Granulocytic Myeloid–Derived Suppressor Cells Increased in Early Phases of Primary HIV Infection Depending on TRAIL Plasma Level

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Background: It has been demonstrated that myeloid-derived suppressor cells (MDSC) are expanded in HIV-1–infected individuals and correlated with disease progression. The phase of HIV infection during which MDSC expansion occurs, and the mechanisms that regulate this expansion remain to be established. In this study, we evaluated the frequency of MDSC in patients during primary HIV infection (PHI) and factors involved in MDSC control.

Methods: Patients with PHI and chronic HIV infection (CHI) were enrolled. PHI staging was performed according to Fiebig classification, and circulating MDSC frequency and function were evaluated by flow cytometry. Cytokine levels were evaluated by Luminex technology.

Results: We found that granulocytic MDSC (Gr-MDSC) frequency was higher in patients with PHI compared with healthy donors, but lower than that in patients with CHI. Interestingly, Gr-MDSC expansion was observed in the early phases of HIV infection (Fiebig II/III), but it was not associated with HIV viral load and CD4 T-cell count. Interestingly, in PHI, Gr-MDSC frequency was inversely correlated with plasmatic level of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), although a direct correlation was observed in CHI. Furthermore, lower level of Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) was observed in PHI compared with that in CHI. In vitro experiments demonstrated that, differently from CHI, recombinant TRAIL–induced apoptosis of Gr-MDSC from PHI, an effect that can be abrogated by GM-CSF.

Conclusion: We found that Gr-MDSC are expanded early during PHI and may be regulated by TRAIL and GM-CSF levels. These findings shed light on the fine mechanisms regulating the immune system during HIV infection and open new perspectives for immune-based strategies.

Key Words: HIV, primary infection, MDSC, immune suppression, Fiebig classification, TRAIL

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INTRODUCTION

HIV-1 infection causes profound immune suppression leading to progressive destruction of the immune system in patients who are untreated. Recently, a subset of myeloid cells, known as myeloid-derived suppressor cells (MDSC), was found to be able to negatively regulate immune functions.[1–4]

MDSC are a heterogenous group of immature and highly differentiated elements belonging to the myeloid lineage, undergoing expansion during pathologic conditions and are characterized by a strong immune suppressive capability.[3,5–7] In humans, the identification of MDSC is not easy because of the lack of specific markers. Moreover, at present, a consensus regarding the combination of markers used to identify MDSC is still lacking. However, in purified peripheral blood mononuclear cells (PBMC), MDSC are identified as HLA-DR$^{-/low}$/CD11b^{++}/CD33^{+}/CD124^{+} and can be divided into 2 main subsets: monocytic MDSC (M-MDSC) and granulocytic MDSC (Gr-MDSC).[8] The monocytic subset contains CD14^{+} cells, whereas the granulocytic subset contains CD14^{-}/CD15^{+} cells.[8] Another MDSC subset HLA-DR$^{-/low}$/CD11b^{+}/CD33^{+}/CD15^{+}/CD16^{-} has been characterized as immature MDSC or promyelocytic MDSC that differs from Gr-MDSC for morphological features and the expression of CD16.[9,10] Suppressive functions are mediated through combination of several major molecular pathways, including inducible nitric oxide synthetase (iNOS), arginase-1 (Arg-1), nicotinamide adenine dinucleotide phosphate oxidase (NOX2), and Transforming growth factor beta.[11–13] MDSC were initially described as bone marrow suppressor cells in mice with metastatic lung tumors,[14] and there has been

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increasing interest in their role in cancer immune regulation,[15] autoimmune disorders,[16] and some infectious diseases.[17–19]

Recently, we and others showed that MDSC are expanded in HIV-1–infected individuals and their frequency correlates with disease progression.[20–22] Although the immunosuppressive ability of MDSC may be beneficial in curbing the damaging effects of persistent immune activation associated with disease progression, a deleterious effect of MDSC has been shown as they also inhibit HIV-1–specific CD8 T-cell response. To date, the phase of HIV infection during which MDSC expansion occurs remains to be established, and no data are available regarding MDSC during primary HIV infection (PHI).

In this study, we evaluated the frequency and the suppressive function of MDSC in patients during the acute phase of HIV infection and investigated the possible mechanism driving their expansion.

METHODS

Study Population

Patients with PHI (n = 51) were enrolled at the National Institute for Infectious Diseases (INMI) "Lazzaro Spallanzani" (Rome, Italy). Patients with chronic HIV infection (CHI, n = 26) and healthy donors (HD, n = 16) were included as controls. All HIV-positive individuals were naive to antiretroviral therapy. Patients with PHI were grouped according to Fiebig classification[23] as follows: II/III, IV, V, and VI. Patients with Fiebig stage VI were defined as patients sampled within 1 year from seroconversion. Patients with CHI were defined as patients sampled after more than 1 year from seroconversion. General characteristics are summarized in Table 1. The procedures followed were in accordance with the ethical standards of the Helsinki Declaration. The study was approved by the Institutional Review Board of the National Institute for Infectious Diseases (INMI) "Lazzaro Spallanzani" (ALPHA and SIREA studies), and signed written informed consent was obtained from all patients.

PBMC Separation and Flow Cytometry

PBMC were isolated from peripheral blood by density-gradient centrifugation (Lympholyte-H; Cederlane). After separation, PBMC were resuspended in RPMI 1640 (EuroClone) supplemented with 10% heat-inactivated fetal bovine serum (EuroClone), 2 mmol/L L-glutamine, 10 mmol/L HEPES buffer (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid), 2 mmol/L penicillin, and 50 μg/mL streptomycin (EuroClone). Evaluation of MDSC percentage was accomplished with 0.5×10^6 PBMC stained with anti-Cluster of Differentiation (CD) 15, anti-CD33, anti-Human Leucocyte Antigen-DR (HLA-DR), anti-CD3, anti-CD56, anti-CD19, anti-CD14, anti-CD11b, anti-CD16 (BD Biosciences), and anti-CD124 monoclonal antibodies (mAbs) (R&D system). CD38 evaluation on T cells was performed using anti-CD3, anti-CD8, and anti-CD38 mAbs (BD Biosciences). Intracellular flow cytometry was performed by using anti-CD3, anti-CD8, and anti-Interferon (IFN)-γ mAbs (BD Biosciences). Acquisition of 100,000 events was performed in the leukocyte-gated population on FACS CANTO II and analyzed with FACS DIVA software (BD Biosciences).

Gr-MDSC Purification and Cell Cultures

Gr-MDSC purification was performed using magnetic selection. Briefly, PBMC from PHI were stained with anti-CD15 FITC–conjugated mAb (BD Biosciences). After washing, cells were labeled with anti-FITC microbeads according to manufacturer's instructions (Miltenyi Biotec). The purity of sorted Gr-MDSC was >95%, as verified by flow cytometry (data not shown). To evaluate MDSC immune suppressive ability, Carboxy Fluorescein Diacetate Succimidyl Ester (CFDA-SE)–labeled PBMC from HD were cultured with purified Gr-MDSC at 1:1 ratio and stimulated with anti-CD3 (0.1 μg/mL, eBiosciences) and anti-CD28 (1 μg/mL, BD Biosciences). Cells were maintained at 37°C in humidified air with 5% CO_2. After 5 days, lymphocyte proliferation was evaluated by flow cytometry. HIV-specific CD8 T-cell response was obtained by stimulating PBMC with a pool of Gag-, Nef-, and Tat-derived peptide (1 μg/mL, National Institutes of Health) for 18 hours. The production of IFN-γ was evaluated by flow cytometry. Gr-MDSC apoptosis was evaluated by flow cytometry analyzing Annexin V-positive cells (Annexin V-FITC Apoptosis Detection kit, Bender MedSysytems). Briefly, cells were treated with recombinant tumor necrosis factor–related apoptosis-inducing ligand (r-TRAIL 100 ng/mL, Gibco, Life technology) and with Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) where indicated (100 ng/mL, Peprotech). The control condition was performed by adding water with 0.1% bovine serum albumin (the buffer recommended by the manufacturer to reconstitute r-TRAIL). A not-treated condition was also used

TABLE 1. Patient Characteristics

		PHI				
	HD	Fiebig II/III	Fiebig IV	Fiebig V	Fiebig VI	CHI
No. individuals	16	13	11	10	17	26
Age in years, median (IQR)	42 (31–48)	38 (32–42)	39 (32–45)	33 (28–39)	26 (23–36)	36 (31–42)
Male sex, %	8 (50)	12 (92.3)	10 (90.9)	10 (100)	16 (94.1)	21 (80.8)
CD4 T cells/μL, median (IQR)	na	536 (342–659)	557 (405–663)	762 (532–1036)	648 (522–841)	450 (149–644)
Plasma HIV RNA copies/mL (Log), median (IQR)	na	6.1 (5.7–7)	5.7 (4.9–6.5)	5.1 (4.3–6)	4.4 (3.7–5.2)	4.8 (4.6–5.3)

na, not applicable; IQR, interquartile range.

(data not shown) to confirm that water with 0.1% bovine serum albumin did not affect MDSC survival in vitro. After 18 hours, the percentage of Annexin V-positive Gr-MDSC was analyzed.

Plasma Cytokine Levels

Plasma samples were obtained after speed centrifugation for 10 minutes at 2000 rpm and immediately aliquoted and stored at −80°C. Plasma samples were assayed using the multiplex bead–based assays Bio-Plex Pro Human group I 19 plex [interleukin (IL)-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, granulocyte colony stimulating factor, GM-CSF, Interferon (IFN)-γ, Monocyte Chemoattractant Protein 1 (MCP-1), macrophage inflammatory protein 1-β, Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), and tumor necrosis factor-α] and Bio-Plex Pro Human group II cytokines (21 plex, BioRad Laboratories). Plates were measured using the Bio-Plex MagPix System and analyzed with the Bio-Plex Manager version 6.0 (BioRad Laboratories).

Statistical Analysis

GraphPad Prism version 4.00 for Windows (GraphPad Software) was used to perform statistical analyses. The nonparametric Mann–Whitney/Wilcoxon tests were used to compare continuous variables. Correlations were evaluated with the nonparametric Spearman test. A P value <0.05 was considered statistically significant.

RESULTS

Gr-MDSC Expansion During PHI

We compared MDSC levels during PHI, CHI, and in HD by flow cytometry. MDSC were identified as CD3- CD19- CD56- (Lin^-) HLA-$DR^{low/-}$ $CD11b^+$ $CD33^+$ (Fig. 1A); monocytic and granulocytic subsets were evaluated by testing the expression of CD14 and CD15, respectively. The expression of IL-4 receptor (CD124) was also analyzed. Gr-MDSC (Lin^- HLA-$DR^{low/-}$ $CD11b^+$ $CD33^+$ $CD14^-$ $CD15^+$ $CD124^+$) frequency was higher in patients with PHI compared with HD (Fig. 1B), but an even higher level of Gr-MDSC was found in patients with CHI (Fig. 1B). To evaluate whether the identified Gr-MDSC were immature/promyelocitic MDSC, we tested the expression of CD16. We found that Gr-MDSC expressed CD16 (Fig. 1A) indicating that the identified subset was not immature/promyelocitic MDSC. In contrast, no expansion of the M-MDSC subset (Lin^- HLA-$DR^{low/-}$ $CD11b^+$ $CD33^+$ $CD14^+$ $CD15^-$ $CD124^+$) was observed in all patient groups (Fig. 1A).

It has been described that MDSC expansion correlates with CD38 expression on CD8 T cells from HIV+ patients.[20,21] We did not find any correlation between the frequency of $CD38^+$ $CD8^+$ T cells and Gr-MDSC in both patients with PHI and CHI (data not shown), suggesting that in PHI, Gr-MDSC expansion is not driven by CD8 T-cell activation. Interestingly, no correlation was observed between Gr-MDSC frequency and HIV viral load or $CD4^+$ T-cell count in both PHI and CHI (data not shown).

Until now, data on the kinetics of MDSC expansion during PHI are lacking. To explore this issue, we grouped patients with PHI according to Fiebig stages, and the MDSC level was evaluated. A higher frequency of Gr-MDSC was observed in Fiebig stages II/III compared with HD (Fig. 1C), indicating that MDSC plays a role soon after infection. Furthermore, the Gr-MDSC level was stably high in all the other stages but in stage V, where it was comparable to HD (Fig. 1C). No significant differences in MDSC frequency were detected among Fiebig stages.

We evaluated whether Gr-MDSC from PHI are able to suppress T-cell function. We found that, when cultured with purified Gr-MDSC, PBMC from HD decreased their proliferation capability in response to anti-CD3/CD28 (Fig. 2A), indicating the suppressive function of these cells. Moreover, in PHI, we found an inverse correlation between Gr-MDSC frequency and the capacity of CD8 T cells to produce IFN-γ when stimulated with HIV peptides (Figs. 2B, C), confirming that this population is able to modulate T-cell function.

Plasmatic TRAIL Correlates With Gr-MDSC Frequency

To evaluate the possible role of soluble mediators in modulating Gr-MDSC frequency during HIV infection, the level of 40 different cytokines and growth factors was analyzed in plasma samples from PHI and CHI. Interestingly, a significant correlation between tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) and Gr-MDSC frequency was found, both in PHI and in CHI (Table 2, Supplemental Digital Content, http://links.lww.com/QAI/A964). Unexpectedly, this correlation was inverse in PHI and direct in CHI. Moreover, in CHI, the Gr-MDSC frequency was positively correlated with several mediators [GM-CSF, IL-3, IL-2Rα, IL-12p40, IL-16, Chemokine (C-C motif) ligand 27 (CCL27), IFN-α, and MCP-1; Table 2, Supplemental Digital Content, http://links.lww.com/QAI/A964], suggesting a possible contribution of inflammation in driving Gr-MDSC expansion during the chronic phase of HIV infection.

We then questioned whether TRAIL, GM-CSF, IL-3, IL-2Rα, IL-12p40, IL-16, CCL27, IFN-α, and MCP-1 levels were differently expressed in PHI and CHI. We found that TRAIL was higher in PHI and CHI than that in HD, but no difference between PHI and CHI was observed (Fig. 3A). Moreover, TRAIL was highly expressed in the plasma of patients with PHI in the early phases of infection (Fiebig II/III and IV) compared with that of HD, and no difference was detected among Fiebig stages (Fig. 3B).

We also found that the unique cytokine differently expressed in PHI and CHI was GM-CSF, which was higher in CHI than in PHI (Fig. 3C), while the other factors were comparable (Table 2, Supplemental Digital Content, http://links.lww.com/QAI/A964).

GM-CSF Makes Gr-MDSC From PHI Resistant to TRAIL-Induced Apoptosis

TRAIL is a member of the tumor necrosis factor superfamily, closely related to Fas ligand, and it is able to induce apoptosis in several cell types,[24] including MDSC.[25] It has been clearly demonstrated that GM-CSF is a pivotal factor

in the modulation of MDSC frequency.[8] Thus, we wondered whether GM-CSF may explain the opposite correlation between TRAIL and Gr-MDSC frequency in PHI and CHI by modulating Gr-MDSC apoptosis. To this aim, we cultured PBMC from PHI and CHI with r-TRAIL and the induction of apoptosis of Gr-MDSC was evaluated by the expression of Annexin V. As previously described,[25] a high level of Gr-MDSC cell death (Annexin V+) was observed after in vitro culture. However, treatment with r-TRAIL was able to increase the expression of Annexin V on the membrane of Gr-MDSC from PHI but not from CHI (Fig. 4A, B), suggesting that TRAIL differently affects Gr-MDSC from PHI and CHI. When we treated PBMC from PHI and CHI with GM-CSF and r-TRAIL, the TRAIL-induced apoptosis was abrogated in PHI (Fig. 4A and B). We could not observe significant effects on Gr-MDSC from CHI. However, GM-CSF in vitro treatment decreased the basal Annexin V expression on Gr-MDSC from both PHI and CHI (even if in CHI the difference was not statistically significant).

DISCUSSION

With the increasing number of HIV-1 studies focusing on HIV-1–associated persistent immune activation on the course of HIV disease, the role of immunoregulatory cell populations becomes more and more important. The immunosuppressive ability of MDSC may be beneficial in curbing the damaging effects of persistent immune activation and consequent systemic inflammation associated with chronic HIV-1 infection. However, the results from previous studies showed also a possible deleterious effect of MDSC, as they inhibit HIV-1–induced proliferation of CD8 T cells. To our

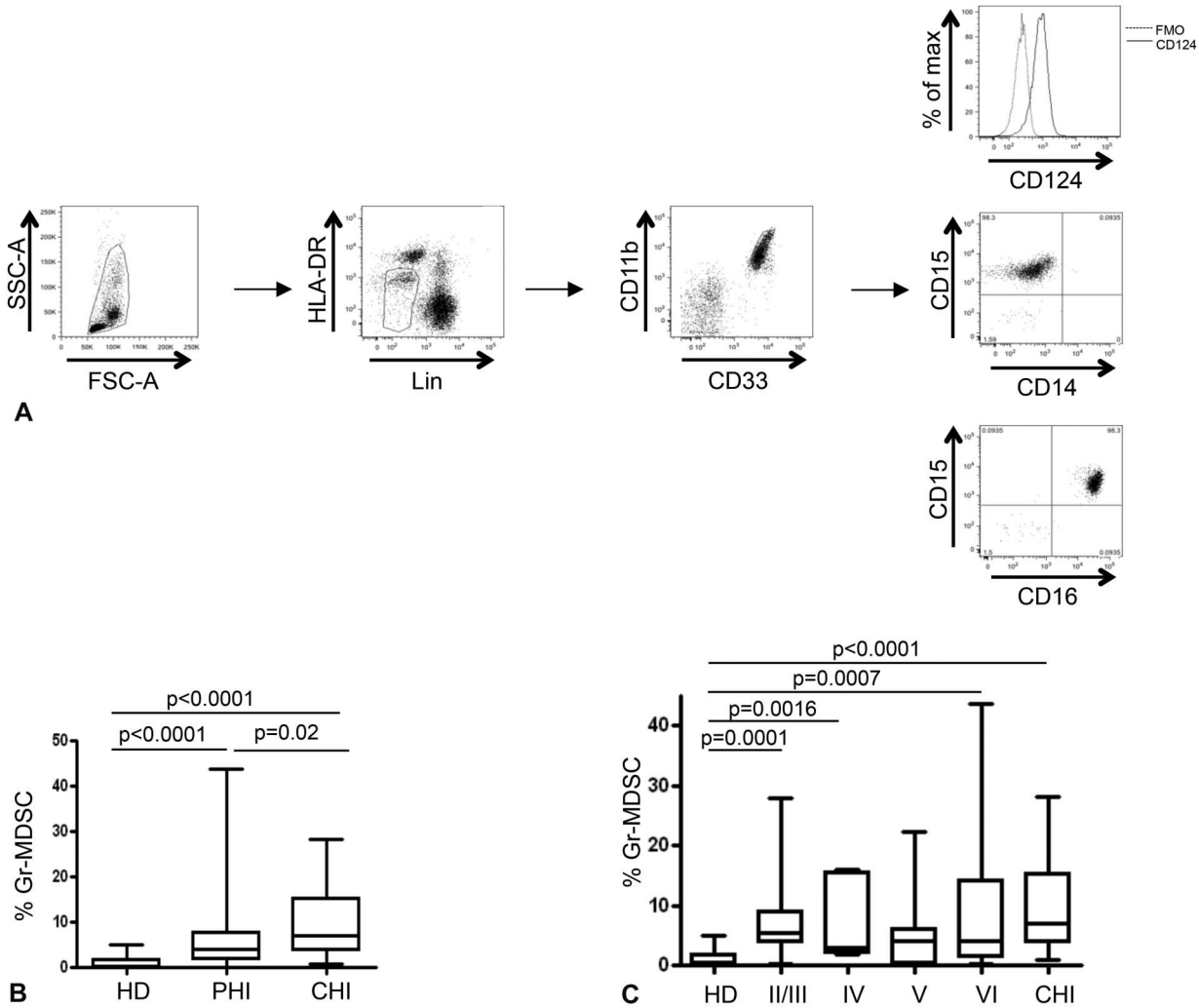

FIGURE 1. MDSC frequency during primary HIV infection. A, Gating strategy used to identify MDSC: in the morphological gate (FSC/SSC) we excluded debris, then we gated Lin$^-$/HLA-DR$^{low/-}$ cells. In this gate, we selected CD11b$^+$CD33$^+$ cells (MDSC). The expression of CD14, CD15, CD16, and CD124 is shown on cells selected from the CD11b$^+$CD33$^+$ gate. The expression of CD124, with its correspondent fluorescence minus one control (FMO), is shown as histogram. B, Gr-MDSC frequency in HD (16), PHI (51), and CHI (26). C, Gr-MDSC frequency in PHI grouped after the Fiebig classification, and CHI. Results are shown as box and whiskers. The Mann–Whitney test was applied, and $P < 0.05$ was considered significant.

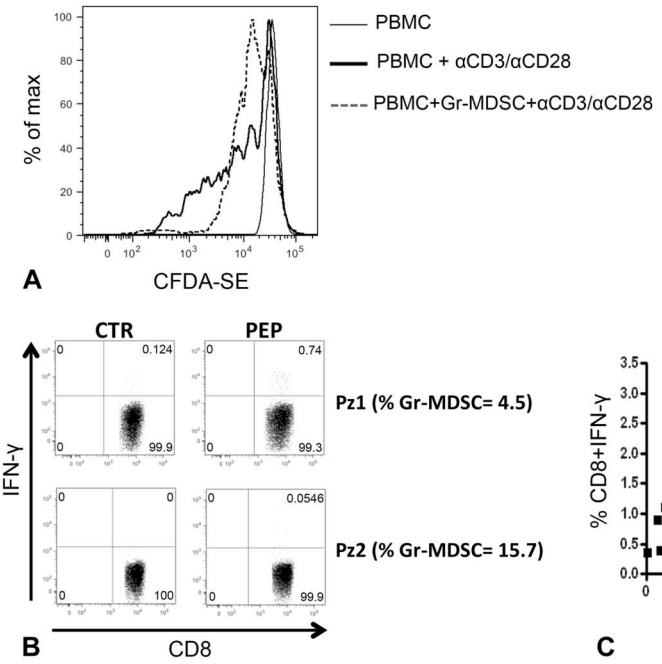

FIGURE 2. Gr-MDSC from PHI inhibits T-cell activation. A, Proliferation rate of stimulated PBMC from HD in the presence of Gr-MDSC from PHI, evaluated by flow cytometry. Representative histogram of 3 independent experiments is shown. B, IFN-γ production by CD8 T cells stimulated with HIV-specific peptides from 2 representative PHI. C, Correlation between Gr-MDSC frequency and the percentage of CD8 T cells producing IFN-γ (evaluated by flow cytometry) from 16 PHI was evaluated by the Spearman test. The $P < 0.05$ was considered statistically significant.

knowledge, no information about the kinetics of MDSC expansion from acute to CHI is available. The present work demonstrates for the first time that Gr-MDSC are already expanded during PHI compared with healthy donors and have suppressive activity, suggesting that this cell dynamics occurs very early during HIV infection. In particular, Gr-MDSC expansion was already observed in the first weeks after infection (Fiebig stages II/III) and remained high over time.

To date, the driving force for Gr-MDSC expansion during HIV infection has not been elucidated. In our study, we did not find any correlation between Gr-MDSC and HIV viral load during the acute phases of infection, suggesting that HIV particles may not directly be involved in expanding MDSC. Furthermore, we could not find correlations between Gr-MDSC frequency and T-cell activation or inflammatory factors, indicating that immune activation is not the major

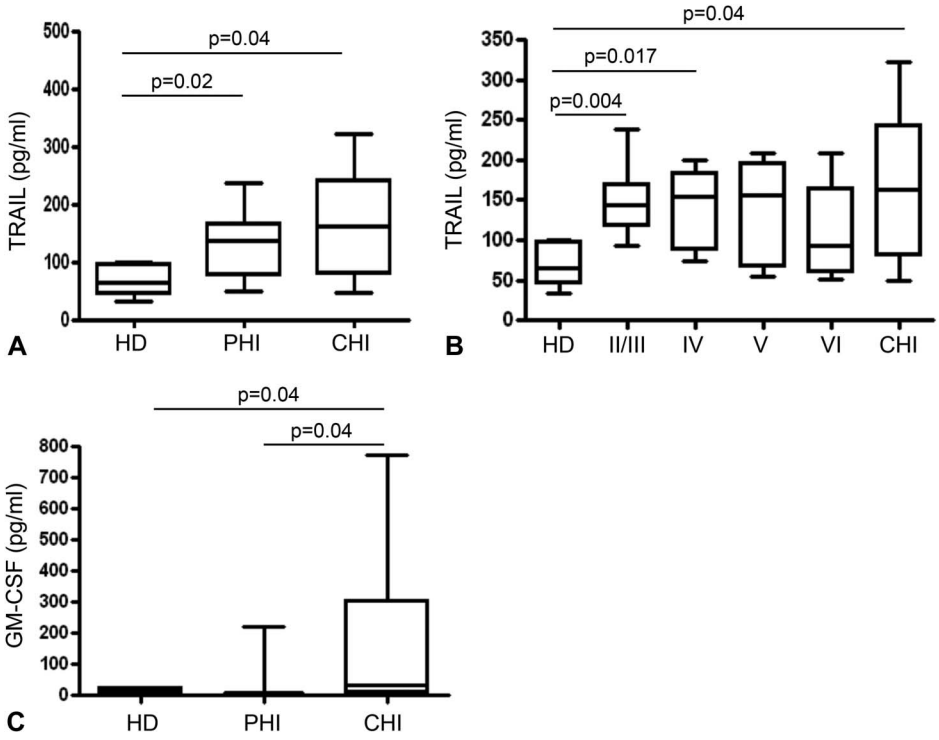

FIGURE 3. TRAIL and GM-CSF plasma level. A, Comparison of TRAIL plasma level in HD (10), PHI (32), and CHI (13). B, Evaluation of TRAIL plasma level in 10 HD, 32 PHI grouped in Fiebig II/III (9), Fiebig IV (6), Fiebig V (7), Fiebig VI (10), and CHI (13). C, GM-CSF plasma level (pg/mL) in HD (10), PHI (32), and CHI (13). Results are shown as box and whiskers. The Mann–Whitney test was applied, and $P < 0.05$ was considered significant.

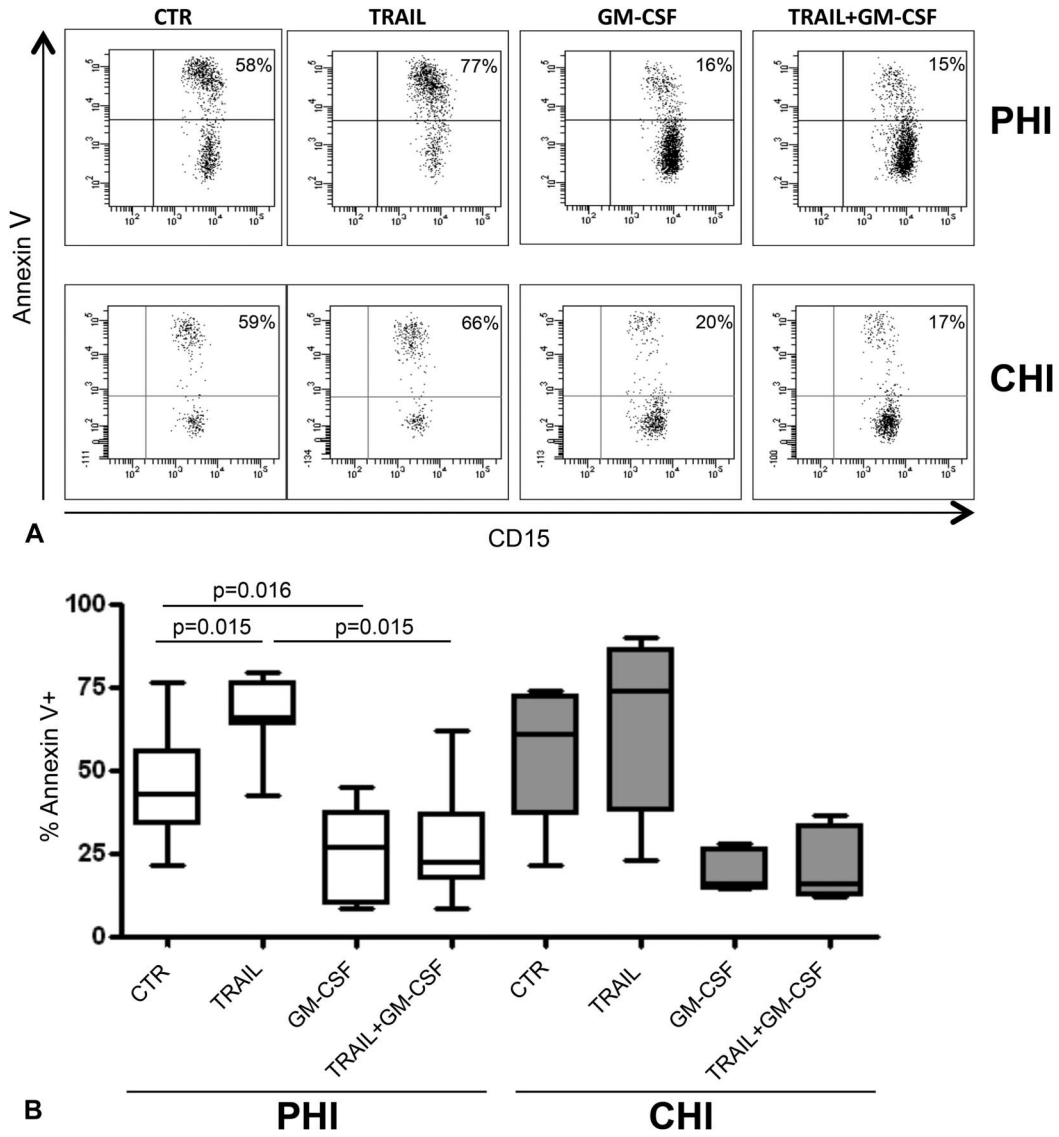

FIGURE 4. GM-CSF and TRAIL effects on Gr-MDSC viability. Percentage of Gr-MDSC from PHI and CHI expressing Annexin V after in vitro treatment with r-TRAIL and GM-CSF was evaluated by flow cytometry. A, Representative dot plots showing Annexin V expression on gated Gr-MDSC (gating strategies in Fig. 1) from one patient with PHI and one patient with CHI. B, Annexin V expression on Gr-MDSC from 8 PHI (2 Fiebig II/III, 1 Fiebig IV, 2 Fiebig V, and 3 Fiebig VI) and 8 CHI. Results are shown as box and whiskers. The Wilcoxon test was applied, and $P < 0.05$ was considered significant. CTR, control condition.

player in regulating MDSC frequency. Differently, in CHI, the immune activation probably contributes to maintaining MDSC. Although Gr-MDSC frequency is not correlated with the expression of CD38 on T cells, the association with soluble IL-2R alpha (clearly associated with T-cell activation in patients infected with HIV[26]) suggests a correlation with T-cell activation. The correlations with the other inflammatory factors also suggest that the activation of different cell types of the innate and adaptive immune system may play a role in modulating Gr-MDSC in CHI.

Whether MDSC may have different roles during acute and CHI has not yet been explored. It has been suggested that MDSC functions may depend on the context in which they expand: Brudecki et al[27] showed, in a murine model of sepsis, that MDSC can enhance or suppress severe inflammation in early and late sepsis, respectively. As such, it is important to further define the multifactorial immunoregulatory network in HIV-1 infection. As stated above, our data indicate that, unlike CHI, Gr-MDSC frequency from PHI is not associated with proinflammatory or anti-inflammatory plasma cytokines; however, they are inversely correlated with TRAIL level. Interestingly, a direct correlation was found in CHI. These data suggest a different role of TRAIL during acute and chronic infection: in PHI, TRAIL is able to induce apoptosis in Gr-MDSC, whereas in CHI it is not, probably because of the presence of other cytokines contributing to MDSC survival. In particular, GM-CSF present in CHI and not in

PHI may play a role in this context. In fact, it has been demonstrated that MDSC expansion is regulated by several soluble factors such as GM-CSF, granulocyte colony stimulating factor, macrophage colony-stimulating factor, IL-6, and others.[28] Herein, our in vitro experiments suggest that GM-CSF treatment of Gr-MDSC from PHI makes these cells resistant to spontaneous and TRAIL-induced apoptosis. The effect is less significant on Gr-MDSC from CHI probably because of the presence of GM-CSF in vivo. The role of recombinant TRAIL on Gr-MDSC survival was clearly demonstrated[25]; however, contrary to the prevailing view that "death receptor" engagement is invariably proapoptotic, accumulated evidence suggests that death receptors frequently initiate potent prosurvival signals, some of which are cytokine/chemokine mediated (reviewed in[29]). Because of the opposite correlations between Gr-MDSC and TRAIL level in PHI and CHI, we may speculate that during acute HIV infection, when GM-CSF is very low, the expanded Gr-MDSC are susceptible to TRAIL-induced apoptosis. On the contrary, during CHI, the presence of GM-CSF not only counteracts the proapoptotic function of TRAIL but also transforms it into a survival factor for Gr-MDSC. This hypothesis is in agreement with the data showing a higher frequency of Gr-MDSC in CHI than that in PHI, and the direct correlation between TRAIL and Gr-MDSC in CHI.

As we did not find any correlation between MDSC frequency and viral load, or $CD4^+$ T-cell count, it would be very interesting to understand whether MDSC expansion may revert after successful antiretroviral therapy, possibly by modulating cytokines levels.

The identification of specific pathways able to induce Gr-MDSC, or interactions between these immunoregulatory cells and other components of the immune system, is pivotal not only to understand the actual role of MDSC during HIV infection but also to design highly effective HIV-1 treatment and immune-based strategies. In fact, it has been demonstrated that simian immunodeficiency virus vaccine–induced MDSC inhibit protective cellular immunity in a nonhuman primate model.[30] Moreover, the frequency of MDSC is markedly elevated in patients infected with HIV-1 receiving a dendritic cell–based HIV-1 vaccine.[31] It has been demonstrated that MDSC are able to crosstalk with different cell types of innate immune system, such as dendritic cells, macrophages, and natural killer cells,[32–34] which play a central role in controlling microbes and in activating adaptive immune response. To understand whether MDSC are involved in the deregulation of these cells during HIV infection is mandatory.

In conclusion, we found that Gr-MDSC are expanded during the very first stages of PHI, indicating that this cell population may play a relevant role in modulating the immune response soon after virus acquisition. Importantly, TRAIL may play a role in controlling Gr-MDSC expansion. Further studies are mandatory to elucidate the mechanisms modulating frequency and functions of MDSC during the diverse phases of HIV infection with the aim to identify future immune-based treatment and vaccination strategies.

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