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Measurement of soluble inflammatory mediators in cerebrospinal fluid of human immunodeficiency virus–positive patients at distinct stages of infection by solid-phase protein array

Thorsten Nolting,[1] Antje Lindecke,[2] Eleni Koutsilieri,[3] Matthias Maschke,[4] Ingo-W Husstedt,[5] Sieghart Sopper,[6] Olaf Stüve,[1,7] Hans-Peter Hartung,[1] Gabriele Arendt,[1] and the Competence Network HIV/AIDS

[1]Department of Neurology; [2]Biomedical Research Center (BMFZ), Heinrich-Heine-University, Duesseldorf, Germany.; [3]Department of Virology and Immunobiology, University of Würzburg, Würzburg, Germany; [4]Department of Neurology, University Hospital of Duisburg-Essen, University of Duisburg-Essen, Essen, Germany; [5]Department of Neurology, University of Münster, Münster, Germany; [6]German Primate Center, Göttingen, Germany; and [7]University of Texas, Southwestern Medical Center, Dallas, Texas, USA

The objective of this study was to evaluate immune cytokine expression in cerebrospinal fluid (CSF) of patients with human immunodeficiency virus-1 (HIV-1)–associated dementia (HAD) using a novel cytokine array assay. HIV-1 induces a condition resembling classical subcortical dementia, known as HAD. The immune mechanisms contributing to HAD have not been elucidated. Cytokine expression in CSF was determined by solid-phase protein array in 33 neurologically asymptomatic HIV-positive male patients and were compared to levels in non-HIV controls and patients with HAD. Neurological examinations and lumbar and venous punctures were conducted in all patients and controls. Interleukin (IL)-1, IL-4, and IL-10, were up-regulated in all treated acquired immunodeficiency syndrome (AIDS) patients independent of neurological status compared to controls. In contrast, interferon gamma (IFN-γ), IL-1α, IL-15, and tumor necrosis factor alpha (TNF-α) were highly expressed in patients with HAD compared to undemented HIV-positive patients. These results show that solid-phase protein array can detect immunological changes in patients infected with HIV. Cytokine expression levels differ in different disease stages and in patients on different treatment paradigms. Pending further validation on a larger number of patients, this method may be a useful tool in CSF diagnostics and the longitudinal evaluation of patient with HAD. *Journal of NeuroVirology* (2010) **15**, 390–400.

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Address correspondence to Thorsten Nolting, MD, Department of Neurology, Heinrich-Heine-University, University Hospital Düsseldorf, Moorenstrasse 5, 40225 Düsseldorf, Germany. E-mail: thorsten.nolting@uni-duesseldorf.de

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Introduction

Human immunodeficiency virus-1 (HIV-1) is associated with a primarily subcortical brain disease, HIV-associated dementia (HAD) (Arendt and von Giesen, 2002; Navia and Rostasy, 2005). This type of dementia is almost never seen in HIV-negative young individuals (Kaul and Lipton, 2006; McArthur, 2004; McArthur *et al*, 2005). It is unlikely that direct HIV-related central nervous system (CNS) cell damage is the only cause of the slowly progressing brain damage. In addition, immune responses to viral antigens or CNS autoantigens may contribute to the pathogenesis of HAD. HIV-associated dementia is likely preceded by asymptomatic neuropsychological impairment (ANI) and mild neuropsychological deficit (MND; formerly minor cognitive motor disorder (MCMD)) (Antinori *et al*, 2007), which are detectable by neurological examination and standardized neuropsychological tests (e.g., Grooved Pegboard, Stroop Color Test, Digit Symbol Test, and fine motor test battery after Arendt et al. [1994]). These clinical precursors to HAD can affect one or more cognitive subsystems (e.g., psychomotor speed, fine motor control, attention, speech, and memory [Lojek and Bornstein, 2005]).

Since the introduction of highly active antiretroviral therapy (HAART) in 1996, the incidence of HAD has decreased. Nevertheless, neuropsychological deficits occur in up to 50% of neurological HIV cohorts (Cysique *et al*, 2004; McArthur *et al*, 2003). Ironically, due to the success of HAART, the prevalence of late-occuring neuropsychological deficits appears to be on the rise (McArthur, 2004; McArthur *et al*, 2003; Valcour *et al*, 2004).

Routine diagnostic paraclinical markers, including HIV viral load in peripheral blood and CD4+ cell count, have not been useful to diagnose or track HAD, ANI, or MND (Antinori *et al*, 2007). Although neurological testing is reproducible and cost-effective in most instances, most of the currently used tests are affected by the subjects' education level, literacy, language, visual and mathematical skills, are time consuming, and not infrequently influenced by the examining and rating physician. Furthermore, neuropsychological tests do not provide mechanistical insight into HAD-assoicated cognitive deficits. Consequently, there is a need for new alternative markers of neuropsychological deficits in HIV patients, especially in patients treated with HAART. According to recent guidelines, a patient is regarded as effectively treated if the viral load in blood is lowered beyond the limit of detection (LOD) and CD4+ cell counts are stably rising at least above 200 cells per microliter (Hammer *et al*, 2006).

The expression of soluble inflammatory mediators, including cytokines, is altered in CSF and blood in HIV disease (Cysique *et al*, 2004; Gallo *et al*, 1989), which influences T-lymphocyte and monocyte/macrophage activation and migration into the CNS (Aquaro *et al*, 2005). Bystander T-cell activation, the balance between Th1/Th2 cell activation and suppression, autoimmune mechanisms, and CNS-intrinsic sources of cytokines may also be important in the evolution of neuropsychological impairment (Bangs *et al*, 2006; Galli *et al*, 2001; Heeney and Plotkin, 2006; Killebrew *et al*, 2005; Schutzer *et al*, 2003). Methodological problems in qualitative and quantitative cytokine measurements have rendered assessment of cytokine contribution to HAD difficult. However, the relatively recent introduction of antibody chip arrays, which can detect a broad variety of cytokines in small tissue samples with high interexperiment accuracy, offers a new method for qualitative and semiquantitative cytokine detection (Nielsen and Geierstanger, 2004).

In the current study, we assessed the value of cytokine arrays in demonstrating altered immunological responses in cerebrospinal fluid (CSF) of HIV-positive patients and explored the role that such cytokine arrays may have as para-clinical tools in monitoring patients infected by HIV, all of whom are at risk for developing neuropsychological deficits.

Results

Patient demographics

All patients were homo- or bisexual Caucasian men born in Germany. All were neurologically and neuropsychologically asymptomatic (see recent nomenclature [Antinori *et al*, 2007]) on initial examination. There were no significant differences between groups with regard to age or CD4+ cell count. Due to antiretroviral therapy, subjects in groups 2 and 4 had lower viral loads in the blood and CSF (nearly all below LOD) and a longer duration of known HIV-positivity (see Table 1 legends for details). Patients with HAD (Memorial Sloan Kettering Scale: 2–4) were diagnosed in the pre-HAART treatment era, before 1995/1996. Seven cases presented with motor deficits, four with speech deficits, five with headache, three with affective disorders, one with disorientation; combinations of symptoms in a given patent were usual. Seven out of 10 patients performed poorly in the "motor test battery." Most had deficits in the "syndrome short test" and scored above 12 points in the Hamilton Depression Scale. Neuropsychological tests were not performed in all HAD subjects due to severe HAD symptoms at the time of examination. Due to the sometimes delayed diagnosis of HIV infection in 1990s in Germany, these patients had a very short disease history after their initial positive HIV test and very low CD4 counts, because they already had established acquired immunodeficiency syndrome (AIDS) (see Table 1). No additional HAD patients with a more recent diagnosis were included in this study

Table 1 Demographic data of the examined patients and controls

	n	Age (years)	Years since HIV diagnosis	CD4+ cell count	Viral load in blood (log_{10})	Viral load in CSF (log_{10})
Non-AIDS, no HAART (Group 1)	10	40.4 (± 14.5)	3.4 (± 4.1)	531.8 (± 165.3)	4.1 (± 0.5)	2.9 (± 1.1)
Non-AIDS, HAART (Group 2)	10	41.7 (± 3.9)	10.1 (± 4.9)	673.3 (± 265.5)	0.5 (± 0.8)	0.8 (± 1.1)
AIDS, no HAART (Group 3)	3	41.7 (± 8.9)	7.0 (± 6.6)	707.7 (± 602.5)	3.2 (± 1.3)	1.3 (± 2.3)
AIDS, HAART (Group 4)	10	49.3 (± 10.9)	12.2 (± 5.4)	407.1 (± 216.7)	1.0 (± 1.4)	0.4 (± 0.9)
HAD patients	10	46.5 (± 12.3)	2.9 (± 2.7)	135.2 (± 186.2)	4.8 (± 5.8)	n.a.
Controls (HIV-negative, Group 5)	5	45.6 (± 9.9)				

Note. Groups are different with respect to human immunodeficiency virus (HIV) duration on a significance level of $P = .007$ and viral load in blood on a significance level of $P = .016$. Groups are comparable in CD4+ cell count ($P = .126$), except HAD ($P < .05$) patients. HIV duration is given as the time period after the first confirmed positive test result. n.a.: not available.

because of the low prevalence of HAD in our clinic populations.

Cytokine levels in CSF

If not otherwise specified, cytokine levels in the patient groups were characterized as up-regulated or down-regulated in comparison with levels in the non-HIV patient group. Due to limited sample size ($n = 3$), results for untreated AIDS patients are given within parentheses. If not otherwise indicated, this patient group was not included in post hoc analysis. Only cytokines with detectable variations between groups are discussed. Signature cytokines of a Th1 and Th2 T-cell responses are shown in Figure 1. All markers of general immune activation are presented in Figure 2.

Comparison of pooled CSF samples from HAD patients and pooled CSF samples from neuropsychologically asymptomatic (no abnormalities in standard neuropsychological test battery) HIV-positive patients revealed an up-regulation of interferon (IFN)-γ (1.60×), interleukin (IL)-1α (1.73×), IL-15 (2.30×), and tumor necrosis factor (TNF)-α (1.71×) in the HAD patients (Figure 3). These observations suggest that these four assays may be most informative as a possible early diagnostic tool for HAD. Other cytokines showed no trend related to HAD status (data not shown in Figure 3).

Correlation analysis of cytokines and other CSF parameters

Correlations between cytokines and other CSF parameters are presented in Table 2. Some markers of immune activation positively correlate with nonspecific CSF markers of CNS inflammation (cells, protein immunoglobulin G [IgG] index, oligoclonal bands (OCB)) in treated HIV patients (groups 2 and 4) only. Also, there was a positive correlation between some markers of immune activation and CSF viral load in untreated early-stage patients.

Discussion

The CNS is a unique environment with regard to immune responses to foreign pathogens. The present study tested HIV-relevant cytokine levels in CSF of HIV-positive individuals, using a novel technique of cytokine detection. As the technology that was applied in this study is currently not able to detect all known cytokines today, only cytokines with feasible relationship to CNS infection with HIV were chosen (Nolting and Arendt, 2008). We were able to detect Th1 and Th2 T-cell activation, bystander T-cell activation, regulatory T-cell (Treg) function, and specific cytokines expression (IL-7, IL-18, intercellular cell adhesion molecule [ICAM]-1, and GRO) as independent markers of possible HIV-associated CNS damage. Our results suggest a potential value of the novel cytokine array in assessing immune function in the CSF in HIV. However, given the sample size of each study group, our findings should be considered preliminary and have to be validated in a larger cohort.

Th1 and Th2 T-cell activation

Our findings present no clear evidence for an increased Th1- or Th2-driven T-cell response in treated or untreated non-AIDS patients. Although this observation is consistent with findings of earlier investigations as well as those from recent animal studies (Buch *et al*, 2001; Clerici and Shearer, 1993), the conclusion here may be compromised by our small sample size.

Bystander T-cell activation

Bystander T-cell activation occurs without involvement of cognate T-cell receptors and may be one of several autoaggressive mechanisms that is less likely to be controlled by the common T-cell regulatory machinery (Bangs *et al*, 2006). There is controversy regarding the role of bystander T-cell activation in HIV. Bystander T cells are usually considered to be activated by high levels of inflammatory cytokines, particularly IL-2, IL-6, TNF-α, and IL-15 (secreted by activated monocytes and macrophages; Bangs *et al*, 2006; Ehl *et al*, 1997). Our study shows signs of early bystander activation (increased levels of IL-2 [data not shown], IL-6, and TNF-α) in untreated non-AIDS patients and delayed bystander activation in treated AIDS patients (increased levels of IL-15 and potentially IL-2 and IL-6). The reason for this

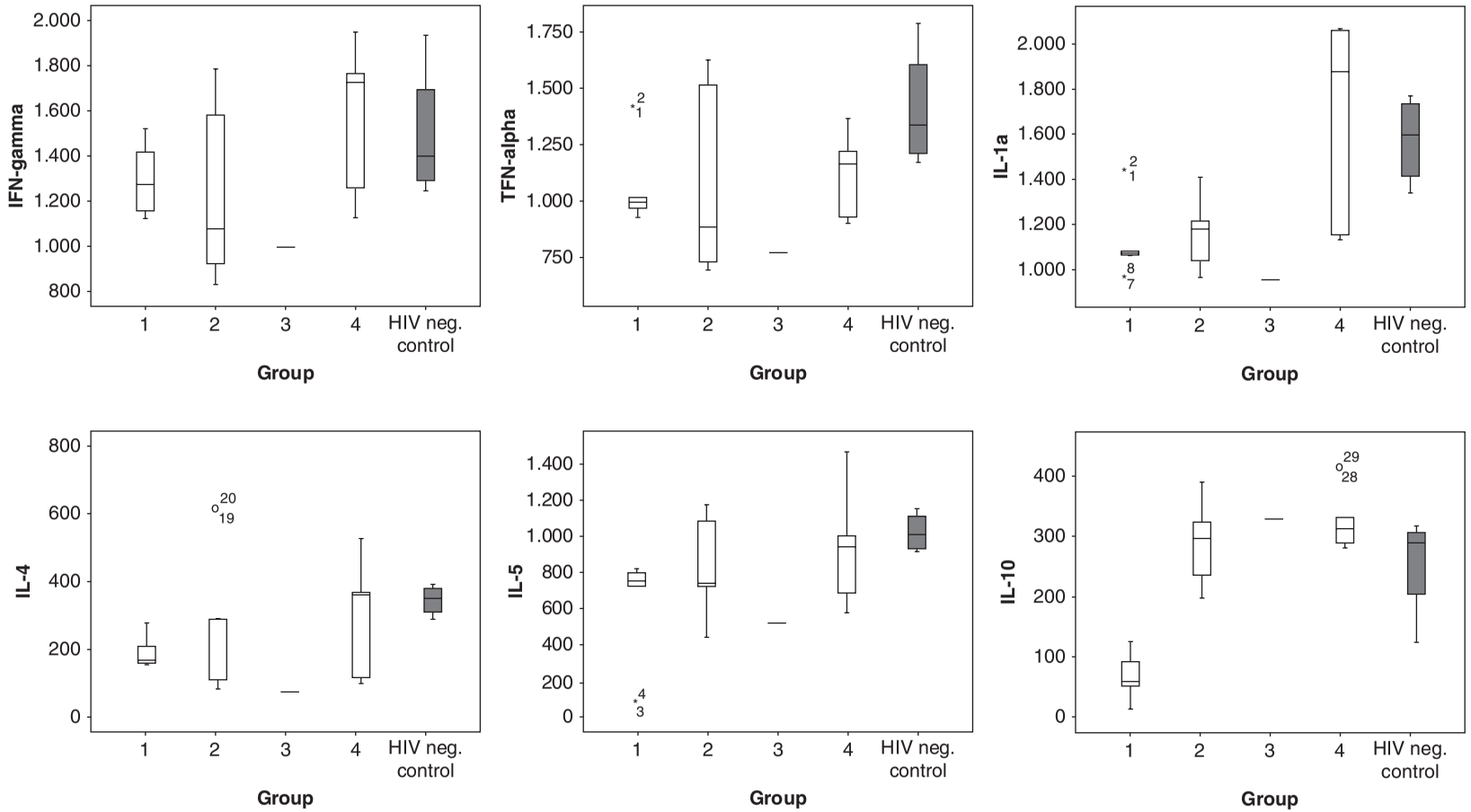

Figure 1 Relative levels of Th1- and Th2-cytokines. The y-axis represents the normalized fluorescence value of each cytokine. Box plots show the median, the upper and lower quartile; whiskers are the 1.5 product of the inter-quartile distance in maximum. Extreme results are marked by numbered stars. P-values are listed in the results section. A Th1-type reaction is induced by IL-18, is marked by high levels of IL-2, IFN-gamma and TNF-alpha and induces a cytotoxic immune reaction. A Th2-type reaction is induced by IL-1. High levels of IL-4, IL-5, IL-10 and IL-13 enhance the production of IgG-antibodies in activated plasma cells. For the expression of Th1 cytokines, including IFN-gamma and TNF-alpha, there were significant differences between treated and untreated early and late stage HIV patients ($p = 0.021$); in post-hoc analysis TNF-α was significantly down-regulated in untreated non-AIDS patients ($p = 0.036$) compared to controls. For Th2, interleukin (IL)-1alpha was significantly down-regulated in untreated and treated non-AIDS patients ($p < 0.001$); IL-5 levels were significantly decreased in untreated non-AIDS patients compared to treated AIDS patients and healthy controls ($p = 0.022$; $p = 0.026$); IL-10 expression was significantly reduced in untreated non-AIDS patients ($p < 0.001$); IL-10 levels were slightly, but not significantly up-regulated in the other groups and IL-4 was not significantly regulated differentially between patient groups ($p = 0.086$).

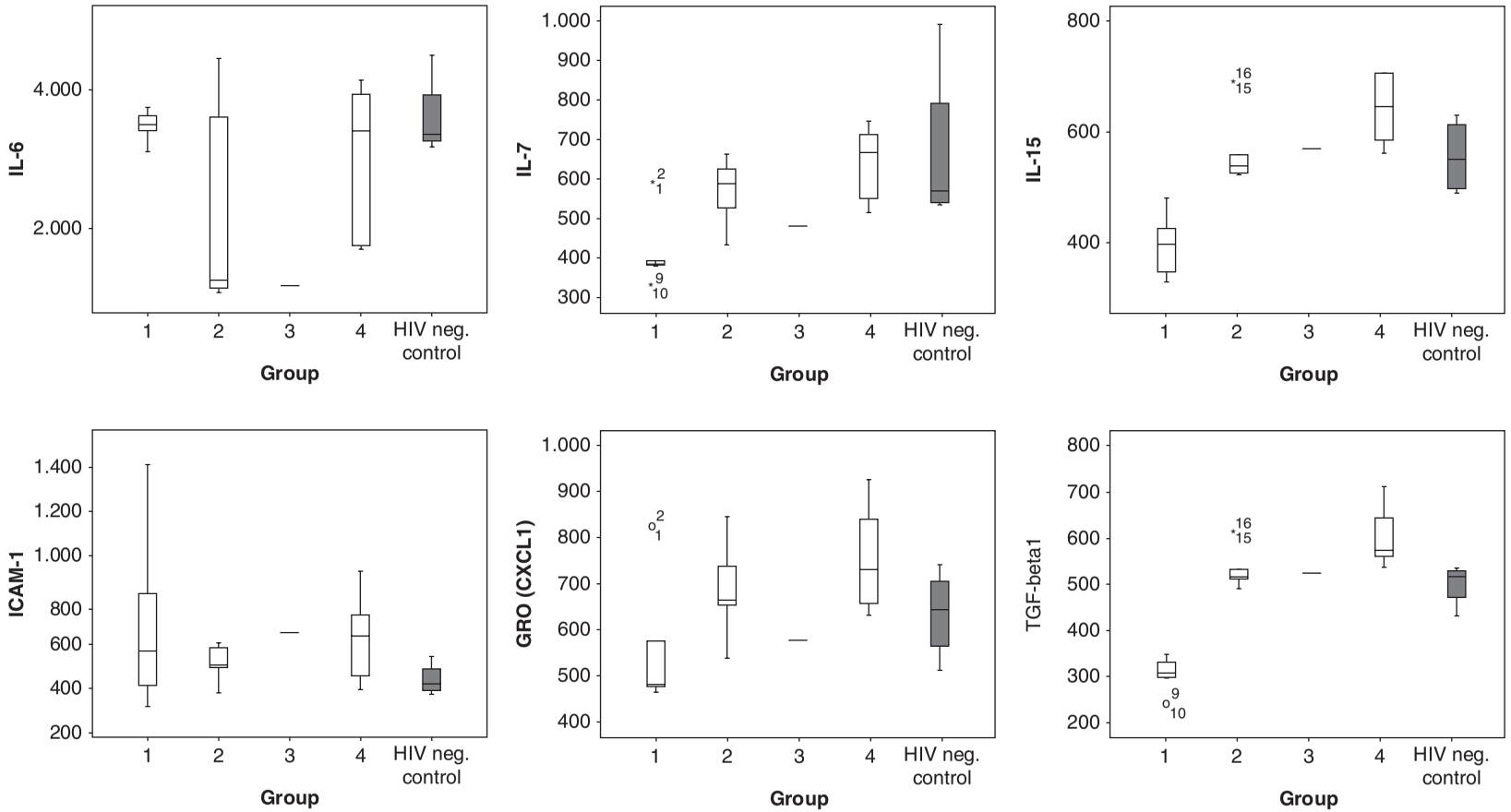

Figure 2 Markers of immune activation and potential factors of HAART surveillance. Markers of general immune activation and regulation which have the highest potential for effective surveillance of HAART in CSF are shown in Fig. 2. IL-6 was significantly down-regulated in treated non-AIDS and untreated AIDS patients (Group 2 and Group 3; $p = 0.040$ and [$p = 0.004$]) compared to HIV-negatives in post-hoc analysis. IL-6 up-regulation in untreated non-AIDS patients and down-regulation in treated AIDS patients (Group 4) did not reach significance compared to controls. IL-7 levels were down-regulated in untreated early stage patients ($p < 0.001$), and within the normal range in groups 2-4. TGFβ was significantly down-regulated in untreated non-AIDS patients (group 1; $p < 0.001$), but levels were in the normal range in treated non- AIDS patients (group 2). TGFβ was upregulated in treated AIDS patients ($p = 0.001$). GRO (CXCL1) levels were in the normal range in all groups of HIV-infected patients when compared to healthy controls, but GRO was slightly down-regulated in untreated non-AIDS patients when compared to treated non-AIDS and AIDS patients ($p = 0.027$ and $p = 0.001$). ICAM-1 (CD54) tended to be higher in every group except for treated non-AIDS patients. IL-15 was significantly down-regulated in untreated non-AIDS patients ($p = 0.006$) and up-regulated in treated AIDS patients ($p = 0.020$).

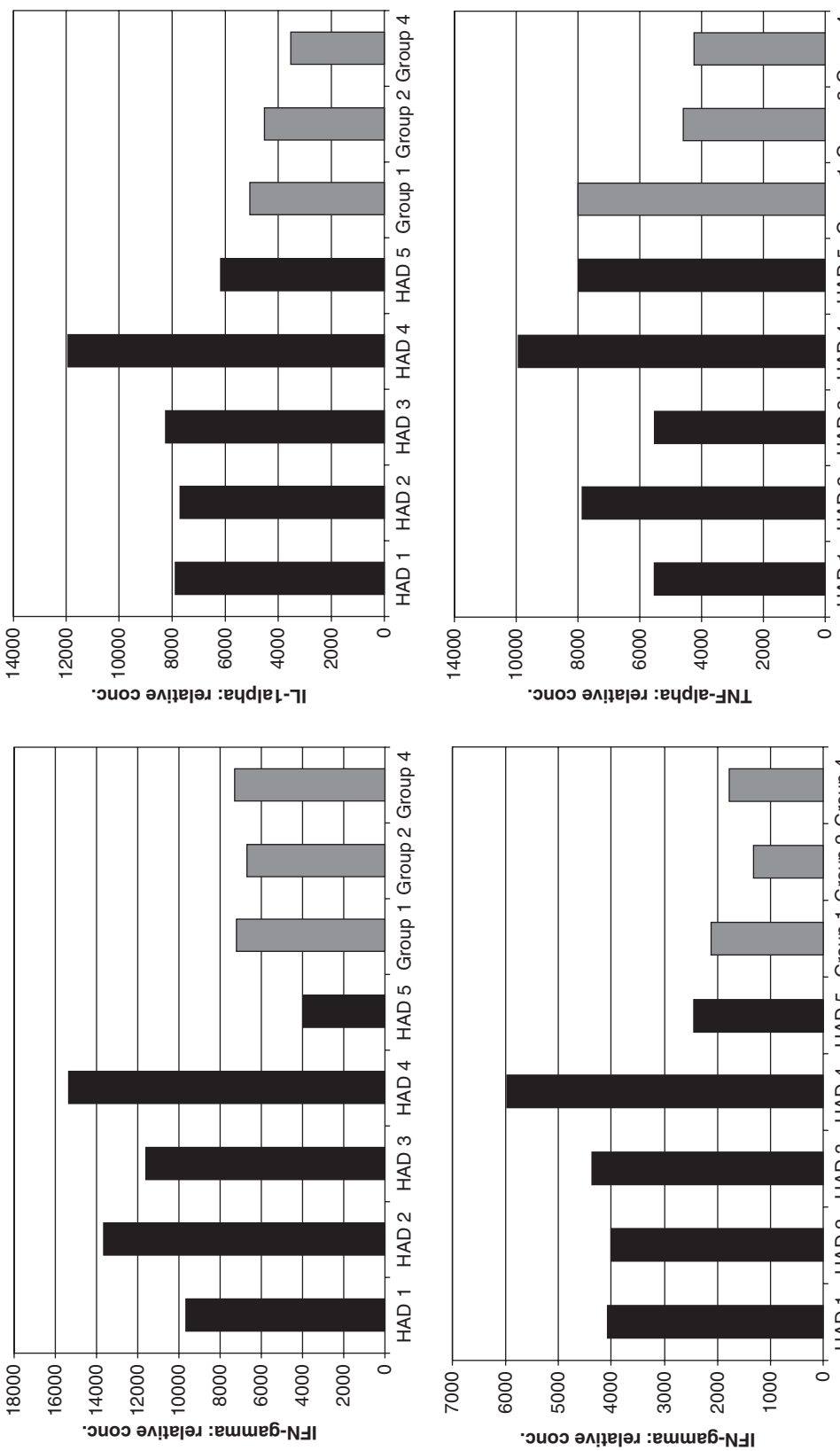

Figure 3 Semi-quantitative analysis of patients with Human Immunodeficiency Virus-1 (HIV-1)-associated dementia (HAD 1-5; 2 patients per pooled probe) compared to groups of non-demented HIV-infected patients. Interferon gamma (IFNγ), interleukin (IL)-1alpha, IL-15, and tumor necrosis factor alpha (TNF-α) are elevated in HAD patients. Group 1: untreated early stages; Group 2: treated early stages; Group 4: treated (late stages) patients with the acquired immune deficiency syndrome (AIDS) (3 patients per pooled probe).

Table 2 Results of the correlation analysis (Pearson's method) of cytokine levels and the parameters in cerebrospinal fluid (CSF) cells, protein content, IgG index, lactate level, and viral load

	CXCL16	GRO	IL-1α	IL-2	IL-5
CSF cells	1: ns	1: ns	1: ns	1: ns	1: ns
	2: $r = -.73$, $P = .15$	2: $r = .668$, $P = .035$	2: $r = .669$, $P = .034$	2: ns	2: ns
	4: ns	4: $r = .79$, $P = .02$	4: ns	4: $r = .826$, $P = .025$	4: $r = .772$, $P = .025$
CSF protein	1: ns	1: ns	1: ns	1: ns	1: ns
	2: $r =. 817$, $P = .004$	2: ns	2: ns	2: ns	2: ns
	4: $r = -.712$, $P = .021$	4: ns	4: ns	4: ns	4: ns
IgG index	1: ns	1: ns	1: ns	1: ns	1: ns
	2: $r = .747$, $P = .033$)	2: ns	2: ns	2: ns	2: ns
	4: ns	4: $r = .763$, $P = .01$	4: ns	4: ns	4: ns
CSF lactate	1: ns	1: ns	1: ns	1: ns	1: ns
	2: $r = -.673$, $P = .033$	2: ns	2: ns	2: ns	2: ns
	4: ns	4: ns	4: ns	4: ns	4: ns
OCB	1: ns	1: ns	1: ns	1: ns	1: ns
	2: $r = .67$, $P = .034$	2: ns	2: ns	2: ns	2: ns
	4: ns	4: ns	4: ns	4: $r = .666$, $P = .035$	4: ns
	Fractalkine	ICAM-1	IL-15		
VL-CSF	1: $r = .644$, $P = .045$	1: $r = .718$, $p = .19$	1: $r = .651$, $P = .041$		
	2: ns	2: ns	2: ns		
	4: ns	4: ns	4: ns		

Note. Only cytokines with significant results are presented. Cytokines that are not presented showed no significant correlation with CSF parameters. Data of group 3 are not shown, as the sample size ($n = 3$) was too small for the analysis. ns: not significant. OCB: oligoclonal bands.

contradictory development of bystander T-cell activation in two entirely different groups of HIV-infected patients, one group untreated and the other treated, remains unclear, and demonstrates the need for confirmatory studies with enlarged sample size.

T-regulatory cell markers

The role of T-regulatory cells in immune function has not been fully elucidated. It appears that these cells are potential suppressors of immune activation via cell-cell interaction or release of IL-10 and transforming growth factor beta (TGFβ) (Romagnani, 2006). IL-10 is responsible for down-regulation of Th1-type cytokines and down-regulation of major histocompatibility complex (MHC) class II on the cell surface. This may be important in HAD, because it is a stimulant for myeloid cells, the major viral host in the CNS (Gallo *et al*, 1994). TGFβ may be involved in extracellular matrix modulation in HIV infection. It was shown to down-regulate tissue inhibitor of metalloproteinases (TIMP)-1. TGFβ is normally found in high concentrations in HAD (Dhar *et al*, 2006; Wahl *et al*, 1991). In this study, levels of both IL-10 and TGFβ were elevated (TGFβ significantly) in treated and untreated AIDS patients. This kind of Th2-biased immune modulation has been demonstrated to be beneficial in the very early stages of asymptomatic HIV infection (Rouse *et al*, 2006). Conversely, the overproduction of signature cytokines of T-regulatory cell activation may indicate an inability of T-regulatory cells to effectively control elevated bystander activation, which has been shown in mouse studies (Ehl *et al*, 1997).

Immune markers of therapeutic response in Neuro-AIDS

IL-7 is a potential candidate for monitoring the efficacy of pharmacotherapies in HIV patients. High levels of IL-7 are associated with poor outcome in HIV infection, possibly due to IL-7 receptor abnormalities and receptor down-regulation by HIV-Tat/Nef (Faller *et al*, 2006; Marchetti *et al*, 2006). Treated AIDS patients in our cohort had elevated IL-7 levels (Figure 2). This is consistent with a possible loss of immunological effectiveness of HAART in the CNS of HIV-infected patients (Malaspina *et al*, 2006). Surprisingly, this finding is not supported by the virological findings in our cohort: Nearly all of the treated AIDS patient in our study showed an excellent treatment response (Table 1). The reasons for this are unclear. Similarly, GRO (CXCL1) is thought to be associated with increased HIV replication in monocyte-derived macrophages and T lymphocytes (Lane *et al*, 2001; Villard *et al*, 1995). GRO was up-regulated in our cohort of treated AIDS patients, suggesting viral replication even in the presence of antiretroviral substances. Again, this finding was not supported by our virological data from the treated AIDS-patients group (Table 1) and results from untreated patients, where GRO levels seem to be low and viral replication is abundantly detectable (Table 1 and Figure 2). Again, this should be retested in larger cohorts. The Ig superfamily adhesion molecule ICAM-1 imediates cell trafficking across biological membranes (Yonekawa and Harlan, 2005). It increases monocyte adhesion to IL-1–activated vascular endothelium and may thereby attract HIV-infected monocytes to the CNS. We found that

ICAM-1 tended to be elevated throughout HIV infection (Figure 2), which might be important for cell-mediated entry of HIV into the CNS (Polman *et al*, 2006; Rudick *et al*, 2006). Interestingly, some inflammatory markers such as the chemokine CXCL16 (ligand of BONZO; HIV-1 coreceptor) and the cytokines IL-1, IL-2, and IL-5 positively correlate with CSF cell count, protein content, and IgG index in treated non-AIDS and AIDS patients (Table 2). These observations underscore the possibility of an autochthonous inflammatory process in these patients, occurring independent of productive viral replication. In untreated patients, viral load in CSF (VL-CSF) is correlated with fractalkine (CX3CL1). Fractalkine may protect against viral infection by virtue of its competition with HIV-1 for the isoforms of chemokine receptor CX3CR1 (Garin *et al*, 2003), but its role in neuronal protection regarding attraction of infected monocytes/marophages is unclear (Tong *et al*, 2000).

Use of cytokine arrays in neurological HIV diagnostics

Our study intended to examine the usefulness of a novel solid phase cytokine array in neurological HIV diagnostics, particularly related to development of HAD. Although limited by our small study population, the results may serve as pilot for future studies with larger cohorts. The study was able to confirm results from basic clinical studies related to treatment status from the pre- and HAART era. Our study supports the hypothesis that activation of the innate and the adaptive immune system takes part in HIV-positive individuals, and could be essential for the development of HAD and its precursors. A common feature seems to be an early inflammatory response with heightened activation of the adaptive immune system in untreated non-AIDS patients. This mechanism is probably directly driven by HIV-1 because immune activation is down-regulated by efficient HAART in non-AIDS patients. We found high levels of IFN-γ, IL-1α, IL-15, and TNF-α in the CSF of HAD patients. In particular, elevation of TNF-α in HAD, comparable to levels in untreated early stage patients, may be an important immunologic marker of HIV CNS infection in humans.

It should be emphasized that all patients in the presented study were neuropsychologically inconspicuous (except HAD patients) according to recent guidelines (Antinori *et al*, 2007). Thus, the need to further clarify the inflammatory cascade is obvious. Cytokine arrays may be a useful tool in this regard. This novel technology may also help to address questions of immunesurveillance and immune repsonses within the CSF and brain parenchyma, and the impact of early or delayed antiretroviral therapy. Potential study outcomes may eventually assist in the assessment of the practical and ethical issues surrounding treatment decisions for early versus delayed start of an antiretroviral therapeutic regimen in HIV-positive patients.

In our study, the solid-state cytokine array technique provided a robust and easy to use system for CSF research. Signals were reproducible and detectable for weeks after the incubation steps were preformed. In environments where necessary laser scanning equipment is not available, chips can be stored in a dry and cool environment for shipment and further processing at a remote location. In addition, only small volume specimens are required for analysis, an important fact in working with CSF.

In conclusion, cytokine arrays provide an affordable and robust system in CSF diagnostics in HIV-positive patients. Nevertheless, additional confirmatory studies with an enlarged sample size are needed to gain deeper insight into the applicability of this technology and the immunological processes involved in the development of CNS damage in HIV infection.

Methods

Study design and patient population and CSF collection

Thirty-three male, HIV-positive patients (homo- or bisexual transmission of HIV-1) were recruited for a prospective, cross-sectional analysis of cytokine levels in CSF. Prior to any experimental studies, approval was obtained from the appropriate institutional review board. Patients with dementia or self-reported neuropsychological deficits, history or current drug and alcohol abuse, CNS infection, antidepressant or antipsychotic medication, or excessively low or high body mass index (BMI; <18 and >30) were excluded. Controls included five HIV-negative subjects with noninflammatory neurological conditions of the CNS to whom similar inclusion and exclusion criteria applied. CSF samples were obtained between 11 AM and 2 PM with consent during routine diagnostic procedures in an outpatient clinic setting of a tertiary academic medical center. All CSF samples were cell-depleted and freshly frozen at -80°C.

Subjects for this single-sample analysis were divided into five groups (Table 1): Early-stage patients without HAART (Group 1; CDC A1+2, B1+2), early-stage patients with HAART (Group 2; CDC A1+2, B1 +2), late-stage patients without HAART (Group 3; CDC A3, B3, C1–3), late-stage patients with HAART (Group 4; CDC A3, B3, C1–3), and HIV-negative controls. The group of HIV-positive patients was stratified according to HIV disease stage and treatment status. The scientific basis for stratification was based on substantial CSF cell count differences between categories that had been determined in a prior study (Arendt *et al*, 2007).

To facilitate the evaluation of the cytokine array technology as a potential diagnostic marker for

neuropsychological impairment in HIV-positive patients, nine frozen CSF samples of untreated and one of AZT monotherapy-treated HAD patients (with two CSF samples mixed equally to form one probe) were tested against pooled samples (three patients per sample) from four of the five HIV-patient groups described above (lacking only untreated late stage patients).

Neuropsychological testing
All patients underwent neuropsychological testing by the same trained and experienced investigator. The following tests were applied to each subject: Stroop Color Test, Wisconsin Card Sorting Task, fine motor test battery (Arendt *et al*., 1994), Grooved Pegboard, Trail-Making Test A + B, formal and semantical word fluency lists (German versions), HIV Dementia Scale (German version), Digit Symbol Test, Rows of numbers, Test of Vocabulary (MWT-b; German), Mosaic Test, Logical Memory Test 1 + 2 (German version), Rey's Figure, syndrome short test (SKT; German), number connection test 1 + 2, Hamilton Depression Scale (HAM-D; German), and Beck Depression Inventory (BDI).

Cytokine arrays
Customized glass chip–based antibody arrays, suitable for serum and CSF probes, were purchased from RayBiotech (Norcross, GA, USA). The chips were designed to detect soluble proteins, including ALCAM (CD166), CCL28, CNTF, CXCL16, Fas (CD95), fractalkine (CX3CL1), GMCSF, GRO (CXCL1), I-309, ICAM-1 (CD54), interferon gamma (IFN-γ), interleukin (IL)-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12(p40p70), IL-15, IL-18, MCP-1 (CCL2), MCP-3, MIF, MIP-3α, 9 RANTES (CCL5), SDF-1 (CXCL2), TGF-β1, TIMP-1, TIMP-2, TNFα, TNFβ (LTA), TRAIL R1 (DR4), and TRAIL R3. Pooled samples of HAD patients were utilized. CSF samples were processed as described above with the "Human Cytokine Antibody Array 2000 G-Series" from RayBiotech. Per patient and chip, 100 µl of CSF were applied. After the initial standard solution preparation and blocking steps, the chips were incubated at room temperature for 2 h with the CSF sample. After standard washing steps, they were incubated with the appropriate biotinylated antibody preparation for 10 h overnight at 4°C. Following the standardized washing steps at room temperature the next day, the chips were incubated for 2 h with Cy3-conjugated streptavidin (diluted 2000-fold) at room temperature in a dark box. All incubation steps were performed under gentle rotation on an orbital shaker. Chips were finally rinsed and dried at 1000 rpm (relvolutions per minute) in a centrifuge at 20°C. The chips were read directly with an Axon GenePix laser scanning device (Axon Instruments; Molecular Devices, Sunnyvale, CA, USA) using the Cy3 channel at maximum resolution and 600× pixel enhancement. All chips were scanned twice, to test results for reproducibility. Raw fluorescence intensities were calculated using the GenePix 6.0 software package (Axon Instruments) and were normalized for further analysis against the first positive-control signal intensity on the first chip using the RayBiotech analysis tool.

HIV-1 viral load
HIV-1 viral load was measured in a reference laboratory using the Quantiplex HIV RNA Assay (bDNA method, v3.0' Bayer Diagnostics, Munich, Germany) in a high-throughput laboratory setting. The LOD was below 40 copies/ml.

Statistical analysis
Statistical analysis was performed using the SPSS 14.0 software package (Chicago, IL, USA). Normalized cytokine levels were compared using one-way analysis of variance (ANOVA) with LSD (least significant difference) post hoc adjustment. Correlations between cytokine levels and CSF parameters were calculated using the Pearson method (Pearson's rho; metric data with gaussian distribution).

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