.* July 2012 2012;14(7):841-848.

43. Giometto B, Marchiori GC, Nicolao P, et al. Sub-acute cerebellar degeneration with anti-Yo autoantibodies: immunohistochemical analysis of the immune reaction in the central nervous system. *Neuropathology and applied neurobiology.* Dec 1997;23(6):468-474.

44. Storstein A, Krossnes BK, Vedeler CA. Morphological and immunohistochemical characterization of paraneoplastic cerebellar degeneration associated with Yo antibodies. *Acta Neurol Scand.* Jul 2009;120(1):64-67.

45. Albert ML, Darnell JC, Bender A, Francisco LM, Bhardwaj N, Darnell RB. Tumor-specific killer cells in paraneoplastic cerebellar degeneration. *Nat Med.* Nov 1998;4(11):1321-1324.

46. Sutton IJ, Steele J, Savage CO, Winer JB, Young LS. An interferon-gamma ELISPOT and immunohistochemical investigation of cytotoxic T lymphocyte-mediated tumour immunity in patients with paraneoplastic cerebellar degeneration and anti-Yo antibodies. *J Neuroimmunol.* May 2004;150(1-2):98-106.

47. Cross SA, Salomao DR, Parisi JE, et al. Paraneoplastic autoimmune optic neuritis with retinitis defined by CRMP-5-IgG. *Ann Neurol.* Jul 2003;54(1):38-50.

48. Brieva-Ruiz L, Diaz-Hurtado M, Matias-Guiu X, Marquez-Medina D, Tarragona J, Graus F. Anti-Ri-associated paraneoplastic cerebellar degeneration and breast cancer: an autopsy case study. *Clinical neurology and neurosurgery.* Dec 2008;110(10):1044-1046.

49. Hormigo A, Dalmau J, Rosenblum MK, River ME, Posner JB. Immunological and pathological study of anti-Ri-associated encephalopathy. *Ann Neurol.* Dec 1994;36(6):896-902.

50. Geis C, Weishaupt A, Hallermann S, et al. Stiff person syndrome-associated autoantibodies to amphiphysin mediate reduced GABAergic inhibition. *Brain.* Nov 2010;133(11):3166-3180.

51. Bernal F, Shams'ili S, Rojas I, et al. Anti-Tr antibodies as markers of paraneoplastic cerebellar degeneration and Hodgkin's disease. *Neurology.* Jan 28 2003;60(2):230-234.

52. Shams'ili S, Grefkens J, de Leeuw B, et al. Paraneoplastic cerebellar degeneration associated with antineuronal antibodies: analysis of 50 patients. *Brain.* Jun 2003;126(Pt 6):1409-1418.

53. van Coevorden-Hameete MH, de Graaff E, Titulaer MJ, Hoogenraad CC, Sillevis Smitt PA. Molecular and cellular mechanisms underlying anti-neuronal antibody mediated disorders of the central nervous system. *Autoimmun Rev.* Mar 2014;13(3):299-312.

54. Keime-Guibert F, Graus F, Broet P, et al. Clinical outcome of patients with anti-Hu-associated encephalomyelitis after treatment of the tumor. *Neurology.* Nov 10 1999;53(8):1719-1723.

55. Graus F, Dalmou J, Rene R, et al. Anti-Hu antibodies in patients with small-cell lung cancer: association with complete response to therapy and improved survival. *J Clin Oncol.* Aug 1997;15(8):2866-2872.

56. Monstad SE, Drivsholm L, Storstein A, et al. Hu and voltage-gated calcium channel (VGCC) antibodies related to the prognosis of small-cell lung cancer. *J Clin Oncol.* Mar 1 2004;22(5):795-800.

57. Darnell RB, DeAngelis LM. Regression of small-cell lung carcinoma in patients with paraneoplastic neuronal antibodies. *Lancet.* Jan 2 1993;341(8836):21-22.

58. Shams'ili S, de Beukelaar J, Gratama JW, et al. An uncontrolled trial of rituximab for antibody associated paraneoplastic neurological syndromes. *J Neurol.* Jan 2006;253(1):16-20.

59. van Broekhoven F, de Graaf MT, Bromberg JE, et al. Human chorionic gonadotropin treatment of anti-Hu-associated paraneoplastic neurological syndromes. *J Neurol Neurosurg Psychiatry.* Dec 2010;81(12):1341-1344.

60. Vernino S, O'Neill BP, Marks RS, O'Fallon JR, Kimmel DW. Immunomodulatory treatment trial for paraneoplastic neurological disorders. *Neuro-oncology.* Jan 2004;6(1):55-62.

61. Graus F, Vega F, Delattre JY, et al. Plasmapheresis and antineoplastic treatment in CNS paraneoplastic syndromes with antineuronal autoantibodies. *Neurology.* Mar 1992;42(3 Pt 1):536-540.

62. Uchuya M, Graus F, Vega F, Rene R, Delattre JY. Intravenous immunoglobulin treatment in paraneoplastic neurological syndromes with antineuronal autoantibodies. *J Neurol Neurosurg Psychiatry.* Apr 1996;60(4):388-392.

63. Orange D, Frank M, Tian S, et al. Cellular immune suppression in paraneoplastic neurologic syndromes targeting intracellular antigens. *Archives of neurology.* Sep 1 2012;69(9):1132-1140.

64. Honnorat J, Cartalat-Carel S, Ricard D, et al. Onco-neural antibodies and tumour type determine survival and neurological symptoms in paraneoplastic neurological syndromes with Hu or CV2/CRMP5 antibodies. *J Neurol Neurosurg Psychiatry.* Apr 2009;80(4):412-416.
65. Pittock SJ, Lucchinetti CF, Lennon VA. Anti-neuronal nuclear autoantibody type 2: paraneoplastic accompaniments. *Ann Neurol.* May 2003;53(5):580-587.
66. Dalmau J, Graus F, Villarejo A, et al. Clinical analysis of anti-Ma2-associated encephalitis. *Brain.* Aug 2004;127(Pt 8):1831-1844.
67. Hoffmann LA, Jarius S, Pellkofer HL, et al. Anti-Ma and anti-Ta associated paraneoplastic neurological syndromes: 22 newly diagnosed patients and review of previous cases. *J Neurol Neurosurg Psychiatry.* Jul 2008;79(7):767-773.
68. Rosenfeld MR, Eichen JG, Wade DF, Posner JB, Dalmau J. Molecular and clinical diversity in paraneoplastic immunity to Ma proteins. *Ann Neurol.* Sep 2001;50(3):339-348.
69. Murinson BB, Guarnaccia JB. Stiff-person syndrome with amphiphysin antibodies: distinctive features of a rare disease. *Neurology.* Dec 9 2008;71(24):1955-1958.
70. Pittock SJ, Lucchinetti CF, Parisi JE, et al. Amphiphysin autoimmunity: paraneoplastic accompaniments. *Ann Neurol.* Jul 2005;58(1):96-107.
71. Sommer C, Weishaupt A, Brinkhoff J, et al. Paraneoplastic stiff-person syndrome: passive transfer to rats by means of IgG antibodies to amphiphysin. *Lancet.* Apr 16-22 2005;365(9468):1406-1411.
72. Dalakas MC. B cells as therapeutic targets in autoimmune neurological disorders. *Nature clinical practice.* Oct 2008;4(10):557-567.
73. Voltz R. Intravenous immunoglobulin therapy in paraneoplastic neurological syndromes. *J Neurol.* Sep 2006;253 Suppl 5:V33-38.
74. Institute NC. Common Toxicity Criteria version 2.0. 1999; http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcv20_4-30-992.pdf.
75. Leone MA, Brainin M, Boon P, et al. Guidance for the preparation of neurological management guidelines by EFNS scientific task forces - revised recommendations 2012. *Eur J Neurol.* Mar 2013;20(3):410-419.
76. Camdessanche JP, Lassabliere F, Meyronnet D, et al. Expression of the onconeural CV2/CRMP5 antigen in thymus and thymoma. *J Neuroimmunol.* May 2006;174(1-2):168-173.
77. Meinl E, Krumbholz M, Hohlfeld R. B lineage cells in the inflammatory central nervous system environment: migration, maintenance, local antibody production, and therapeutic modulation. *Ann Neurol.* Jun 2006;59(6):880-892.
78. Ransohoff RM, Engelhardt B. The anatomical and cellular basis of immune surveillance in the central nervous system. *Nature reviews.* Aug 10 2012;12(9):623-635.
79. Cohen JA, Barkhof F, Comi G, et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. *The New England journal of medicine.* Feb 4 2010;362(5):402-415.
80. Stuve O, Marra CM, Jerome KR, et al. Immune surveillance in multiple sclerosis patients treated with natalizumab. *Ann Neurol.* May 2006;59(5):743-747.
81. Coles AJ, Twyman CL, Arnold DL, et al. Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. *Lancet.* Oct 31 2012.
82. Cohen JA, Coles AJ, Arnold DL, et al. Alemtuzumab versus interferon beta 1a as first-line treatment for patients with relapsing-remitting multiple sclerosis: a randomised controlled phase 3 trial. *Lancet.* Oct 31 2012.

83. Ketterl TG, Messinger YH, Niess DR, Gilles E, Engel WK, Perkins JL. Ofatumumab for refractory opsoclonus-myoclonus syndrome following treatment of neuroblastoma. *Pediatr Blood Cancer.* Dec 2013;60(12):E163-165.

84. Dalmau J, Graus F, Rosenblum MK, Posner JB. Anti-Hu—associated paraneoplastic encephalomyelitis/sensory neuronopathy. A clinical study of 71 patients. *Medicine (Baltimore).* Mar 1992;71(2):59-72.

85. Lucchinetti CF, Kimmel DW, Lennon VA. Paraneoplastic and oncologic profiles of patients seropositive for type 1 antineuronal nuclear autoantibodies. *Neurology.* Mar 1998;50(3):652-657.

86. Peterson K, Rosenblum MK, Kotanides H, Posner JB. Paraneoplastic cerebellar degeneration. I. A clinical analysis of 55 anti-Yo antibody-positive patients. *Neurology.* Oct 1992;42(10):1931-1937.

87. Yu Z, Kryzer TJ, Griesmann GE, Kim K, Benarroch EE, Lennon VA. CRMP-5 neuronal autoantibody: marker of lung cancer and thymoma-related autoimmunity. *Ann Neurol.* Feb 2001;49(2):146-154.

88. Buckanovich RJ, Posner JB, Darnell RB. Nova, the paraneoplastic Ri antigen, is homologous to an RNA-binding protein and is specifically expressed in the developing motor system. *Neuron.* Oct 1993;11(4):657-672.

89. Antoine JC, Absi L, Honnorat J, et al. Antiamphiphysin antibodies are associated with various paraneoplastic neurological syndromes and tumors. *Archives of neurology.* Feb 1999;56(2):172-177.

90. Chan KH, Vernino S, Lennon VA. ANNA-3 anti-neuronal nuclear antibody: marker of lung cancer-related autoimmunity. *Ann Neurol.* Sep 2001;50(3):301-311.

91. Adamus G, Ren G, Weleber RG. Autoantibodies against retinal proteins in paraneoplastic and autoimmune retinopathy. *BMC ophthalmology.* 2004;4:5.

92. Ohguro H, Yokoi Y, Ohguro I, et al. Clinical and immunologic aspects of cancer-associated retinopathy. *American journal of ophthalmology.* Jun 2004;137(6):1117-1119.

93. Saiz A, Blanco Y, Sabater L, et al. Spectrum of neurological syndromes associated with glutamic acid decarboxylase antibodies: diagnostic clues for this association. *Brain.* Oct 2008;131(Pt 10):2553-2563.

94. Oosterhuis HJ. The natural course of myasthenia gravis: a long term follow up study. *J Neurol Neurosurg Psychiatry.* Oct 1989;52(10):1121-1127.

95. Klein CM, Vernino S, Lennon VA, et al. The spectrum of autoimmune autonomic neuropathies. *Ann Neurol.* Jun 2003;53(6):752-758.

96. Titulaer MJ, McCracken L, Gabilondo I, et al. Treatment and prognostic factors for long-term outcome in patients with anti-NMDA receptor encephalitis: an observational cohort study. *Lancet Neurol.* Feb 2013;12(2):157-165.

97. Lai M, Huijbers MG, Lancaster E, et al. Investigation of LGI1 as the antigen in limbic encephalitis previously attributed to potassium channels: a case series. *Lancet neurology.* Aug 2010;9(8):776-785.

98. Irani SR, Alexander S, Waters P, et al. Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia. *Brain.* Sep 2010;133(9):2734-2748.

99. Irani SR, Michell AW, Lang B, et al. Faciobrachial dystonic seizures precede Lgi1 antibody limbic encephalitis. *Ann Neurol.* May 2011;69(5):892-900.

100. Krastinova E, Vigneron M, Le Bras P, Gasnault J, Goujard C. Treatment of limbic encephalitis with anti-glioma-inactivated 1 (LGI1) antibodies. *J Clin Neurosci.* Nov 2012;19(11):1580-1582.

101. Lancaster E, Huijbers MG, Bar V, et al. Investigations of caspr2, an autoantigen of encephalitis and neuromyotonia. *Ann Neurol.* Feb 2011;69(2):303-311.

102. Irani SR, Pettingill P, Kleopa KA, et al. Morvan syndrome: clinical and serological observations in 29 cases. *Ann Neurol.* Aug 2012;72(2):241-255.

103. Becker EB, Zuliani L, Pettingill R, et al. Contactin-associated protein-2 antibodies in non-paraneoplastic cerebellar ataxia. *J Neurol Neurosurg Psychiatry.* Apr 2012;83(4):437-440.

104. Lai M, Hughes EG, Peng X, et al. AMPA receptor antibodies in limbic encephalitis alter synaptic receptor location. *Ann Neurol.* Apr 2009;65(4):424-434.

105. Bataller L, Galiano R, Garcia-Escrig M, et al. Reversible paraneoplastic limbic encephalitis associated with antibodies to the AMPA receptor. *Neurology.* Jan 19 2010;74(3):265-267.

106. Lancaster E, Lai M, Peng X, et al. Antibodies to the GABA(B) receptor in limbic encephalitis with seizures: case series and characterisation of the antigen. *Lancet neurology.* Jan 2010;9(1):67-76.

107. Boronat A, Sabater L, Saiz A, Dalmau J, Graus F. GABA(B) receptor antibodies in limbic encephalitis and anti-GAD-associated neurologic disorders. *Neurology.* Mar 1 2011;76(9):795-800.

108. Jeffery OJ, Lennon VA, Pittock SJ, Gregory JK, Britton JW, McKeon A. GABAB receptor autoantibody frequency in service serologic evaluation. *Neurology.* Sep 3 2013;81(10):882-887.

109. Hoftberger R, Titulaer MJ, Sabater L, et al. Encephalitis and GABAB receptor antibodies: novel findings in a new case series of 20 patients. *Neurology.* Oct 22 2013;81(17):1500-1506.

110. Marignier R, Chenevier F, Rogemond V, et al. Metabotropic glutamate receptor type 1 autoantibody-associated cerebellitis: a primary autoimmune disease? *Archives of neurology.* May 2010;67(5):627-630.

111. Sillevis Smitt P, Kinoshita A, De Leeuw B, et al. Paraneoplastic cerebellar ataxia due to autoantibodies against a glutamate receptor. *The New England journal of medicine.* Jan 6 2000;342(1):21-27.

112. Lancaster E, Martinez-Hernandez E, Titulaer MJ, et al. Antibodies to metabotropic glutamate receptor 5 in the Ophelia syndrome. *Neurology.* Nov 1 2011;77(18):1698-1701.

113. Iorio R, Damato V, Mirabella M, et al. Cerebellar degeneration associated with mGluR1 autoantibodies as a paraneoplastic manifestation of prostate adenocarcinoma. *J Neuroimmunol.* Oct 15 2013;263(1-2):155-158.

114. Mat A, Adler H, Merwick A, et al. Ophelia syndrome with metabotropic glutamate receptor 5 antibodies in CSF. *Neurology.* Apr 2 2013;80(14):1349-1350.

115. Hutchinson M, Waters P, McHugh J, et al. Progressive encephalomyelitis, rigidity, and myoclonus: a novel glycine receptor antibody. *Neurology.* Oct 14 2008;71(16):1291-1292.

116. Mas N, Saiz A, Leite MI, et al. Antiglycine-receptor encephalomyelitis with rigidity. *J Neurol Neurosurg Psychiatry.* Dec 2011;82(12):1399-1401.

117. Honnorat J, Didelot A, Karantoni E, et al. Autoimmune limbic encephalopathy and anti-Hu antibodies in children without cancer. *Neurology.* Jun 11 2013;80(24):2226-2232.

8

A prospective open-label study of sirolimus for the treatment of anti-Hu associated paraneoplastic neurological syndromes

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ABSTRACT

Background

Several lines of evidence suggest a T cell-mediated immune response in paraneoplastic neurological syndromes with anti-Hu antibodies (Hu-PNS). In order to investigate whether suppression of T cell-mediated immune responses in Hu-PNS patients improved their neurological outcome, we performed a prospective open-label, single-arm study on sirolimus.

Methods

Seventeen progressive Hu-PNS patients were treated with sirolimus with an intended treatment duration of 8 weeks. Primary outcome measures were (i) functional improvement defined as a decrease of one or more points on the modified Rankin Scale (mRS), and (ii) improvement of neurological impairment defined as an increase of one or more points on the Edinburgh Functional Impairment Tests (EFIT).

Results

One patient showed improvement on both clinical scales (mRS and EFIT). This patient presented with limbic encephalitis and improved dramatically from an mRS score of 3 to mRS 1. Another patient with subacute sensory neuronopathy remained stable at mRS 2, and improved one point on the EFIT scale. The other patients showed no improvement on the primary outcome measures. Median survival was 21 months.

Conclusion

We conclude that treatment of Hu-PNS patients with sirolimus may improve or stabilize their functional disabilities and neurological impairments. However, the effects of this T cell-targeted therapy were not better than reported in trials on other immunotherapies for Hu-PNS.

Trial Registration https://www.clinicaltrialsregister.eu/ctr-search/trial/2008-000793-20/NL

INTRODUCTION

Among paraneoplastic neurological syndromes (PNS) with well-characterized onconeural antibodies, anti-Hu antibody-associated PNS (Hu-PNS) are the most frequent.[1,2] Hu-PNS have a very poor prognosis: more than half of the patients become bedridden (modified Rankin scale [mRS] score ≥4), only 2%-6% of patients improve, and median survival is < 12 months.[3,4] Hu-PNS are thought to result from an immune response against Hu-expressing tumor cells that additionally destroys Hu-expressing neurons.[5] There are several reasons why neuronal destruction in Hu-PNS is more likely caused by T cells than anti-Hu antibodies (Hu-Ab): Hu proteins are intracellular proteins,[6] animal models failed to demonstrate Hu-Ab -induced neurological disease,[7] and autopsy studies showed T cell infiltrates with frequent appositions of cytotoxic T cells to neurons with associated neuronal loss.[8-10]

Sirolimus (Rapamune ®) is an immunosuppressive drug that specifically inhibits activated T cells.[11] Sirolimus binds to FKBP-12, an intracellular protein, to form an immunosuppressive complex that inhibits the regulatory kinase mammalian target of rapamycin (mTOR).[11] This inhibition suppresses cytokine-mediated T cell proliferation. As a result, T cells cannot proliferate following antigenic stimulation, and T cell-mediated immune responses are strongly suppressed. In addition, sirolimus has anti-oncogenic and anti-angiogenic effects that may prevent growth of the underlying tumor.[12]

We hypothesized that cellular immune suppression with sirolimus could stop neuronal damage by the presumed T cell-mediated immune response in Hu-PNS, and may improve neurological outcome. To test this hypothesis, we prospectively treated 17 progressive Hu-PNS patients with sirolimus, and recorded neurological function and impairment using well-defined clinical scales in a prospective open-label, single-arm study.

METHODS

Eligibility Criteria

Inclusion criteria included high serum titers of Hu-Ab (≥400 by indirect immunofluorescence, confirmed by Western blotting) and progression of neurological symptoms defined as neurological deterioration over the last four weeks. From May 2008 to October 2012, we identified 68 patients with high-titer Hu-Ab. Four of these patients died before test results became available. Eighteen patients did not meet the inclusion criteria according

to their treating physician: 12 had no progressive disease over the last 4 weeks, while 6 patients had other potential causes for their neurological symptoms (toxic neuropathy or brain metastasis). Of the remaining 46 patients who were invited to participate in this study, 29 declined participation (mainly because of severe illness or unwillingness to travel to our institution) or did not respond to our invitation. Seventeen patients gave written informed consent and participated in this trial. Clinical characteristics of the 17 included patients are summarized in Table 1. All patients were evaluated throughout the study by one of two clinical investigators (JEB, PASS). The study was approved by the Erasmus University Medical Center Institutional Review Board.

Treatment Plan

Sirolimus treatment was initiated with an oral loading dose of 6 mg sirolimus per day for 3 consecutive days, followed by oral maintenance dosing of 3 mg/day. The dosing was adjusted weekly to maintain trough concentrations of 8-12 ng/mL. If the patient was under co-therapy with drugs that induce or inhibit the activity of CYP3A4, the sirolimus dose was adjusted accordingly, under the guidance of the clinical pharmacologist. Patients were informed that any planned change in co-medication needed to be reported to the treating physician, in order to avoid adverse events due to drug-drug interactions.

Laboratory Tests

Serum and CSF were sampled at baseline and after 4 weeks of sirolimus treatment. Serum was additionally sampled at weeks 8 and 16. Sirolimus blood trough levels were determined in the clinical chemistry laboratory (target level 8-12 ng/ml) using a chromatographic assay. IgG titers of the Hu-Ab were determined, as described.[3]

Assessment of Efficacy

The primary endpoints of the study were the functional and neurological improvement after 8 weeks of sirolimus. Functional improvement was defined as an decrease of one point or more on the modified Rankin scale (mRS) as compared with the baseline evaluation. Improvement of neurological impairment was assessed with the Edinburgh Functional Impairment Tests (EFIT), which incorporate objective measures of upper and lower limb function, memory and a rating scale for dysphasia.[13] Overall EFIT = 0 indicates no change, EFIT > 0 indicates significant neurological improvement and EFIT < 0 indicates significant neurological deterioration.

Secondary endpoints included reduction of CSF protein and white blood cells (WBCs), decrease in Hu-Ab titers in serum and CSF, and improvement in activities of daily living (ADL) as evaluated by means of the Barthel index (BI).[14] We additionally added a post-hoc analysis to enable comparison with previous studies. In this post-hoc analysis,

Table 1. Patient characteristics

No.	Age (years)	Sex	PNS	Symptoms to Diagnosis (mo)	Tumor	Symptoms to Tumor (mo)	Tumor Stage	Tumor Treatment	Tumor Response	Symptoms to last FU (months)	Dead/ Alive	Cause of Death
1	68	M	PEM (LE)	7	SCLC	8	ED	Chemo + RT	PD	27	Dead	Tumor
2	67	M	BE	9	SCC parotid	-8	T1N2bM0	Chemo + RT + surg	PD	16	Dead	PNS
3	77	F	PEM (SSN), LEMS	4	SCLC	6	LD	No	NA	8	Dead	PNS
4	60	M	PCD	4	No[A]	NA	NA	No	NA	10	Dead	PNS
5	52	F	SSN	11	SCLC	-4	LD	Chemo + RT	CR	43	Alive	NA
6	75	F	PCD	2	SCLC	4	LD	Chemo + RT	CR	33	Alive	NA
7	46	F	PEM (SSN)	1	Lung[B]	1	ED	No	NA	2	Dead	PNS
8	66	F	PLE	7	Lung[C]	8	LD	No	NA	21	Dead	PNS
9	77	F	SSN	5	No[D]	NA	NA	No	NA	24	Alive	NA
10	73	F	SSN	2	SCLC	4	LD	Chemo + RT	CR	21	Alive	NA
11	65	F	SSN, AN	4	Chondro-sarcoma	-312	Metastatic	No	NA	14	Dead	PNS
12	59	F	MN	2	SCLC	-24	LD	Chemo + RT	CR	9	Dead	PNS
13	64	F	SSN	0	SCLC	-4	LD	Chemo + RT	CR	16	Alive	NA
14	64	M	PLE	0	SCLC	1	LD	Chemo + RT	PR	7	Dead	Tumor
15	62	M	SSN	2	SCLC	2	LD	Chemo + RT	CR	11	Alive	NA
16	66	M	PCD	4	Lung[E]	5	LD	No	NA	10	Alive	NA
17	67	F	SSN	1	SCLC	2	LD	Chemo	CR	7	Alive	NA

Abbreviations: Ab, antibody; PNS, paraneoplastic neurological syndrome; FU, follow-up; M, male; F, female; Amp, amphiphysin; PEM, paraneoplastic encephalomyelitis;BE, brainstem encephalitis; LEMS, Lambert-Eaton myasthenic syndrome; PCD, paraneoplastic cerebellar degeneration;SSN, subacute sensory neuronopathy; AN, autonomic neuropathy; PLE, paraneoplastic limbic encephalitits; MN, motor neuronopathy; SCLC, small-cell lung cancer; SCC parotid, small-cell cancer of parotid gland; NA, not applicable; ED, extensive disease; LD, limited disease; Chemo, chemotherapy; RT, radiotherapy; surg, surgery; PD, progressive disease; CR, complete response; PR, partial response. In PEM patients, the predominant syndromes are indicated in parentheses.

[A] CT-thorax and 2-fluoro-2-deoxy-D-glucose-PET-CT scan negative

[B] Extensive mediastinal and pleural lesions on CT-scan

[C] Mediastinal 2-fluoro-2-deoxy-D-glucose positive lymphnodes on PET-CT scan

[D] CT-thorax/abdomen unchanged

[E] Mediastinal 2-fluoro-2-deoxy-D-glucose positive lymphnodes on PET-CT scan (not accessible for biopsy).

outcome was considered 'successful' when a patient with an mRS score ≤ 3 improved or stabilized (i. e. remained ambulatory) and when a patient with an mRS score ≥ 4 (bedridden patient) improved to ≤ 3 (ambulatory), after the 8th week of sirolimus treatment as compared with baseline, as defined by Keime-Guibert et al.[15]

Statistical Methods

We compared WBC, total protein concentration and Hu-Ab titers in baseline CSF and CSF obtained after 4 weeks of treatment by means of the Wilcoxon matched pairs test. We used the same test to compare Hu-Ab serum titers at baseline and end of study. P-values were 2-sided and a significance level $\alpha = 0.05$ was used. All statistical analyses were performed using GraphPad Prism version 5 software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Patient Flow and Treatment

A total of 17 patients started treatment with sirolimus, 11 of whom completed the full 8 weeks of treatment (Table 2). Six patients of these 11 patients received sirolimus for 9-11 weeks because of low sirolimus blood levels during the titration phase. In 6 patients, sirolimus treatment was terminated before 8 weeks. In 2 of these patients serious adverse events (SAE) related to sirolimus were reported (patients 8 and 15; see below). In 2 patients, withdrawal was requested for fear of increased hematological toxicity by oncologists in other hospitals who wanted to start treatment with chemotherapy. In 1 patient, sirolimus was withdrawn after 4 weeks because of obvious neurological deterioration and 1 patient died after 1 week of treatment of the neurological disorder. The range of the average daily sirolimus dose was 2.3 – 5.3 mg/day resulting in average trough levels of 3.0 – 11.7 µg/L (Table 2).

Toxicity

As stated above, 2 patients were unable to complete the treatment because of serious adverse events. Patient 8 developed severe epistaxis that caused a drop in hemoglobin levels (from 5.3 to 4.8 mmol/l) requiring hospitalization and transfusion of 2 units of packed red cells. Sirolimus was stopped and the patient recovered completely (hemoglobin 6.0 mmol/L). Patient 15 developed a generalized erythrodermia for which the consultation of a dermatologist was necessary. Sirolimus was withdrawn and topical corticosteroids were prescribed. Two weeks later, the patient was seen again by the dermatologist and the skin was recovering. One week later, the patient was admitted to the hospital because of a *Staphylococcus aureus* bacteriemia with the skin as possible

porte d'entrée. The patient recovered completely from the erythrodermia and the bacteriemia. Patient 1 developed oral mucositis, thrombocytopenia and hypokalemia from which he recovered completely.

Table 2. Sirolimus treatment duration and intensity

No.	Sirolimus			Reason Early Termination	Concurrent
	Weeks	Average Daily Dose (mg)	Average Trough Level (µg/L)		Chemotherapy
1	8	3.0	7.1	NA	Yes
2	3	3.0	3.1	Initiation chemotherapy	No
3	9	3.0	4.8	NA	No
4	8	5.0	3.4	NA	No
5	9	3.0	5.2	NA	Yes
6	5	3.8	3.9	Neurological deterioration	No
7	1	3.0		Died of PNS	No
8	4	3.8	4.1	SAE, severe epistaxis	No
9	9	2.3	11.5	NA	No
10	3	4.7	5.5	Initiation chemotherapy	No
11	9	5.3	7.4	NA	No
12	11	4.8	6.1	NA	No
13	8	4.1	10.2	NA	No
14	10	4.7	8.0	NA	Yes
15	2	3.0	4.2	SAE, severe erythrodermia	Yes
16	8	2.3	8.9	NA	No
17	8	3.4	11.6	NA	Yes

Abbreviations: SEA, serious adverse event; NA, not applicable.

Primary Endpoints

Only in patient 14 did the mRS score improve (from 3 to 1) with concomitant improvement of one point on the EFIT score (Table 3). This patient suffered from limbic encephalitis (memory deficits, sexual disinhibition and visual hallucinations) and received concomitant treatment with chemotherapy for the underlying small-cell lung cancer. After treatment, his memory and behaviour had improved dramatically, and he had no more hallucinations. Patient 5, with subacute sensory neuronopathy, improved one point on the EFIT scale while remaining stable at an mRS score of 2. Overall, 10 patients had stable mRS scores (59%), while 6 patients (35%) showed further functional deterioration. The neurological outcome (EFIT) was stable in 7 of 14 evaluable patients (50%) and deteriorated in 5 (36%).

Table 3. Primary outcome measures

No.	Sirolimus (weeks)	Concurrent Chemotherapy	Functional Outcome	mRS Baseline	mRS Change	Neurological Outcome	EFIT Baseline	EFIT Overall
1	8	Yes	Stable	3	0	Stable	2	0
2	3	No	Worse	2	1	Worse	0	-1
3	9	No	Worse	3	2	Worse	3	-1
4	8	No	Stable	3	0	Worse	4	-1
5	9	Yes	Stable	2	0	**Improved**	2	1
6	5	No	Worse	3	1	Worse	2	-1
7	1	No	Worse	3	3	NE[A]	2	
8	4	No	Stable	3	0	Stable[B]	2	0
9	9	No	Stable	4	0	Stable	2	0
10	3	No	Stable	3	0	NE[C]	1	
11	9	No	Stable	4	0	Stable	2	0
12	11	No	Worse	3	1	Stable	2	0
13	8	No	Stable	4	0	Stable	3	0
14	10	Yes	**Improved**	3	-2	**Improved**	2	**1**
15	2	Yes	Stable	3	0	NE[C]	1	
16	8	No	Worse	4	1	Worse	4	-2
17	8	Yes	Stable	5	0	Stable	2	0

Abbreviations: NE, not evaluable.

[A] Patient died because of progressive PNS

[B] Patient's PNS remained stable on sirolimus. After discontinuation because of side effects, her PNS progressed.

[C] EFIT score was not determined, mRS was determined by contacting patient and treating physician. Functional and neurological outcome after up to 8 weeks of treatment with sirolimus. Abbreviations: mRS, modified Rankin scale; EFIT, Edinburgh Functional Impairment Tests; NE, not evaluable. An mRS change <0 indicated functional improvement; an mRS change of 0 indicated stable functional outcome, an mRS change >0 indicated functional deterioration. An overall EFIT of > 0 indicated neurological improvement, an overall EFIT of 0 indicated stable neurological function, while an overall EFIT < 0 indicated neurological progression.

Secondary Endpoints

Improvement in activities of daily living (a rise of 5 points or more on the Barthel index) was seen in patients 11 (+15) and 13 (+20). Both patients had a stable mRS score of 4 and unchanged EFIT score. Laboratory evaluations showed in CSF a median of 5 WBC/μL at baseline versus 5 WBC/μL after 4 weeks of treatment (P=0.38, n=12). The median CSF protein concentration was 0.42 g/L at baseline and 0.35 g/L after 4 weeks (P=0.12). The median CSF Hu-Ab titer changed from 32 at baseline to 64 at week 4 (P=0.47). In serum, the median Hu-Ab titer at baseline was 3200, at 4 weeks 2400 (P=0.16 vs baseline, n=12), at 8 weeks 1600 (P=0.03 vs baseline, n=11) and at 16 weeks 800 (P=0.50 vs baseline, n=5).

Post-hoc Analyses

According to the criteria of Keime-Guibert and colleagues, treatment response would be classified as 'successful' in 7 patients (41%; patients 1, 4, 5, 8, 10, 14 and 15).[15] Median survival was 21 months.

DISCUSSION

One patient showed a dramatic clinical improvement during sirolimus treatment with a decrease of 2 points on the mRS (from 3 to 1) and concomitant improvement on the overall EFIT of +1. Of the remaining patients, 59% had a stable functional outcome, while 35% showed further deterioration. Treatment response was 'successful' in 7 patients (41%).[15]

Six other studies have reported treatment results in Hu-PNS patients using the mRS as the primary outcome measure, and have recently been reviewed elsewhere.[16] In 3 retrospective studies, treatment with plasma exchange and methylprednisolone with or without cyclophosphamide was successful in 0 of 6 (0%),[17] intravenous immunoglobulin treatment was successful in 6 of 17 (35%),[18] while treatment with intravenous immunoglobulin, cyclophosphamide and methylprednisolone was successful in 2 of 9 (22%)[15] evaluable Hu-PNS patients. In a prospective study, treatment with plasma exchange combined with either cyclophosphamide or chemotherapy (in patients with a tumor) was successful in 3 of 5 (60%) Hu-PNS patients.[19] In two prospective studies, rituximab was successful in 4 of 8 (50%)[20] and human chorionic gonadotropin in 7 of 15 (47%)[21] Hu-PNS patients. The rate of success in the current study is within the range of the success rates of previous studies.

The current study suggests that immunosuppressive and immuomodulatory therapy may modify the course of Hu-PNS. However, several confounding factors may have contributed to these results. The patient who showed improvement on both primary outcome measures (patient 14) suffered limbic encephalitis, a syndrome that appears most responsive to treatment and can rarely spontaneously resolve, even in the presence of Hu-Abs.[3] In addition, this patient received concomitant chemotherapy, and several studies have demonstrated that effective treatment of the tumor is important to at least stabilize Hu-PNS. [3,4] Four of the other 6 patients with a 'successful' outcome received chemotherapy with a complete response in 3 of them. Patient 5 had a long interval from symptom onset to diagnosis (11 months), which is also associated with a more favorable outcome.[3]

During sirolimus treatment, we did not find significant changes in the CSF WBC count, protein concentration, nor Hu-Ab titers. Also, serum titers were not significantly affected by sirolimus. The significant decrease in the serum titer at 8 weeks (P=0.03) probably reflects multiple testing.

To summarize, this study and other studies suggest that immunomodulation may modify the course of Hu-PNS. However, we can currently not conclude that T cell-targeted therapies should be preferred above other immunotherapies, and more effective treatments are clearly needed.

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REFERENCES

1. de Beukelaar JW, Sillevis Smitt PA. Managing paraneoplastic neurological disorders. *The oncologist.* Mar 2006;11(3):292-305.
2. Giometto B, Grisold W, Vitaliani R, Graus F, Honnorat J, Bertolini G. Paraneoplastic neurologic syndrome in the PNS Euronetwork database: a European study from 20 centers. *Archives of neurology.* Mar 2010;67(3):330-335.
3. Sillevis Smitt P, Grefkens J, De Leeuw B, et al. Survival and outcome in 73 anti-Hu positive patients with paraneoplastic encephalomyelitis/sensory neuronopathy. *J Neurol.* 2002;249(6):745-753.
4. Graus F, Keime-Guibert F, Rene R, et al. Anti-Hu-associated paraneoplastic encephalomyelitis: analysis of 200 patients. *Brain.* 2001;124(Pt 6):1138-1148.
5. Roberts WK, Darnell RB. Neuroimmunology of the paraneoplastic neurological degenerations. *Curr Opin Immunol.* Oct 2004;16(5):616-622.
6. Dalmau J, Furneaux HM, Cordon-Cardo C, Posner JB. The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues. *Am J Pathol.* 1992;141(4):881-886.
7. Sillevis Smitt PAE, Manley GT, Posner JP. Immunization with the paraneoplastic encephalomyelitis antigen HuD does not cause neurologic disease in mice. *Neurology.* 1995;45(10):1873-1878.
8. Bernal F, Graus F, Pifarre A, Saiz A, Benyahia B, Ribalta T. Immunohistochemical analysis of anti-Hu-associated paraneoplastic encephalomyelitis. *Acta Neuropathol (Berl).* 2002;103(5):509-515.
9. Bien CG, Vincent A, Barnett MH, et al. Immunopathology of autoantibody-associated encephalitides: clues for pathogenesis. *Brain.* May 2012;135(Pt 5):1622-1638.
10. Voltz R, Dalmau J, Posner JB, Rosenfeld MR. T-cell receptor analysis in anti-Hu associated paraneoplastic encephalomyelitis. *Neurology.* 1998;51(4):1146-1150.
11. Powell JD, Delgoffe GM. The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. *Immunity.* Sep 24 2010;33(3):301-311.
12. LoRusso PM. Mammalian target of rapamycin as a rational therapeutic target for breast cancer treatment. *Oncology.* 2013;84(1):43-56.
13. Clyde Z, Chataway SJ, Signorini D, Gregor A, Grant R. Significant change in tests of neurological impairment in patients with brain tumours. *J Neurooncol.* Aug 1998;39(1):81-90.
14. Mahoney FI, Barthel DW. Functional evaluation: the Barthel index. *Md State Med J.* Feb 1965;14:61-65.
15. Keime-Guibert F, Graus F, Fleury A, et al. Treatment of paraneoplastic neurological syndromes with antineuronal antibodies (Anti-Hu, anti-Yo) with a combination of immunoglobulins, cyclophosphamide, and methylprednisolone. *J Neurol Neurosurg Psychiatry.* Apr 2000;68(4):479-482.
16. de Jongste AH, van Rosmalen JM, Gratama JW, Sillevis Smitt PA. Current and future approaches for treatment of paraneoplastic neurological syndromes with well characterized onconeural antibodies. *Expert Opinion on Orphan Drugs.* 2014;5(2): 483-495.
17. Graus F, Vega F, Delattre JY, et al. Plasmapheresis and antineoplastic treatment in CNS paraneoplastic syndromes with antineuronal autoantibodies. *Neurology.* Mar 1992;42(3 Pt 1):536-540.

18. Uchuya M, Graus F, Vega F, Rene R, Delattre JY. Intravenous immunoglobulin treatment in paraneoplastic neurological syndromes with antineuronal autoantibodies. *J Neurol Neurosurg Psychiatry.* Apr 1996;60(4):388-392.

19. Vernino S, O'Neill BP, Marks RS, O'Fallon JR, Kimmel DW. Immunomodulatory treatment trial for paraneoplastic neurological disorders. *Neuro-oncol.* Jan 2004;6(1):55-62.

20. Shams'ili S, de Beukelaar J, Gratama JW, et al. An uncontrolled trial of rituximab for antibody associated paraneoplastic neurological syndromes. *J Neurol.* Jan 2006;253(1):16-20.

21. van Broekhoven F, de Graaf MT, Bromberg JE, et al. Human chorionic gonadotropin treatment of anti-Hu-associated paraneoplastic neurological syndromes. *J Neurol Neurosurg Psychiatry.* Dec 2010;81(12):1341-1344

9

Summary and discussion

This thesis aimed to unravel the immunopathogenesis of paraneoplastic neurological syndromes with anti-Hu antibodies (Hu-PNS), and to contribute to the development of effective immunotherapy.

DETECTION OF HUD-SPECIFIC T LYMPHOCYTES

Since passive immunization of animals resulted in high titers of anti-Hu antibodies but no disease and because of the intracellular localization of the Hu-antigens, it was hypothesized that anti-Hu antibodies are not pathogenic.[1] Instead, neuronal destruction in Hu-PNS seems more likely caused by cytotoxic $CD8^+$ T lymphocytes, as supported by the $CD8^+$ T lymphocyte infiltrates around damaged neurons found at autopsy.[1,2] Based on this hypothetical $CD8^+$ T lymphocyte response, others and we have performed several studies to detect HuD-specific cytotoxic $CD8^+$ T lymphocytes, but these studies often showed conflicting results.[3-10] More recently, Roberts et al. described in three patients T lymphocyte responses to the HuD-derived T lymphocyte epitopes Hu133 and Hu157, and reported 'type 2' $CD8^+$ T lymphocytes that secreted the type 2 cytokines IL-4, IL-5 and IL-13.[6] The detection and further characterization of any HuD-specific T lymphocytes is of potential relevance to patients with Hu-PNS, since these lymphocytes could serve as a target for specific therapies. Therefore we aimed to confirm the presence of HuD-specific T lymphocytes in a relatively large group of 17 Hu-PNS patients in **chapter 2**. We used three highly sensitive assays to detect classical 'type 1' cytotoxic T lymphocytes and 'type 2' $CD8^+$ T lymphocytes. To gain maximal sensitivity, all procedures included the use of dendritic cells (DCs) to expand the number of any HuD-specific T lymphocytes. Despite using these 3 highly sensitive assays, we did not detect any HuD-specific T lymphocytes. The discrepancies between our results and those of Roberts et al.[6] could be caused by methodological differences or differences in patient characteristics (especially a delay in patients inclusion). However, since our methods and patient characteristics were very similar to those of Roberts et al., we consider it unlikely that these factors would have accounted for our negative results. There are several possible explanations for our negative results. First, HuD-specific T lymphocytes may be preferentially found in or around target tissues such as CNS, dorsal roots or SCLC rather than in peripheral blood. As a consequence, the numbers of HuD-specific T cells in the blood might be extremely low and not detectable with even the most sensitive techniques. Second, HuD itself might not be the target of the hypothetical auto-aggressive T lymphocytes in Hu-PNS. Although the high expression of HuD in both neurons and SCLC lymphocytes makes HuD the most likely candidate, other proteins, such as the Hu-protein Hel-N1 which is also expressed by neurons and SCLC cells, may be involved.[11] Even proteins that do not belong to the

family of Hu proteins may be involved, despite the presence of high titers of Hu-Ab in Hu-PNS patients. An example is celiac disease, in which a B lymphocyte response is seen that is targeted against the enzyme tissue transglutaminase itself, while T lymphocyte responses are targeted at the products of this enzyme.[12] In summary, we propose that auto-aggressive T lymphocytes in Hu-PNS either do not target HuD, or are extremely rare in the blood which makes their detection extremely demanding and certainly not feasible for clinical application. Finally, Hu-PNS might not be caused by auto-reactive T cells at all.

CELLULAR COMPOSITION OF CEREBROSPINAL FLUID IN HU-PNS

In **chapter 3**, we reviewed the use of flow cytometry to study the cellular components of cerebrospinal fluid (CSF). In the clinic, CSF flow cytometry is used for detection of leptomeningeal localizations of hematological malignancies. In research settings, flow cytometry is used to study the cellular composition of CSF in neuro-inflammatory diseases such as multiple sclerosis and PNS. In order to reliably compare the cellular composition of CSF of Hu-PNS patients with the cellular composition of control patients, we first investigated the effect CMV carrier status on T lymphocyte subsets in the CSF of patients with non-inflammatory disorders in **chapter 4**. In the blood, CMV carrier status is known to significantly increase the numbers of effector memory and late memory T lymphocytes.[13] We did not find a significant effect of CMV carrier status on T lymphocyte subsets in CSF. Hence, there was no need to stratify for CMV carrier status when comparing T lymphocyte subsets in CSF of Hu-PNS patients with those of (i) patients with other inflammatory neurological disorders (IND) of non-paraneoplastic origin and (ii) patients with other non-inflammatory neurological disorders (OND) in **chapter 5**. In this chapter, we found higher numbers of regulatory T lymphocytes, central memory T lymphocytes, class-switched memory B lymphocytes and dendritic cells in CSF of Hu-PNS patients than in OND patients. These findings in the absence of similar findings in the blood of Hu-PNS patients indicate that their immune system is locally activated and support a role for cytotoxic T lymphocytes, T helper lymphocytes and B lymphocytes as well as regulatory T lymphocytes and dendritic cells in the pathogenesis of Hu-PNS. In all studies on CSF described above, we stabilized CSF samples with serum-containing medium, since otherwise, rapid cellular decay would occur directly after withdrawal of CSF from the body.[14] The serum-containing medium we used is not commercially available, and has a limited shelf life of 3 months. Recently, a novel CSF-stabilizing agent came on the market that has a shelf life of 12 months (TransFix™).[15] In **chapter 6**, we studied the effects of this commercially available CSF-stabilizing agent on cellular loss in CSF and the detection of leptomeningeal

localizations of hematological malignancies as detected by flow cytometry. We found that TransFix™ prevented cellular loss, and enhanced the flow-cytometric detection of leptomeningeal localizations of hematological malignancies after 18 hours of storage. Hence, TransFix™ may be an attractive alternative for our serum-containing medium in future studies.

TREATMENT OF HU-PNS

In **chapter 7**, we reviewed the available evidence on immunotherapy in Hu-PNS and other PNS with well characterized onconeural antibodies. To improve the poor functional outcome in these patients, various immunotherapies have been used including plasma exchange, ivIg, rituximab, steroids and cyclophosphamide.[16-21] In patients with Hu-PNS, treatment with immunotherapy resulted in functional improvement, defined as an improvement of ≥1 point on the modified rankin scale (mRS), in 11% (7/61) of patients,[16-21] and median survival from the start of immunotherapy was 10 months (range 0 to >48).[16-21] In patients with PNS with anti-Yo antibodies (Yo-PNS), 8% (2/26) of patients showed functional improvement (of ≥ 1 point on the mRS), and survival from the start of immunotherapy was 18 months (range, 2 to >53),[16,17,19,21] Also in PNS with other well characterized onconeural antibodies, the effects of immunotherapy were very limited. In patients with an identified tumor, immediate antitumor therapy is probably the most important factor contributing to better functional outcome. The available evidence underlined the need for more effective immunotherapies for PNS. In **chapter 8**, we describe the results of a clinical trial in which we treated Hu-PNS patients with sirolimus, an inhibitor of activated T lymphocytes. In this trail, 1 patient showed functional improvement (of ≥ 1 point on the mRS) and improvement of neurological impairment (as measured with the Edinburgh Functional Impairment Tests) while another patient only showed improvement of neurological impairment. If we apply the criteria of Keime-Guibert[17] and collegues, than treatment response would be classified as successful in 41% (7/17) patients, which is within the range of previous studies.[16-21] Thus, sirolimus may modify the course of Hu-PNS but our results might be influenced by several confounding factors, such as the predominant paraneoplastic syndrome (i.e. limbic encephalitis versus other PNS such as subacute sensory neuronopathy), concomitant chemotherapy and long interval from symptom onset to diagnosis.[22,23] To conclude, there is still insufficient evidence to recommend immunotherapy for all patients with Hu-PNS or other PNS with well characterized onconeural antibodies. Immunotherapy may be considered in individual patients, especially patients with progressive PNS despite tumor treatment and patients with-

out detectable tumor. In those patients, steroids and ivIg are well tolerated while cyclophosphamide or rituximab could be used as a second-line therapy.[18,24]

DIRECTIONS FOR FUTURE RESEARCH

Future research should focus on the role of the effector T lymphocytes found in the CSF and nervous tissue of Hu-PNS and their interplay with other components of the immune system, including regulatory T lymphocytes, B lymphocytes and dendritic cells. Hopefully, novel techniques such as single-cell PCR and the ability to isolate functionally active lymphocytes at autopsy will help to further elucidate the immune response in Hu-PNS.[25] Regarding the development of immunotherapy, we propose two treatment strategies that should be further investigated. First, therapies should be investigated that potently suppress T lymphocyte mediated immune responses in the CNS. Examples are fingolimod, a drug that prevents egress of T lymphocytes from the lymph nodes by down regulation of the sphingosine 1-phosphate receptor,[26] and natalizumab, a drug that prevents adhesion of T lymphocytes and other leukocytes to endothelial cells by binding to very late activation antigen 4 (VLA-4) thereby preventing their extravasation and migration to the central nervous system.[27] Second, combined suppression of T lymphocyte-mediated immune responses and B lymphocyte-mediated immune responses might be more effective than suppression of T lymphocyte-mediated immune responses alone. An example of a single agent that suppresses both, T lymhocytes and B lymphocytes, is alemtuzumab (anti-CD52). Alemtuzumab leads to reduced numbers of circulating T lymphocytes and B lymphocytes and has shown effective in multiple sclerosis.[28,29] Alternatively, the combination of T lymphocyte-targeted therapy and rituximab treatment could be investigated.[18] Finally, there is a need for larger, controlled clinical trials, which will be a formidable challenge given the rarity and heterogeneity of Hu-PNS.

REFERENCES

1. Sillevis Smitt PA, Manley GT, Posner JB. Immunization with the paraneoplastic encephalomyelitis antigen HuD does not cause neurologic disease in mice. *Neurology.* Oct 1995;45(10):1873-1878.
2. Roberts WK, Darnell RB. Neuroimmunology of the paraneoplastic neurological degenerations. *Curr Opin Immunol.* Oct 2004;16(5):616-622.
3. Rousseau A, Benyahia B, Dalmau J, et al. T cell response to Hu-D peptides in patients with anti-Hu syndrome. *J Neurooncol.* Feb 2005;71(3):231-236.
4. Benyahia B, Liblau R, Merle-Beral H, Tourani JM, Dalmau J, Delattre JY. Cell-mediated autoimmunity in paraneoplastic neurological syndromes with anti-Hu antibodies. *Ann Neurol.* Feb 1999;45(2):162-167.
5. Tanaka M, Maruyama Y, Sugie M, Motizuki H, Kamakura K, Tanaka K. Cytotoxic T cell activity against peptides of Hu protein in anti-Hu syndrome. *J Neurol Sci.* Sep 15 2002;201(1-2):9-12.
6. Roberts WK, Deluca IJ, Thomas A, et al. Patients with lung cancer and paraneoplastic Hu syndrome harbor HuD-specific type 2 CD8+ T cells. *J Clin Invest.* Jul 2009;119(7):2042-2051.
7. Plonquet A, Garcia-Pons F, Fernandez E, et al. Peptides derived from the onconeural HuD protein can elicit cytotoxic responses in HHD mouse and human. *J Neuroimmunol.* Sep 2003;142(1-2):93-100.
8. DeLuca I, Blachere NE, Santomasso B, Darnell RB. Tolerance to the neuron-specific paraneoplastic HuD antigen. *PLoS One.* 2009;4(6):e5739.
9. de Beukelaar JW, Verjans GM, van Norden Y, et al. No evidence for circulating HuD-specific CD8+ T cells in patients with paraneoplastic neurological syndromes and Hu antibodies. *Cancer Immunol Immunother.* Sep 2007;56(9):1501-1506.
10. de Beukelaar JW, Milikan JC, Verjans GM, et al. No evidence for the presence of HuD-specific T cells in the cerebrospinal fluid of patients with Hu-associated paraneoplastic neurological syndromes. *J Neurol.* Feb 2009;256(2):279-282.
11. King PH. Differential expression of the neuroendocrine genes Hel-N1 and HuD in small-cell lung carcinoma: evidence for down-regulation of HuD in the variant phenotype. *International journal of cancer.* Aug 22 1997;74(4):378-382.
12. Schuppan D, Junker Y, Barisani D. Celiac disease: from pathogenesis to novel therapies. *Gastroenterology.* Dec 2009;137(6):1912-1933.
13. Gratama JW, Naipal AM, Oosterveer MA, et al. Effects of herpes virus carrier status on peripheral T lymphocyte subsets. *Blood.* Aug 1987;70(2):516-523.
14. de Graaf MT, van den Broek PD, Kraan J, et al. Addition of serum-containing medium to cerebrospinal fluid prevents cellular loss over time. *J Neurol.* Aug 2011;258(8):1507-1512.
15. Barnett D, Granger V, Mayr P, Storie I, Wilson GA, Reilly JT. Evaluation of a novel stable whole blood quality control material for lymphocyte subset analysis: results from the UK NEQAS immune monitoring scheme. *Cytometry.* Sep 15 1996;26(3):216-222.
16. Graus F, Vega F, Delattre JY, et al. Plasmapheresis and antineoplastic treatment in CNS paraneoplastic syndromes with antineuronal autoantibodies. *Neurology.* Mar 1992;42(3 Pt 1):536-540.
17. Keime-Guibert F, Graus F, Fleury A, et al. Treatment of paraneoplastic neurological syndromes with antineuronal antibodies (Anti-Hu, anti-Yo) with a combination of immuno-

globulins, cyclophosphamide, and methylprednisolone. *J Neurol Neurosurg Psychiatry.* Apr 2000;68(4):479-482.

18. Shams'ili S, de Beukelaar J, Gratama JW, et al. An uncontrolled trial of rituximab for antibody associated paraneoplastic neurological syndromes. *J Neurol.* Jan 2006;253(1):16-20.

19. Uchuya M, Graus F, Vega F, Rene R, Delattre JY. Intravenous immunoglobulin treatment in paraneoplastic neurological syndromes with antineuronal autoantibodies. *J Neurol Neurosurg Psychiatry.* Apr 1996;60(4):388-392.

20. van Broekhoven F, de Graaf MT, Bromberg JE, et al. Human chorionic gonadotropin treatment of anti-Hu-associated paraneoplastic neurological syndromes. *J Neurol Neurosurg Psychiatry.* Dec 2010;81(12):1341-1344.

21. Vernino S, O'Neill BP, Marks RS, O'Fallon JR, Kimmel DW. Immunomodulatory treatment trial for paraneoplastic neurological disorders. *Neuro-oncology.* Jan 2004;6(1):55-62.

22. Graus F, Keime-Guibert F, Rene R, et al. Anti-Hu-associated paraneoplastic encephalomyelitis: analysis of 200 patients. *Brain.* Jun 2001;124(Pt 6):1138-1148.

23. Sillevis Smitt P, Grefkens J, de Leeuw B, et al. Survival and outcome in 73 anti-Hu positive patients with paraneoplastic encephalomyelitis/sensory neuronopathy. *J Neurol.* Jun 2002;249(6):745-753.

24. Voltz R. Intravenous immunoglobulin therapy in paraneoplastic neurological syndromes. *J Neurol.* Sep 2006;253 Suppl 5:V33-38.

25. Verjans GM, Hintzen RQ, van Dun JM, et al. Selective retention of herpes simplex virus-specific T cells in latently infected human trigeminal ganglia. *Proceedings of the National Academy of Sciences of the United States of America.* Feb 27 2007;104(9):3496-3501.

26. Cohen JA, Barkhof F, Comi G, et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. *The New England journal of medicine.* Feb 4 2010;362(5):402-415.

27. Stuve O, Marra CM, Jerome KR, et al. Immune surveillance in multiple sclerosis patients treated with natalizumab. *Ann Neurol.* May 2006;59(5):743-747.

28. Coles AJ, Twyman CL, Arnold DL, et al. Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. *Lancet.* Oct 31 2012.

29. Cohen JA, Coles AJ, Arnold DL, et al. Alemtuzumab versus interferon beta 1a as first-line treatment for patients with relapsing-remitting multiple sclerosis: a randomised controlled phase 3 trial. *Lancet.* Oct 31 2012.

Appendices

SAMENVATTING

Het onderzoek beschreven in dit proefschrift had tot doel om de onderliggende mechanismen die leiden tot paraneoplastische syndromen met anti-Hu antistoffen (Hu-PNS) te ontrafelen. Hu-PNS zijn effecten-op-afstand van kanker, die niet direct veroorzaakt worden door de tumor of metastasen. Waarschijnlijk worden Hu-PNS veroorzaakt door een immuunrespons tegen de tumor, die zich ook richt tegen het zenuwweefsel. Een mogelijk doelwit van deze immuunrespons is HuD, een eiwit dat zowel in bepaalde kankercellen als in neuronen tot expressie komt.

DETECTIE VAN HUD-SPECIFIEKE T CELLEN

Op basis van de observatie dat passieve immunisatie van proefdieren wel leidde tot hoge anti-Hu antistoftiters maar niet tot ziekte, kwam men tot de hypothese dat Hu-PNS niet wordt veroorzaakt door anti-Hu antistoffen, maar door HuD-specifieke T cellen. In **hoofdstuk 2** beschrijven wij ons onderzoek gericht op het detecteren van deze hypothetische HuD-specifieke T cellen in het bloed van patiënten met Hu-PNS. We gebruikten drie zeer gevoelige detectiemethoden, maar detecteerden desondanks geen HuD-specifieke T cellen. Mogelijke verklaringen hiervoor zijn onder andere: (1) dat Hu-PNS wel worden veroorzaakt door HuD-specifieke T cellen, maar dat deze zich vooral in kanker- en zenuwweefsels bevinden en niet in het bloed; (2) dat Hu-PNS wel worden veroorzaakt door T cellen, maar dat deze T cellen niet HuD-specifiek zijn; of (3) dat Hu-PNS toch niet worden veroorzaakt door anti-neuronale T cellen.

CELLULAIRE COMPOSITIE VAN LIQUOR CEREBROSPINALIS IN HU-PNS

Hoofdstuk 3 geeft een overzicht van de toepassingen van flowcytometrie van liquor cerebrospinalis. Met flowcytometrie kunnen leukocyten in liquor cerebrospinalis worden geteld en getypeerd. Deze techniek wordt gebruikt voor het detecteren van leptomeningeaal gelocaliseerde hematologische maligniteiten en voor wetenschappelijk onderzoek naar neurologische ontstekingsziekten zoals multipele sclerose en Hu-PNS. In **hoofdstuk 4** onderzochten we het effect van cytomegalovirus(CMV)-dragerschap op de het aantal effector- T cellen in liquor cerebrospinalis. In de tegenstelling tot in het bloed was het aantal effector-T cellen in liquor cerebrospinalis van proefpersonen die drager zijn van het CMV virus niet verhoogd. Deze kennis was nodig om een betrouwbare vergelijking te kunnen maken van de cellulaire compositie van liquor cerebrospinalis van Hu-PNS patiënten met die van controlepatiënten in **hoofdstuk 5**.

Liquor cerebrospinalis van Hu-PNS patiënten bevat verhoogde aantallen van regulatoire T cellen, central memory-T cellen, memory-B lymfocyten en dendritische cellen. Deze cellen spelen mogelijk een rol in de pathogenese van Hu-PNS. In **hoofdstuk 4 en 5** maakten we gebruikt van een mediumvloeistof om ervoor te zorgen dat leukocyten intact bleven tijdens transport van de patiënt naar ons laboratorium. In **hoofdstuk 6** onderzochten we een nieuwe commerciele vloeistof om deze leukocyten te stabiliseren, genaamd TransFix™. Het blijkt dat dit middel voordelen heeft om leukocyten te stabliseren tijdens transport naar een flowcytometrie laboratorium. De cellen bleven langer gestabiliseerd waardoor ze ook na 18 uur bewaren beter onderzocht konden worden. Dit is nuttige kennis voor toekomstige onderzoeken naar leukocyten in liquor cerebrospinalis.

BEHANDELING VAN HU-PNS

Hoofdstuk 7 is een review van alle studies die met immunotherapie hebben geprobeerd om het functioneren van patiënten met Hu-PNS te verbeteren. Helaas is er nog geen bewezen werkzame immunotherapie voor Hu-PNS. Slechts 11% van de patiënten ging in functioneren vooruit, en de mediane overleving was 10 maanden. In **hoofdstuk 8** beschrijven we de resultaten van onze studie met het middel sirolimus, een remmer van geactiveerde T cellen. De resultaten van deze studie suggereerden een beperkt effect, vergelijkbaar met effecten die beschreven waren in voorgaande studies. Er is dus nog steeds onvoldoende bewijs om standaard immunotherapie toe te passen bij patiënten met Hu-PNS, en behandeling van de onderliggende tumor is de aangewezen therapie. In individuele gevallen kan wel immunotherapie overwogen worden, met name wanneer er progressie is ondanks behandeling van de onderliggende tumor, of wanneer er geen tumor kan worden aangetoond.

DANKWOORD

Allen die een bijdrage hebben geleverd aan dit proefschrift wil ik hiervoor hartelijk bedanken, een aantal van hen wil ik hieronder graag noemen.

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PHD PORTFOLIO

	Year	Workload (ECTS)
General courses		
Basiscursus Regelgeving Klinisch Onderzoek, examen Good Clinical Practice	2010	1.5
Introduction to Data-Analysis, Netherlands Institute for Health Sciences (overall mark 8.5)	2010	1.5
English Biomedical Writing and Communication	2010-2011	4
In-depth courses		
Geavanceerde beeldvormende technieken voor dokters	2009	0.5
Biomedical research techniques IX, day 1 and 2	2010	0.5
Advanced Course "Molecular Immunology"	2010	1.5
European Association of Neuro Oncology Educational Day	2010	0.5
7th course on Leukemia/lymphoma immunophenotyping, European Society for Clinical Cell Analysis, Dublin, Ireland	2011	0.5
Cursus Neuro-anatomie en radiologie	2013	1
National conferences		
Wetenschappelijke dag, Landelijke Werkgroep Neuro-Oncologie	2010	0.5
Symposium Medische Immunologie	2010	0.5
Landelijke werkgroep Neuro-Oncologie investigators	2011	0.5
Wetenschappelijke vergadering, Nederlandse Vereniging voor Neurologie	2012	0.5
Molecular Medicine Day, Molecular Medicine Postgraduate School	2012	0.5
International conferences		
9th meeting of the European Association of Neuro Oncology, Maastricht, the Netherlands	2010	21
Measuring Antigen-Specific Immune Responses, Mykonos, Greece	2010	1.5
11th Euroconference, European Society for Clinical Cell Analysis, Dublin, Ireland	2011	1.5
12th Euroconference, European Society for Clinical Cell Analysis, Budapest, Hungary	2012	1.5
Oral presentations		
Josephine Nefkens Institute meeting	2010	0.5
Wetenschappelijke dag, Landelijke Werkgroep Neuro-Oncologie	2010	0.5
9th meeting of the European Association of Neuro Oncology	2010	0.5
Landelijke werkgroep Neuro-Oncologie investigators	2011	0.5
12th Euroconference, European Society for Clinical Cell Analysis, Budapest, Hungary	2012	0.5

Poster presentations		
Measuring Antigen-Specific Immune Responses, Mykonos, Greece	2010	0.5
10th Euroconference, European Society for Clinical Cell Analysis, Valencia, Spain	2010	0.5
11th Euroconference, European Society for Clinical Cell Analysis, Dublin, Ireland	2011	0.5
Molecular Medicine Day, Molecular Medicine Postgraduate School	2012	0.5
Seminars and workshops		
Workshop on Photoshop and Illustrator CS5	2011	0.3
Workshop Writing Successful Grant Proposals	2012	0.5
Teaching activities		
Teaching nursing staff	2011	0.5
Teaching students MSc Neuroscience	2011-2012	1.0
Teaching students MSc Infection and Immunity	2011-2012	1.0
Reviewing papers for international peer-reviewed journals	2012-2014	1.0

CURRICULUM VITAE

Adriaan Hendrik Cornelis de Jongste (Arjen) was born on March 2nd, 1984 in Rotterdam. He attended secondary school at Sint-Laurenscollege in Rotterdam, and graduated in 2002. He was admitted to the Medical Faculty of the Erasmus University Medical Center (Erasmus MC) in the same year. He obtained his bachelor ('propedeuse') *Cum Laude* in 2003. During his medical study, Arjen additionally followed a research master in Neuroscience at the department of Neuroscience of the Erasmus MC (head: prof. dr. C.I. de Zeeuw) and conducted research on the translational vestibulo-ocular reflex under supervision of prof.dr. J. van der Steen. In 2006 he obtained MSc degrees in Medicine and in Neurosciences. After an internship Internal Medicine at the Sint-Fransiscus Gasthuis in Rotterdam in 2008, he obtained his medical degree. In the same year he worked as a resident ('ANIOS') at the department of Neurology of the Erasmus MC (head: prof.dr. P.A.E. Sillevis Smitt). In 2009, he started the work described in this thesis. He followed a training in special laboratory techniques at the Diabetes and Autoimmunity Research laboratory (INSERM U1016) in Paris, France (head: dr. R. Mallone). He was awarded a travel scholarship from the European Society for Clinical Cell Analysis in 2011 for his oral presentation at the 12th Euroconference in Budapest, Hungary. At present, Arjen is continuing his residency ('AIOS') in Neurology at the Erasmus MC.

LIST OF PUBLICATIONS

1. Houben MM, Goumans J, **de Jongste AH**, van der Steen J. Angular and linear vestibulo-ocular responses in humans. *Annals of the New York Academy of Sciences.* Apr 2005;1039:68-80.
2. **de Jongste AH,** Tilanus AM, Bax H, Willems MH, van der Feltz M, van Hellemond JJ. New insights in diagnosing Schistosoma myelopathy. *J Infect.* Mar 2010;60(3):244-247.
3. de Graaf MT, **de Jongste AH**, Kraan J, Boonstra JG, Smitt PA, Gratama JW. Flow cytometric characterization of cerebrospinal fluid cells. *Cytometry.* May 12 2011(80B):271-281.
4. **de Jongste AH**, de Graaf MT, Martinuzzi E, et al. Three sensitive assays do not provide evidence for circulating HuD-specific T cells in the blood of patients with paraneoplastic neurological syndromes with anti-Hu antibodies. *Neuro Oncol.* July 2012;14(7):841-848.
5. **de Jongste AH**, de Graaf MT, van den Broek PD, Kraan J, Smitt PA, Gratama JW. Elevated numbers of regulatory T cells, central memory T cells and class-switched B cells in cerebrospinal fluid of patients with anti-Hu antibody associated paraneoplastic neurological syndromes. *J Neuroimmunol.* May 15 2013;258(1-2):85-90.
6. **de Jongste AH**, de Graaf MT, van den Broek PD, Kraan J, Sillevis Smitt PA, Gratama JW. Effector memory and late memory T cells accumulate in the blood of CMV-carrying individuals but not in their cerebrospinal fluid. *Cytometry.* Jul-Aug 2013;84(4):218-221.
7. **de Jongste AH**, Kraan J, van den Broek PD, et al. Use of TransFix cerebrospinal fluid storage tubes prevents cellular loss and enhances flow cytometric detection of malignant hematological cells after 18 hours of storage. *Cytometry.* Jul 2014;86(4):272-279.
8. **de Jongste AH**, van Rosmalen JM, Gratama JW, Sillevis Smitt PA. Current and future approaches for treatment of paraneoplastic neurological syndromes with well characterized onconeural antibodies. *Expert Opinion on Orphan Drugs.* 2014;5(2): 483-495.
9. **de Jongste AH**, van Gelder T, Bromberg JE, et al. A prospective open-label study of sirolimus for the treatment of anti-Hu associated paraneoplastic neurological syndromes. *Neuro-oncology.* Epub ahead of print, doi:10.1093/neuonc/nou126