

Chronic progressive HIV-1 infection is associated with elevated levels of myeloid-derived suppressor cells

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Objectives: Myeloid-derived suppressor cells (MDSC) have been described as suppressors of T cell functions in many tumor models. However MDSC in HIV-1 infection have not been studied to date. As impaired T cell function is a hallmark of chronic progressive HIV-1 infection, we hypothesized that MDSC also play a role here.

Methods: Surface staining and FACS analysis were performed on freshly isolated PBMC of HIV-infected individuals and compared to healthy controls and individuals with lung carcinoma. MDSC of late-stage HIV-infected subjects were isolated using magnetic beads and co-cultured with the respective CD8 T cells for evaluation of proliferative capacity.

Results: We found that chronically HIV-infected HAART-naïve individuals had significantly higher CD11b+CD14-CD33+CD15+ MDSC levels than healthy controls ($p=0.01$). MDSC frequencies showed a positive correlation with viral load ($r^2=0.24$, $p=0.0002$) and a negative correlation with CD4 count ($r^2=0.29$, $p<0.0001$). Initiation of HAART led to a rapid drop in MDSC levels. MDSC from HIV-infected progressors restricted the proliferative capacity of CD8 T cells from healthy donors and of Gag/Nef-specific CD8 T cells from HIV-controllers *in vitro*. Furthermore CD11b+CD14-CD33+CD15+ MDSC induced the expansion of CD4+CD25+FoxP3+ regulatory T cells when co-incubated with PBMC from controllers *in vitro*.

Conclusion: We conclude that chronic uncontrolled HIV-infection is associated with elevated levels of MDSC which potentially contribute to the impaired T cell responses characteristic for the progressive disease stage.

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Introduction

In the natural course of HIV infection, viremia is controlled by the majority of infected subjects for a certain time. However loss of control almost always occurs at some point [1]. HIV-specific CD8 T cells are an important component of the adaptive immune response against HIV, yet these cells become ineffective in most chronically infected individuals [2,3]. The reasons for this change in effectiveness are not completely understood so far.

Myeloid-derived suppressor cells (MDSC) accumulate in various malignant diseases and were shown to exert suppressive regulatory functions especially on T cells [4–6]. In the mouse model MDSC are defined by the expression of the markers CD11b and Gr1 [5]. Lacking the marker Gr1, MDSC in humans are less well defined. There are mainly two different phenotypes that have been described in cancer patients: CD11b+CD14-CD33+CD15+ [7,8] and CD14+HLA-DR^{-low} [9,10].

We hypothesized that MDSC also contribute to T cell dysfunction in chronic progressive HIV infection, analogous to what is seen in cancer. We therefore assessed MDSC frequencies in HIV infected subjects of various disease stages in comparison to healthy controls. Additionally we examined MDSC function of HIV infected individuals in vitro.

Methods

Study subjects

97 individuals participated in the study after signing informed consent. The study was approved by the Institutional Review Board of the Ludwig-Maximilians-Universität, Munich. The study subjects were divided into the following groups (supplementary digital content S1): 16 healthy controls; 20 HIV-infected subjects on highly active antiretroviral treatment (HAART) with undetectable viral load (VL; < 50 cp/ml) for at least 2 years; 49 HIV-infected HAART-naïve subjects (CD4 counts: 422/μl, range 16–1237; VL: 11,532 cp/ml, range 59–570,156 cp/ml; two subjects were HCV co-infected), 4 HIV-infected subjects with a failing salvage HAART without further treatment options and 8 subjects with advanced non-small cell lung carcinoma (NSCLC) as positive controls. 7 of the 49 HAART-naïve subjects were reassessed ≥6 weeks after starting HAART. HIV-infected individuals with proven or suspected malignancy based on clinical, biochemical or radiological findings were excluded from the study.

Cell isolation and separation

PBMC were isolated from freshly obtained blood by Ficoll density gradient centrifugation. For isolation of

CD11b+CD14- MDSC, PBMC were purified by a negative selection of CD14+ cells followed by a positive selection of CD11b+ cells using magnetic microbeads (Stemcell Technologies, France) according to manufacturer's instructions. Purity for bead-isolated CD11b+CD14-CD33+CD15+ MDSC was 60–96%. MDSC-depleted PBMC contained 0.7–4.0% MDSC.

Flow cytometry analysis

Extracellular staining with fluorescent antibodies was performed as described previously [11]. The following antibodies were used: CD14-APC, CD11b-FITC, CD33-PE, CD15-PerCP, HLA-DR-PerCP, CD38-PE, CD25-FITC, CD8-PerCP, CD4-APC (Biolegend, Germany), CD38-PE (BD, Germany), IL4Ralpha-PE and isotype control (R&D Systems, Germany). Intracellular staining with anti-FoxP3-PE (Biolegend) was performed using the FoxP3 staining buffer set (eBiosciences, USA) (as described [12]). Cells were collected on a FACSCalibur (BD, Germany). Data was analyzed using FlowJo software (TriStar, USA). For gating strategies, see supplementary digital content S2. As monocyte frequencies vary widely in subjects with a chronic viral infection, we expressed MDSC as percentage of PBMC.

Mixed lymphocyte reaction and proliferation assays

Allogeneic PBMC from healthy controls or from HIV controllers were labeled with CFSE as described [13]. They were then stimulated with PHA (1.25 μg/ml; healthy controls) or a pool of Gag/Nef overlapping peptides (100 μg/ml; HIV controllers) and incubated alone, with bead-isolated MDSC or with study subject's PBMC depleted of MDSC (ratio = 2:1) in RPMI media with 10%FCS (PAA, Austria). After 72–96 hours, cells were stained for CD8-PerCP (Biolegend) and analyzed. For analysis of regulatory T cell frequencies, cells were stained for CD4, CD25 and FoxP3 after co-incubation.

Statistical analysis

GraphPad Prism Version 5 was used to analyze data. Mann-Whitney tests were used to compare groups and linear regression analysis for correlations. Paired t test was used for paired comparisons. A p-value < 0.05 was considered statistically significant.

Results

CD11b+CD14-CD33+CD15+ MDSC are associated with chronic progressive HIV-1 infection

To determine if MDSC might play a role in chronic HIV-1 infection, we compared MDSC frequencies in HIV-infected individuals to those in healthy controls and to subjects with advanced NSCLC. We found that HIV-

infected HAART-naïve individuals had significantly higher CD11b+CD14-CD33+CD15+ MDSC frequencies than healthy controls ($p = 0.01$; Fig. 1a).

We next assessed whether there was an association of MDSC levels with different stages of HIV infection. As MDSC levels of HAART-naïve progressors and subjects with failure of salvage regimen were comparable (Fig. 1d), we combined the two groups for this analysis. Controllers (defined as $VL < 5,000$ cp/ml; HAART-naïve) on average had significantly lower MDSC frequencies than individuals with progressive disease (defined as $VL > 50,000$ cp/ml; $p = 0.0004$; Fig. 1b). Likewise subjects with a CD4 count $> 500/\mu\text{l}$ (HAART-naïve) had significantly less MDSC than individuals with a CD4 count $< 250/\mu\text{l}$ (Fig. 1b; $p = 0.0001$). MDSC levels of individuals with medium viral load (5000 – 50,000 cp/ml) and medium CD4 count (250 – 500/ μl) were not significantly different from those of controllers (Fig. 1b). When looking at all subjects however, there was a statistically significant positive correlation of MDSC frequencies with VL (Fig. 1c; $r^2 = 0.24$, $p = 0.0002$) and a negative correlation with CD4 count (Fig. 1c; $r^2 = 0.29$, $p < 0.0001$). In addition MDSC levels correlated significantly with immune activation, measured as CD38 expression, on bulk CD8 T cells ($r^2 = 0.23$, $p = 0.02$; data not shown) and on HIV-specific CD8 T cells ($r^2 = 0.35$, $p = 0.002$; Fig. 1e).

We then asked the question whether effective HAART affects the MDSC level. We found lower frequencies of MDSC in subjects on HAART with undetectable VL for at least 2 years compared to HAART-naïve individuals with $VL > 5000$ cp/ml (Fig. 1d; $p = 0.004$). Seven of the HAART-naïve individuals could be studied again after initiation of HAART. This analysis showed that HAART led to a rapid drop of MDSC levels (Fig. 1d; $p = 0.003$). There was no difference in MDSC levels between subjects on effective HAART and spontaneous controllers (defined as $VL < 5,000$ cp/ml; HAART-naïve; $p = 0.58$; Fig. 1d).

Taken together we found that CD11b+CD14-CD33+CD15+ MDSC are associated with high viral loads and poor CD4 status in chronic HIV infection.

Another potential phenotype of human MDSC is CD14+HLA-DR^{-low}. Such cells (CD11b+CD14+HLA-DR^{low}CD33+) have recently been described in individuals with chronic HCV infection [14]. We examined levels of CD11b+CD14+HLA-DR^{-low} MDSC in a subgroup of the HAART-naïve group and in the group on effective HAART. We did not find a positive correlation with VL in the HAART-naïve subgroup ($r^2 = 0.0004$, $p = 0.95$; data not shown). Neither was there a statistically significant difference between HAART-naïve subjects and individuals on HAART ($p = 0.64$; data not shown).

CD11b+CD14-CD33+CD15+ MDSC in HIV-infected individuals express IL4Ralpha and impair the proliferative capacity of CD8 T cells of healthy donors

Tumor-derived MDSC with suppressor function have been described to express IL4Ralpha [15]. CD11b+CD14-CD33+CD15+ MDSC of our cohorts also expressed IL4Ralpha suggesting suppressor activity (supplementary digital content S3).

To further characterize MDSC function in progressive HIV-infection, we tested for their suppressive activity on intact CD8 T cells directly ex vivo. Bead-isolated MDSC of 6 different HIV-infected progressors led to significantly reduced proliferation of PHA-stimulated CD8 T cells of healthy donors ($p = 0.004$; Fig. 2b) and Gag/Nef-peptide stimulated CD8 T cells of HIV controllers ($p = 0.004$; Fig. 2c) in co-culture experiments compared to incubation with progressors' MDSC-depleted PBMC. The latter did not significantly alter proliferation compared to the controls where no cells of HIV-infected individuals were added.

MDSC levels correlated highly significantly with the frequency of CD4+CD25+FoxP3+ regulatory T cells (Tregs) in a subgroup of the 49 HAART-naïve individuals ($r^2 = 0.54$, $p < 0.0001$; Fig. 2d) directly ex vivo. This correlation was even stronger than the correlation between Treg levels and viral load ($r^2 = 0.31$, $p = 0.006$; data not shown). Hence we assessed Tregs in co-incubation experiments. Incubation of PBMC of HIV controllers with MDSC of progressors led to significantly increased Treg frequencies compared to incubation of those cells with MDSC-depleted PBMC of progressors ($p = 0.0008$; Fig. 2e).

CD11b+CD14-CD33+CD15+ MDSC of HIV infected individuals are of suppressive function shown by the expression of IL4Ralpha and the reduction of proliferative capacity of bulk and HIV-specific CD8 T cells. Induction of Treg frequencies is therefore proposed to be one mechanism by which MDSC exert their suppressive function.

Discussion

MDSC have been described in various malignant diseases and sepsis as inhibitory regulators of T cells [6,16]. Here we show for the first time that CD11b+CD14-CD33+CD15+ MDSC can be found to be elevated in progressive HIV infection and also proof their suppressive function on CD8 T lymphocytes.

As comparison we used individuals with advanced NSCLC. NSCLC and renal cell carcinoma are those malignancies where CD11b+CD14-CD33+CD15+

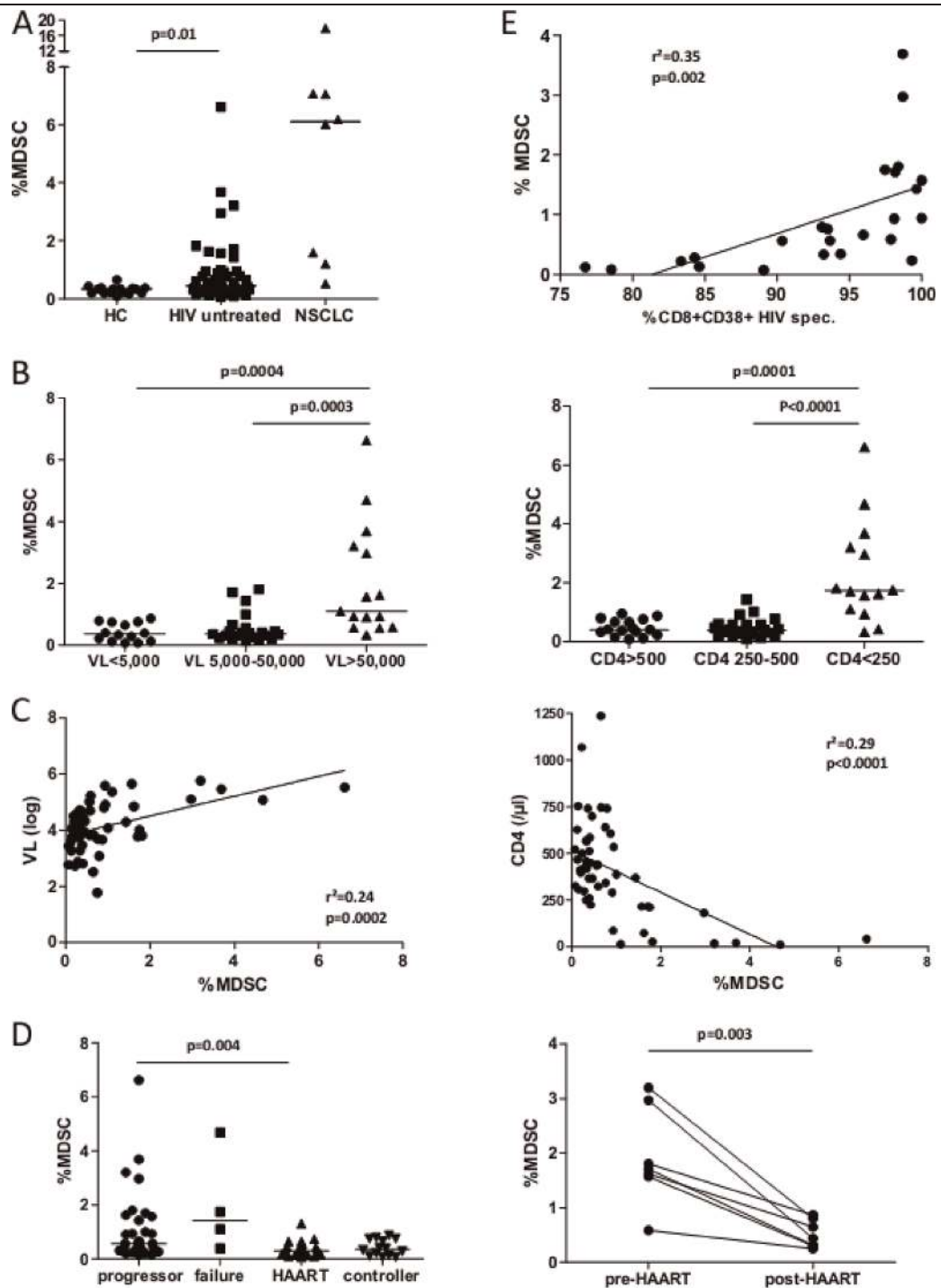


Fig. 1. CD11b+CD14-CD33+CD15+ MDSC frequencies in chronic HIV infection. (a) Comparison between healthy controls (HC), HIV-infected HAART-naïve individuals (HIV untreated) ($p=0.01$; Mann-Whitney-Test) and individuals with non-small cell lung carcinoma (NSCLC). (b) Left: Comparison between low (<5000 cp/ml), medium (5000 – 50,000 cp/ml) and high (>50,000 cp/ml) viral load of HAART-naïve subjects and individuals with failing salvage regimen ($p=0.0004$ and $p=0.0003$ respectively; Mann-Whitney-Test). Right: Comparison between high (>500/ μ l), medium (250 – 500/ μ l) and low (<250/ μ l) CD4 counts ($p=0.0001$ and $p<0.0001$ respectively; Mann-Whitney-Test). (c) Correlations between MDSC levels and VL ($r^2=0.24$, $p=0.0002$; linear regression; left) and CD4 count ($r^2=0.29$, $p<0.0001$; linear regression; right). (d) Left: Comparison between HIV-infected HAART-naïve individuals (=progressor, VL>5000 cp/ml), subjects with failing salvage regimen (=failure), subjects on effective HAART (=HAART) and spontaneous controllers (=controllers, VL < 5000 cp/ml). $p=0.004$ for group 1 and 3, Mann-Whitney-Test. Right: Intra-individual comparison before HAART start and ≥ 6 weeks after effective HAART ($p=0.003$, paired t test). (e) Correlation between HIV-specific CD8+CD38+ T cell frequencies and MDSC levels ($r^2=0.35$, $p=0.002$; linear regression).

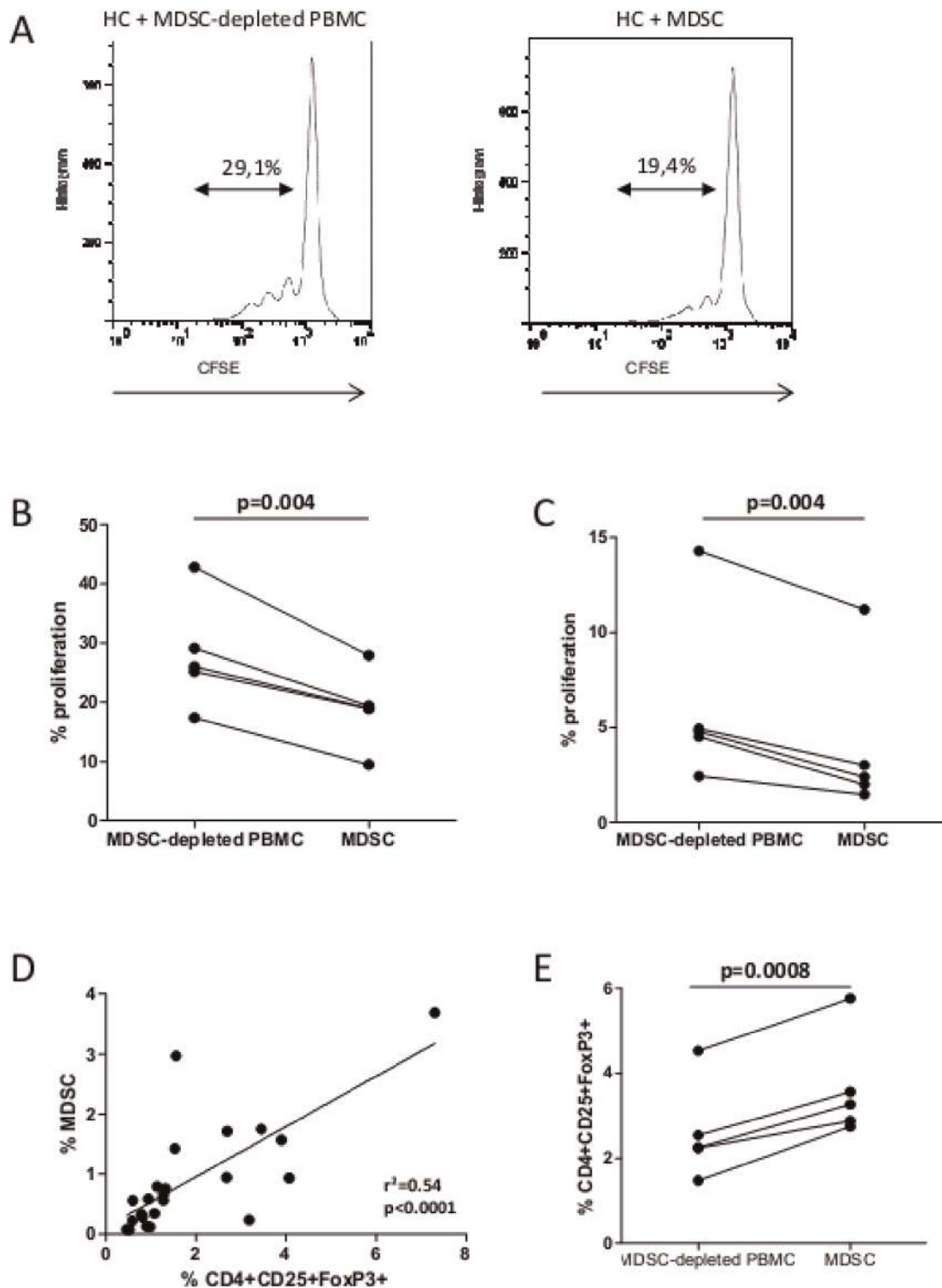


Fig. 2. Proliferative capacity of CD8 T cells and regulatory T cell frequencies. (a) Representative histograms of CFSE proliferation assays of PHA-stimulated CD8 T cells of healthy control (HC). MDSC-depleted PBMC = PBMC of HIV-infected progressor depleted of MDSC. MDSC = bead-isolated MDSC of HIV-infected progressor. (b) Proliferation of PHA-stimulated CD8 T cells of healthy controls incubated with MDSC-depleted PBMC or MDSC of HIV-progressors ($p = 0.004$, paired t test). (c) Proliferation of Gag/Nef-stimulated CD8 T cells of HIV-controllers incubated with MDSC-depleted PBMC or MDSC of progressors ($p = 0.004$, paired t test). (d) Correlation between CD4+CD25+FoxP3+ regulatory T cell frequencies and MDSC levels ($r^2 = 0.54$, $p < 0.0001$; linear regression). (e) CD4+CD25+FoxP3+ regulatory T cell frequencies in PBMC of controller after incubation with MDSC-depleted MDSC or MDSC of HIV progressor ($p = 0.0008$; paired t test).

MDSC have been characterized in most detail [7,8]. The median level of MDSC in NSCLC was higher than in untreated HIV infection not selected for disease status. Comparing NSCLC patients with HIV-infected progressors with very advanced disease status, the difference becomes less apparent. Disease status seems to be an important factor for the existence of MDSC. This is also reflected in the correlation of MDSC levels with well defined clinical markers of HIV infection – viral load and CD4 count.

Initiation of HAART resulted in a prompt reduction of MDSC frequencies. In addition subjects on effective HAART had MDSC levels comparable to HIV-controllers and healthy controls. Therefore HIV viremia seems to be a direct inducer of MDSC. This and other possible mechanisms by which these cells are induced should be the goal of future studies.

Recently it has been shown that hepatitis C virus is an inducer of MDSC with a different phenotype [14]. We were not able to find differences between various disease stages in HIV infection for this phenotype. However in the report by Tacke et al., PBMC of only 5 subjects with HCV infection were studied directly *ex vivo* – as we did with our subjects. Clinical data, e.g. viral load, for these subjects were not mentioned. A more detailed analysis with the same method is necessary to finally compare the two findings.

When testing for the suppressive capacity of MDSC, proliferation of the target cell is often used as read-out. Loss of proliferative capacity of T cells has been described as an effect of human MDSC in hepatocellular carcinoma [10] and renal cell carcinoma [8] and of murine MDSC in mouse tumor models [17]. In addition, the expression of IL4Ralpha has been associated with suppressive function of MDSC in mice [18] and humans [15]. Therefore we assume that the MDSC population isolated from HIV progressors is of suppressive function. As one mechanism by which MDSC exert their suppressive function, the induction of regulatory T cells has been proposed [10]. We confirmed this for HIV-derived MDSC in co-incubation experiments. The relevance of this finding is also stressed by the observation that the correlation between Tregs and MDSC was stronger than the correlation between Tregs and viral load. Nevertheless we expect additional mechanisms of action to be used by MDSC in HIV infection which should be assessed in future studies.

In summary this is the first report of an association of elevated CD11b+CD14-CD33+CD15+ MDSC levels with chronic progressive HIV-1 infection. As impaired CD8 T cell function is a hallmark of progressive HIV infection, reducing MDSC production or function could potentially become an adjunct therapeutic approach in this disease stage.

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Conflicts of interest

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