Activated monocytes and markers of inflammation in newly diagnosed multiple sclerosis

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Abstract
In multiple sclerosis (MS), the inflammation and demyelination of the central nervous system (CNS) develop in distinct ways. This makes diagnosing patients difficult, imperative to initiating early and proper treatment. Several common features exist, among them a profound infiltration of monocytes into the CNS mediating demyelination and tissue destruction. In the periphery, monocytes are divided into three subsets depending on expression of CD14 and CD16, representing different stages of activation and differentiation. To investigate their involvement in MS, peripheral blood mononuclear cells (PBMCs) from 61 patients with incipient, untreated MS and 22 symptomatic control (SC) patients as well as 6 patients with radiologically isolated syndrome (RIS) were characterized ex vivo. In addition, paired serum and cerebrospinal fluid (CSF) samples were analyzed with a panel of biomarkers. In PBMC samples, we demonstrate decreased levels of nonclassical monocytes with a concomitant significant decrease of human endogenous retrovirus (HERV) H3 envelope epitopes on this monocyte subset compared with SC and RIS. The observed HERV expression is present on nonclassical monocytes irrespective of MS and thus presumably a result of the inflammatory activation. For the other surface markers analyzed, we found significantly decreased expression between classical and nonclassical monocytes. In matched samples of CSF a highly significant increase in levels of soluble markers of activation and inflammation is shown, and notably this is not the case for the serum samples. Of the soluble markers investigated, interleukin (IL)-12/IL-23p40 had the highest discriminatory power in differentiating patients with MS from SC and RIS, almost comparable to the immunoglobulin G index.

INTRODUCTION
Multiple factors influence the initiation and progression of the neuroinflammatory disease multiple sclerosis (MS). Intense research efforts have so far not succeeded in identifying a single, defined biomarker for the disease.¹,² Studies of families with MS have defined genetic variants that may account for up to one-third of the inherent susceptibility,³ but environmental factors such as Epstein–Barr virus infection or smoking still account for the majority of risk factors for MS.⁴ Without a predominant exogenous risk factor, it thus remains an open question whether MS is triggered in the periphery or in the central nervous system (CNS).⁵ Despite the considerable heterogeneity of MS, several disease subtypes have been recognized, and generally, patients experience either relapsing-remitting (RRMS) or progressive (PMS) disease courses. Patients who do not have spreading of symptoms in both time and space are diagnosed with clinically isolated syndrome (CIS), of which most (eight out of ten) eventually progress to MS.⁶ Newly revised guidelines for MS diagnostics⁷ establish the diagnosis of
Monocytes and markers of inflammation in MS

M Carstensen et al.

Monocytes and markers of inflammation in MS if the first symptoms with objective neurological findings and typical MS abnormalities on magnetic resonance imaging (MRI) are combined with a positive test for oligoclonal bands or an increased immunoglobulin G (IgG) index. This emphasizes the importance of biomarkers in MS diagnostics. A number of potential biomarkers have thus been investigated in various body fluids, but given the complex nature of the disease, only a few candidates have been considered as contributions to the diagnostic criteria of MS.8

In addition to soluble markers, several key surface receptors involved in immune regulation are shed from the surfaces of immune cells as a result of inflammatory stimuli.9–11 A combination of surface-bound and secreted molecules may therefore have potential as diagnostic and prognostic markers of disease and progression.12,13 Multifactorial processes of inflammation and neurodegeneration can thus be accounted for and biomarker panels presently hold the best promise for earlier diagnosis and improved prognostics.14 In biomarker panels, an individual marker may not achieve sufficiently high sensitivity and specificity, but combined with other markers may contribute positively to the overall diagnostic and/or prognostic value of the test.15

Historically, the main focus in the pathogenesis of MS has been on autoreactive T cells, but substantial evidence now also implies other cell types (e.g. peripheral monocytes) as prominent cell types early in disease,16 mediating both proinflammatory and anti-inflammatory responses.17,18 In peripheral blood, monocytes are divided into three subsets, each performing different functions by differential expression of antigens and cytokines,19,20 and it has recently been established that the three subsets are a result of a gradual maturation process.21,22 Expression levels of CD14 and CD16 are used to distinguish classical (CD14+CD16−), intermediate (CD14+CD16+) and nonclassical (CD14−CD16+ ) monocyte subsets,23 and similar subsets exist in other species, for example, in mice based on LY6C, CCR2 and CX3CR1 expression.24

In a number of chronic inflammatory diseases such as arthritis,25 coronary heart disease,26 MS,27 psoriasis28 or HIV encephalitis,29 monocytes exhibit characteristics of activation during periods of disease activity. The different pathways of monocyte activation predetermine the immune response in each individual. This may have led to the inconsistencies in attributing which subsets are proinflammatory and which ligands elicit migration toward inflammation.19,30–32 In pathologies involving the CNS, however, it is recognized that specifically CCR1, CCR2 and CCR5 are among the most important receptors for migration.33 These receptors are highly expressed on classical monocytes, but evidence also indicates that intermediate and patrolling nonclassical monocytes are migrating into the CNS and promoting infiltration of other cells as well as tissue destruction.17

We have previously shown that in patients with MS compared with healthy controls, there is a significant increase of nonclassical monocytes with a concomitant reduction of classical monocytes.34 In addition, a significant reduction of several surface receptors was found on the intermediate and nonclassical monocytes, possibly because of increased shedding activity. By contrast, expression of human endogenous retrovirus (HERV) envelope (Env) epitopes, encoded by both HERV-H/F and HERV-W, was localized to the nonclassical monocytes. Expression of HERVs has previously been associated with neurological diseases, notably MS,35 and we have previously demonstrated expression of these HERV epitopes in patients with active MS36,37 as well as an HERV-directed humoral immune response.38–40 In addition to this, our previous studies of monocytes were accompanied by analysis of selected markers of inflammation and neurodegeneration in serum and cerebrospinal fluid (CSF), establishing the potential of combining several biomarkers into a single multivariable model.15,34

To further investigate the role of monocyte subsets as well as HERVs in patients with MS, we here present further analysis of the circulating monocytes and their subsets in newly diagnosed patients with MS. In this study, we compared the newly diagnosed patients with MS to a group of patients presenting with MS-like symptoms but without documented objective neurological findings: symptomatic controls (SCs), enrolled during the diagnostic workup.

Furthermore, as defining biomarkers for MS remains elusive, we have investigated a panel of 17 soluble biomarkers, illustrating essential components in the pathogenesis of MS focusing on inflammation and monocyte/macrophage activity (see Supplementary table 1 for a review of the selected soluble markers).

RESULTS

Patients enrolled in this study were undergoing diagnostic workup for MS and thus largely represent incipient MS. Patients that fulfill some, but not all of the diagnostic criteria for MS are often encountered in the clinic. Here, these patients comprise the SC group if they did not have another defined neurological disease (please refer to the Methods for further details). This is an advantage for the proficiency of the biomarker analysis, as clinically relevant biomarkers ideally should be able to distinguish these patient groups. Initially, 92 patients were enrolled during a period of 2 years (see Supplementary figure 1). Of those, one opted out and two were excluded after subsequent diagnoses of anti-AQP4-positive NMO and
Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation and subjected to flow cytometric analysis to characterize the three distinct monocyte subsets as well as expression of other markers of activation and cell signaling. The gating strategy used for the flow cytometric analyses is shown in Figure 1.

The median levels and range of all the surface markers analyzed are presented in Supplementary table 3, showing that the HERV H3 Env epitopes are expressed at a significantly lower level in patients with MS or CIS than in SC and RIS. Supplementary table 3 also shows the existing overlap in the surface marker levels for the five patient groups. A Kruskal-Wallis test with Dunn’s multiple comparisons test ($\alpha = 0.05$) was used to calculate the significance of differences between groups.

In Supplementary table 4, the variation in cell surface marker expression between MS groups and correlations with clinical disease measures are presented. Overall, no significant differences were seen in surface marker expression between RRMS and PMS, between MS and CIS or between RIS and SC, and we thus further grouped these patients into MS + CIS and SC + RIS. Of particular interest in comparing patients with MS + CIS versus SC + RIS is the significant difference between HERV H3 Env epitopes on the nonclassical monocyte population, making it the only surface marker analyzed that is able to distinguish between these patient groups (Figures 2 and 3). Furthermore, no differences in surface marker expression were found in patients with MS + CIS with regard to gender distribution, oligoclonal band status, progression or medication at follow-up.

Expression of surface markers on the three monocyte subsets is shown in Supplementary figure 2. Interestingly,
the differences in surface marker expression between the monocyte subsets indicate a highly coordinated downregulation or shedding of surface receptors on the intermediate and nonclassical monocytes compared with classical monocytes, except for TACE on the intermediate subset. Mann–Whitney U-tests were used to calculate the significance of differences between surface marker expression on the monocyte subsets.

The analyses of cell surface marker expression on peripheral blood monocytes were accompanied by multiplex measurements of an extensive panel of relevant soluble markers in paired CSF and serum samples from the study cohort. Supplementary tables 5 and 6 illustrate variations in levels of soluble markers in CSF and serum, respectively, between the MS-relevant groups as well as correlations with clinical disease measures. No distinct differences were seen for the soluble markers in CSF between RRMS and PMS, between MS and CIS or between RIS and SC, indicating the suitability of the grouping of patients into MS + CIS and SC + RIS. When comparing these patient groups, however, highly significant differences were seen in CSF for interferon-γ, interleukin (IL)-1β, IL-8, IL-10, tumor necrosis factor alpha (TNFα), IL-7, IL-12/IL-23p40, IL-17A, macrophage inflammatory protein-1β and vascular endothelial growth factor (VEGF), indicating that such biomarkers may be used individually—or preferably in combination—to differentiate patients with MS + CIS from SC + RIS (see...
also Figure 4). As seen from Supplementary table 6, this is not evident for the serum samples, where only serum IL-7 is significantly different between MS + CIS and SC + RIS. In the present study, this illustrates that the levels of biomarkers in serum were not correlated with the inflammatory processes occurring in the CNS, whereas levels of biomarkers in the CSF were highly distinct for the two groups.

Supplementary table 7 shows the median levels and range of the soluble markers analyzed in CSF and serum and the CSF-to-serum ratio. This table illustrates that the biomarker levels differ significantly between the MS
Figure 4. Differences in the soluble marker levels in CSF of patient (CIS, RRMS, PMS) versus control (SC, RIS) samples. The soluble markers were graphed in pg mL$^{-1}$: (a) the markers with a median between 0 and 1 (pg mL$^{-1}$); (b) the markers with a median between 0 and 6 (pg mL$^{-1}$); (c) the markers with a median above 6 (pg mL$^{-1}$). The CSF IgG index = (CSF IgG × serum albumin) × (CSF albumin × serum IgG)$^{-1}$ is included here as the index of local IgG production and it serves as a reference biomarker. Bars represent the median of the population and braces indicate a significant difference (Mann–Whitney U-test) between the median of the patient group (n = 61) and the median of the control group (n = 28). P-values are shown. CIS, clinically isolated syndrome; CSF, cerebrospinal fluid; Env, envelope; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon gamma; Ig, immunoglobulin; IL, interleukin; MCP-1, monocyte chemoattractant protein; MIP-1$\beta$, macrophage inflammatory protein-$\beta$; PMS, progressive multiple sclerosis; Pt, patients; RIS, radiologically isolated syndrome; RRMS, relapsing-remitting multiple sclerosis; SC, symptomatic control; TNF$\alpha$, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.
group and the CS + RIS control group mainly in CSF and CSF-to-serum ratio. Serum IL-2, serum IL-7 and serum IL-12/IL-23p40 do, however, differ significantly between PMS and SC; between RRMS, PMS, RIS and SC and between PMS, RIS and SC, respectively, but only contribute little to the differences in the ratio. The results in Supplementary table 7 also illustrate the existing overlap in the soluble biomarker levels for the five patient groups.

To further investigate the role of the surface and soluble biomarkers, the surface and soluble markers in CSF, serum and their respective ratios were correlated using Spearman’s correlations. Figure 5 shows the rho values of the correlations; both positive (blue) and negative (red) correlations are shown. Correlations with a significant P-value are marked with black frames (see Supplementary table 8 for rho and P-values). Interestingly, several surface markers correlate significantly with the soluble markers and specifically surface-bound CD163 correlates significantly with nine of the soluble markers. Of those, serum IL-7 and CSF VEGF also differ significantly between the patients with MS + CIS and the controls SC + RIS. However, the only surface marker that differs significantly between patients with MS + CIS and SC + RIS is the HERV H3 Env on the nonclassical monocyte population, and HERV H3 Env only correlates significantly with the monocyte chemoattractant protein (MCP)-4 ratio, which does not differ significantly between any of the five patient groups. By contrast, the expression of the receptor CCR-5 correlates significantly with three chemotactic proteins/ligands in serum as well as the proinflammatory INFγ. Similarly, expression of TACE correlates significantly with IL-12/IL-23p40 and TNFα in CSF but not in serum, indicating that there are other sources of these proteins in serum, for example, tissue homing or resident leucocytes at distal sites of inflammation.

To explore the biomarker potential of the surface and soluble markers that are significantly different between patients with MS + CIS and SC + RIS, a logistic regression analysis with receiver operating characteristic curve output was performed. Figure 6 shows the receiver operating characteristic curves for granulocyte-macrophage colony stimulating factor and MCP-1 in CSF as well as their combined contribution to predict disease progression. As seen from the figure, the AUCs for the three graphs are all below 68% and are thus the aforesaid are poor prognostic markers in differentiating patients with MS + CIS that have progressed from those that have not. Similarly, Supplementary figure 6 shows the receiver operating characteristic curves for IL-2 in CSF and serum, IL-12/IL-23p40 in CSF as well as their combined contribution to predict if patients receive medication or not after the diagnosis. As seen from the figure, both IL-2 in serum and IL-12/IL-23p40 in CSF have AUC > 70% and are thus fair markers; however, when combined with IL-2 in CSF, the AUC is 68.8% (95% CI 0.54–0.83) and thus do not contribute positively to a combined AUC. Lastly, the IgG index had an AUC of 57.1% (95% CI 0.42–0.72) for predicting disease progression and 64.5% (95% CI 0.49–0.80) for predicting whether patients with MS + CIS receive medication and did not contribute positively to either of the prognostic assessments.

**DISCUSSION**

Characterizing monocytes and their subsets in incipient nontreated MS facilitates important insight into early mechanisms of disease, and is unaffected by disease progression or modifying treatment. In addition, the inclusion of SCs recruited during the diagnostic workup facilitates the use of any potential, highly specific biomarkers and panels hereof, directly applicable to a clinical setting.

In recent years, many new therapeutics or disease-modifying therapies have been approved for MS therapy, either immunosuppressants or more specific antibodies targeting T and B cells in the periphery. Targeting monocytes or subsets hereof may also have potential in MS therapy as it is indicated in animal models of MS that depletion of monocytes inhibit both initiation of disease and progression.
In the present study, we have analyzed the composition of monocyte subsets and expression of markers of activation and cell signaling in incipient MS, together with a comprehensive panel of inflammation-associated signaling molecules in paired CSF and serum samples. In samples from patients with CIS, RRMS, PMS, RIS or SC variations in the median total number of monocytes were in general low, as were the variation of the different monocyte subsets between these patient groups, except for the nonclassical monocytes where a marked lower median was observed for patients with MS + CIS than for SC + RIS. In our previous study, we noted a marked increase in the nonclassical monocyte subset with a concomitant downregulation of the classical

Figure 5. Correlations between surface and soluble markers of activation and inflammation. Rho values are graphed in a heatmap based on correlations between surface markers and soluble markers in CSF, serum and their respective ratios. Blue indicates positive correlations and red indicates negative correlations. Correlations with a significant P-value are marked with a black frame. CSF, cerebrospinal fluid; GM-CSF, granulocyte-macrophage colony stimulating factor; IFNγ, interferon gamma; IL, interleukin; MCP-1, monocyte chemoattractant protein; MIP-1β, macrophage inflammatory protein-1β; Pt, patients; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.
Figure 6. Logistic regression analyses with receiver operating characteristic curve output of surface and soluble markers. AUC, with 95% CI, is given for each parameter. The surface expression of each parameter for patients \((n = 61)\) is combined as true positives and plotted against controls \((n = 28)\) as true negatives. The diagonal dividing the ROC space represents the random event. In addition, a logistic regression analysis with combined parameter results was performed for “all parameters.” AUC, area under the curve; CSF, cerebrospinal fluid; HERV, human endogenous retrovirus; IFN, interferon; IL, interleukin; ROC, receiver operating characteristic; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

subsets, compared with healthy controls. A detailed comparison, however, revealed a similar pattern of monocyte subsets in the two studies, with a large proportion of patients with MS + CIS having the same number of monocyte subsets as the SC + RIS or healthy controls as well as a number of significant outliers. One striking difference though was the high number of nonclassical monocytes in some of the SC and RIS patients in the current study, emphasizing that SC and RIS are indeed not comparable to healthy controls.

For the surface markers investigated, we found a significantly lower expression of HERV H3 Env epitopes on the nonclassical monocytes in patients with MS + CIS versus SC and MS + CIS versus SC + RIS. In our previous study comprising incipient and progressed patients with MS compared with a healthy control group, the numbers of nonclassical monocytes and the expression levels of HERV Env were higher for the patients with MS. Our present results on monocyte subsets are in line with a recent report which additionally found increased numbers of nonclassical monocytes in the CSF, probably indicating their migration into the CNS. Furthermore, in our previous study one-third of the patients included were treated with immune-modulating therapy, and it is known that nonclassical monocyte populations may increase after treatment initiation.

The HERVs investigated here are associated with a number of neurologic diseases and likely also with the inflammatory processes, and may thus be expressed because of a defective mechanism of chromatin-dependent repression.

Interestingly, in both patients with MS + CIS and SC + RIS, we found a marked downregulation of expression of all the measured surface markers on the nonclassical monocytes compared with intermediate or classical, except for CD40 and TACE, where the intermediate monocytes have higher expression. This pattern of expression is in agreement with our previous study and in the present study, we also show this for CD11b, CD18, CD64, CD86, CCR1 and CCR5. Surprisingly, the expression of CD40 and TACE was
highest in the intermediate monocyte population, indicating that these monocytes indeed have high stimulatory and inflammatory potential.\textsuperscript{51,52}

For the soluble biomarkers investigated in CSF and serum, a highly significant difference was demonstrated between patients with MS + CIS and SC + RIS in 11 of 17 biomarkers measured in CSF. This was also evident in the CSF-to-serum ratios. Levels were significantly higher in MS + CIS samples, except for IL-7, which is a hematopoietic growth factor that promotes generation and survival of lymphoid cells.\textsuperscript{53} In addition, patients with MS + CIS with the highest levels of, for example, INF\textsubscript{y} tended to have high levels of most of the other biomarkers, except granulocyte-macrophage colony stimulating factor and VEGF, for which no differences were seen (data not shown). This illustrates that multifactorial processes of immune regulation are at play in any given time point of MS disease, as most of the biomarkers have relatively short half-lives.\textsuperscript{54} Interestingly, biomarker levels in serum were not directly correlated with the inflammatory process occurring in CNS, and the only significant difference found was for IL-7, which was higher in MS sera compared with SC + RIS. Previously, IL-7 has been shown to be lower in patients with MS, as well as the IL-7 receptor (IL-7R\textsubscript{y}) expression associated with MS pathogenesis,\textsuperscript{55} although the mechanisms are not clear. Furthermore, that study was based on comparisons with healthy controls, which might explain the discrepancy.

In the present correlation analysis, several surface markers were significantly correlated with levels of soluble markers in both CSF and serum as their respective ratios. Notably, surface-bound CD163, which correlated significantly with nine of the soluble markers, is worthy of attention, as we have previously demonstrated a significant association between CD163 and MS.\textsuperscript{15,34} In addition, expression of CCR5 correlated significantly with three proteins for monocyte chemotaxis in serum, and it has been shown that CCR5\textsuperscript{+} mononuclear cells accumulates in the CNS of MS patients.\textsuperscript{56} Finally, expression of TACE correlated significantly with several soluble markers, notably TNF\textsubscript{x} and IL-12/IL-23p40 in CSF but not in serum. This is interesting, as treatment with monoclonal antibodies against TNF\textsubscript{x} in MS has shown increased disease activity,\textsuperscript{57,58} and treatment with neutralizing antibodies toward IL-12/IL-23p40 can be used to treat several autoimmune diseases,\textsuperscript{59} but no effect was seen in MS.\textsuperscript{60} However, further investigations of this last treatment could be indicated in selected patients with high levels of IL-12/IL-23p40 in serum and comorbidity with psoriasis.

In the logistic regression analysis, the HERV H3 Env expression on the nonclassical monocytes as well as several soluble biomarkers performed well in differentiating patients with MS + CIS from HC + RIS, and a combined regression analysis of 11 biomarkers showed excellent performance with an AUC of 98.7%. It is noteworthy that IL-12/IL-23p40 had an excellent performance, even when used alone, with an AUC of 92.4% almost comparable to the IgG index with an AUC of 96.4%.

Notably, when IL-12/IL-23p40 was combined with TNF\textsubscript{x} with an AUC of 85.4%, the combined AUC was 94.7%, but when combined with HERV H3 Env on the nonclassical monocytes with an AUC of 70.1% and the IgG index, the combined AUC was 99.5%, higher than any other combination of the investigated biomarkers. A combination of a surface biomarker and two soluble biomarkers thus performs almost perfectly in differentiating patients with MS + CIS from SC + RIS. However, none of the investigated biomarkers could predict progression, or differentiate between patients with MS + CIS that received immune modulating therapy and not, emphasizing that longer follow-up periods may be needed for some of the patients.

We have previously demonstrated that combinations of specific biomarkers enable pertinent differentiation between patients with MS + CIS and SC\textsuperscript{15} as well as MS + CIS and HC,\textsuperscript{34} illustrating that in a multifactorial diseases such as MS, the combination of multiple biomarkers into a single multivariable model provides a high level of diagnostic discrimination.\textsuperscript{15} Here we demonstrate an AUC of 99.5% with only three biomarkers combined, emphasizing the additional potential of especially IL-12/IL-23p40 as a diagnostic marker in incipient MS.

Taken together, the present results of significant changes in expressed inflammation-related cell surface markers between the three monocyte subsets, as well as the HERV Env epitopes expressed on the nonclassical monocyte populations, are interesting. It substantiates our hypothesis that monocyte subsets recruited to plaque formations in the CNS carrying viral epitopes maybe contribute to the recurrent and/or continued inflammatory activity.\textsuperscript{34} We do also find these HERV epitopes expressed in RIS and SC patients, with the marked difference that these patients likely have intact blood–brain barriers and thus few nonclassical monocytes homing to the CNS.\textsuperscript{61}

Importantly, we also present significant changes in 11 inflammation-related signaling molecules in MS + CIS compared with SC + RIS, illustrating highly coordinated responses in patients with MS + CIS, and that some of these molecules are excellent in differentiating MS-like disease from non-MS.

Overall, the results indicate that clear definition of a multifactorial disease such as MS is only possible using
carefully selected combinations of biomarkers and that development of a small but proficient biomarker panel is possible.

METHODS

Ethics statement

The study was conducted in accordance with the Ethical Declaration of Helsinki and all patients gave written, informed consent. The study and the material for informed consent were approved by The Central Denmark Region Committee on Biomedical Research Ethics (journal number: 1-10-72-202-13).

Patients and controls

Patients admitted to the MS clinic, Department of Neurology, Aarhus University Hospital, were consecutively included from June 2015 to February 2018. A full diagnostic workup included medical history, clinical examination, MRI of the entire neural axis, CSF analyses (cells, protein, IgG index) and evoked potentials as recommended. CSF and MRI examination were evaluated according to the revised MacDonald criteria and an Extended Disability Status Scale score was assessed according to Kurtzke. Patients were excluded if they had other neurologic disease, received glucocorticoids or other immune modulating treatments within the month preceding sampling. One patient was diagnosed with neuromyelitis optica and one with motor neuron disease. This specific definition is described in detail by Teunissen et al. and they do not represent early MS.

Disease activity was ascribed on the basis of a new clinically defined attack or a sustained increase of more than 0.5 on the Extended Disability Status Scale within a follow-up period of 9–41 months (median = 25). Medication at follow-up was ascribed to those receiving any immune-modulating therapy in the follow-up period. In total, 89 patients agreed to participate in this study and in accordance with consensus guidelines, serum and CSF were frozen at −70°C until use, while whole PBMCs were isolated and frozen at −150°C until use.

PBMC isolation

Each collected sample was labeled with a study ID at inclusion and all the analyses were performed blinded to the clinical status of the patients. PBMCs were isolated from heparinized whole blood using Ficoll-Paque PLUS (Amersham Biosciences, Umeå, Sweden, Catalog number 17-1440-02) within a few hours after drawing. In brief, whole blood was mixed with phosphate-buffered saline (PBS) (Sigma, Welwyn Garden City, UK, Catalog number D8537) in 1:1 and 20 mL Ficoll-Paque was layered underneath about 25 mL of the diluted blood. The tubes were centrifuged at 750g for 25 min at room temperature, and PBMCs were collected from the interface layer. Then PBMCs were washed three times with 20 mL PBS and centrifuged at 450g for 15 min, 280g for 10 min and finally at 190g at 10 min, to remove the sugar gradient and impurities (platelets). PBMCs were frozen in Roswell Park Memorial Institute-1640 medium (BioWhittaker, Bornem, Belgium, Catalog number BE12-702F) supplemented with 20% (heat-inactivated and sterile-filtrated) human serum and 10% dimethyl sulfoxide (Sigma, Lyon, France, Catalog number D4540). Before use, each portion of frozen PBMCs was quickly thawed (37°C); washed once in 10 mL ice-cold Roswell Park Memorial Institute-1640 medium containing 10 mM HEPES, 0.03% w v−1 glutamine, 0.2 mio IU L−1 penicillin–streptomycin and 10% (heat-inactivated and sterile-filtrated) human serum and resuspended to a concentration of 40 × 10⁶ cells mL−1.

FLOW analysis

Before staining PBMCs with antibodies, samples were first incubated with the amine-reactive reagent LIVE/DEAD Fixable Near-IR Dead Cell Stain (Life Technologies, Eugene, OR, USA, #L10119), then washed with PBS (pH 7.4) and blocked in PBS + 0.2% human serum albumin (CSL Behring, Marburg, Germany, #109697) + 100 µg mL−1 h-IgG (human serum albumin; Privigen 10%, CSL Behring) + 0.09% NaAz (Ampliqon, Odense, Denmark, #AMPQ52310.0250) for 15 min at 4°C. Samples were then washed two times and labeled by incubating with a mixture of different monoclonal antibodies for 30 min in the dark at 4°C, washed two times in PBS and resuspended in 100 µL PBS + 0.2% human serum albumin + 0.09% NaAz. The following monoclonal mouse antibodies were used to stain 1 × 10⁶ PBMCs in 100 µL PBS: 5 µL anti-CD14 Brilliant Violet 421 (BD, San Jose, CA, USA; clone MoP9, IgG2b, #563743), 10 µL anti-CD16 PC7 (Beckman Coulter, Chaska, MN, USA; clone 3G8, IgG1, #66077118), 10 µL anti-CD18 Per-CP (Abcam, Cambridge, UK; clone GRF1, IgG1
Detection of HERV H3 and HERV W3 expression was performed using polyclonal antisera as previously described. In brief, antibodies against an HERV-H/F Env peptide epitope (HERV H3) and an HERV-W/MSRV Env peptide epitope (HERV W3) were raised in New Zealand White rabbits, and preimmune sera were collected from all rabbits before immunization and used as background staining/control. The peptide epitopes are localized in α-defensin, a defensin homolog.

Flow cytometric analyses were performed using an LSRFortessa (BD) equipped with four lasers [a violet (405 nm), blue (488 nm), yellow (561 nm) and a red (640 nm)]. FlowJo software version 10 (FlowJo LLC, Ashland, OR, USA) was used for data analysis. More than 100 000 events were collected for further analysis. For each surface marker, all of the flow data were collected in one run. To validate the compensation matrices and gating strategy, appropriate fluorescence-minus-one controls were made (not shown).

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Additional supporting information may be found online in the Supporting Information section at the end of the article.