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GRANULOCYTIC MYELOID DERIVED SUPPRESSOR CELLS IN PRIMARY HIV INFECTION

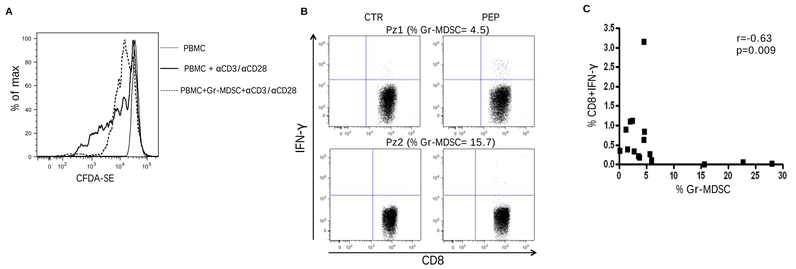
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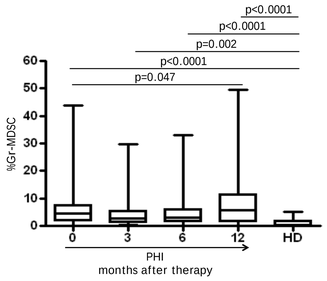
Background It has been demonstrated that Myeloid Derived Suppressor Cells (MDSC) are expanded in HIV-1 chronically infected individuals. The phase of HIV infection during which MDSC expansion occurs, and the mechanisms that regulate this expansion remain to be established. In this study we evaluated the frequency of MDSC in patients during primary HIV infection, and factors involved in MDSC control.
Methods Patients with primary (PHI, n=51) and chronic (CHI, n=26) HIV infection were enrolled before and one year after therapy initiation. At baseline, median CD4 T cell count was 588 cells/mm^3 (IQR 470-830) in PHI, and 499 cells/mm3 (IQR 148-644) in CHI. Median viral load was 5.31 Log RNA copies/ml (IQR 4.33-6.16) in PHI and 4.75 Log RNA copies/ml (IQR 4.44-5.34) in CHI. Circulating MDSC frequency and function were evaluated by flow cytometry. Cytokine levels were evaluated by Luminex technology.
Results We found that, before therapy granulocytic MDSC (Gr-MDSC) frequency was higher in PHI compared to healthy donors ($p<0.0001$), but lower than CHI ($p=0.02$). Interestingly, Gr-MDSC expansion was already observed in the early phases of HIV infection (Fiebig II/III), but it was not associated to HIV viral load and CD4 T cell count. As previously demonstrated in CHI, Gr-MDSC from PHI inhibit HIV-specific T cell response, suggesting a detrimental role of Gr-MDSC. Interestingly, in PHI Gr-MDSC frequency was inversely correlated with plasmatic level of TRAIL ($r= -0.52$, $p= 0.003$), while a direct correlation was observed in CHI ($r= 0.6$, $p= 0.029$). Further, lower level of GM-CSF was observed in PHI compared with CHI ($p= 0.04$). 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.
Antiretroviral therapy was not able to affect Gr-MDSC frequency in both PHI and CHI. However, in PHI an inverse correlation between Gr-MDSC frequency and the number of CD4 T cells was observed ($r= -0.39$, $p= 0.029$), suggesting that Gr-MDSC persistence, after treatment of acute infection, could be associated with a worst CD4 T cell recovery.
Conclusion We found that suppressive Gr-MDSC are expanded very early during PHI, and correlate with CD4 T cell count after therapy. TRAIL and GM-CSF may play a role in regulating Gr-MDSC expansion, thus opening new perspectives for immune-based strategies.

Figure 2. Gr-MDSC frequency inversely correlates with T cell function



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 from HIV+ patients to produce IFN-γ upon stimulation with HIV peptides (Fig. 2B and C), confirming that this population is able to modulate T cell function.

Figure 5. cART was not able to normalize Gr-MDSC frequency



We asked whether cART is able to decrease MDSC frequency to level comparable to HD. To this aim we evaluated MDSC frequency after 1, 3, 6, and 12 months after therapy initiation. We found that cART was not capable to decrease MDSC; rather, after 12 months we observed a slight significant increase of MDSC frequency (Fig. 5). These data indicte that other factors than viral load influence MDSC expansion.

Methods

Patients with primary HIV infection (PHI, n=51) were enrolled at the National Institute for Infectious Diseases (INMI) "Lazzaro Spallanzani" (Rome, Italy). Chronic HIV infected patients (CHI, n=26) and healthy individuals (HD, n=16) were included as controls. PHI patients were grouped according to Fiebig classification as follows: II/III, IV, V, VI.
Fiebig stage VI patients were defined as patients sampled whitin 1 year from seroconversion. CHI patients were defined as sampled after more than 1 year from seroconversion. General patients characteristics are summarized in Table 1.
MDSC percentage and IFNγ production were evaluated by flow cytometry. Gr-MDSC purification was performed using magnetic selection. CFDA-SE labeled PBMC from HD were cultured with purified Gr-MDSC at 1:1 ratio and stimulated with anti-CD3 (0.1μg/ml, eBiosciences) and anti-CD28 (1μg/ml, BD Biosciences). After 5 days, lymphocytes proliferation was evaluated by flow cytometry. HIV-specific CD8 T cell response was performed by stimulating PBMC from HIV+ patients with a pool of Gag, Nef and Tat derived peptide (1μg/ml, NIH) for 18 hours.
Gr-MDSC apoptosis was evaluated analyzing Annexin V positive cells (Annexin V-FITC Apoptosis Detection kit, Bender MedSysytems) by flow cytometry . Plasma samples were assayed by Luminex technology using the multiplex bead-based assays Bio-Plex (BioRad Laboratories). Plates were measured using the Bio-Plex MagPix System and analyzed with the Bio-Plex Manager version 6.0 (BioRad Laboratories).
GraphPad Prism version 4.00 for Windows (GraphPad Software) was used to perform statistical analyses. The non-parametric Mann–Whitney/Wilcoxon tests were used to compare continuous variables. Correlations were evaluated with the non-parametric Spearman test. A p value <0.05 was considered statistically significant.

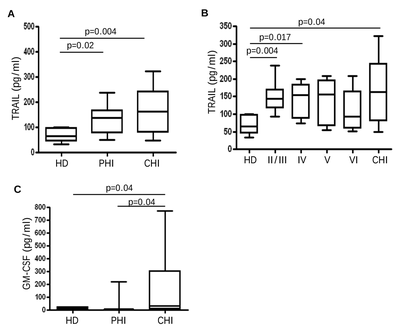
Table 1. Patients characteristics

	HD	PHI				CHI
		Fiebig II/III	Fiebig IV	Fiebig V	Fiebig VI	
Number of 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 gender (%)	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)

Figure 3. Plasmatic TRAIL correlates with Gr-MDSC frequency

Table 2. Statistical analysis of plasmatic cytokines

	Gr-MDSC frequency correlations (Spearman test)		Cytokines levels (Median and IQR) *p<0.05 PHI vs CHI	
	PHI	CHI	PHI	CHI
TRAIL	**r=-0.52 p=0.003**	**r=0.604 p=0.029**	138.8 78.69 - 169.2	158.1 71.3 - 215.6
IL2Ralpha	p>0.05	**p=0.567 p=0.043**	204.8 151.4 - 295.2	196.2 165.2 - 310.9
IL3	p>0.05	**r=0.630 p=0.021**	246.9 186.6 - 357.4	272.2 186.6 - 316.3
IL12p40	p>0.05	**r=0.634 p=0.02**	843.6 638.5-1212	768.4 66.6 - 1049
IL16	p>0.05	**r=0.703 p=0.007**	680.6 454.9 - 954.4	591.3 392.4 - 763.7
CCL27	p>0.05	**r=0.709 p=0.007**	400.7 322- 575.6	466.3 309.5 - 684.0
IFN-α	p>0.05	**r=0.595 p=0.032**	64.0 52.1 - 74.9	67.3 61.9 - 79.1
GM-CSF	p>0.05	**r=0.46 p=0.043**	**7.7* 7.7-7.7**	**32.46 * 7.7 - 304.7**
MCP1	p>0.05	**r=0.691 p=0.009**	35.72 14.9-121.3	120.3 6.6 - 254.8

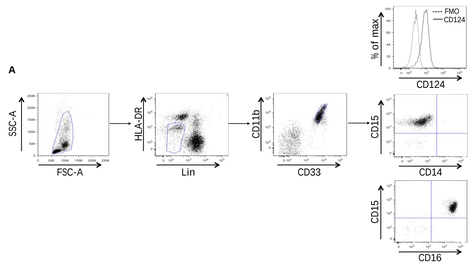


A significant correlation between TRAIL and Gr-MDSC frequency was found, both in PHI and in CHI (Table 2). Unexpectedly, this correlation was inverse in PHI and direct in CHI. Moreover, in CHI the Gr-MDSC frequency was positively correlated with several mediators (GM-CSF, IL-3, IL-2Rα, IL-12p40, IL-16, CCL27, IFN-α and MCP1, Table 2). We found that TRAIL was higher in PHI and CHI compared to HD, but no difference between PHI and CHI was observed (Fig. 3A). Moreover, TRAIL was highly express in the plasma of PHI patients in the early phases of infection (Fiebig II/III, and IV) compared to 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).

Figure 6. After cART MDSC inversely correlates with CD4 T cell count

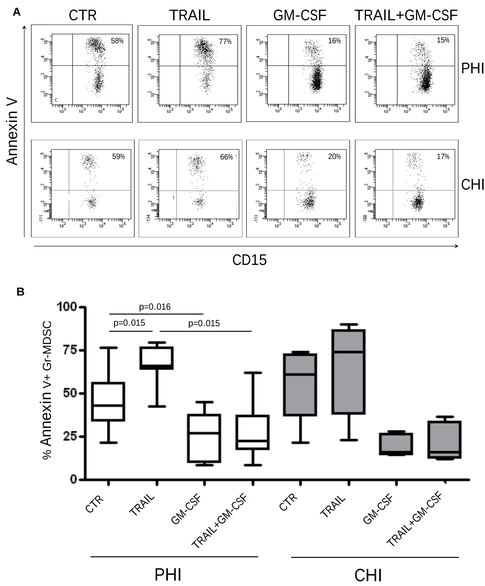
Since MDSC remain high during cART, and an up regulation was observed after 12 months of therapy, we evaluated whether there is a correlation between MDSC frequency and CD4 T cell count after 12 months after therapy. We found an inverse correlation between Gr-MDSC frequency and CD4 T cell count (Fig. 6), suggesting that a worst CD4 T cell reconstitution may be associated with high frequency of MDSC.

Figure 1. Gr-MDSC expand early during primary HIV infection



MDSC were identified as Lin$^-$ HLA-DR$^{low/-}$ CD11b$^+$ CD33$^+$ (Fig. 1A). Gr-MDSC (Lin- HLA-DRlow/- CD11b+ CD33+ CD14- CD15+ CD124+) frequency was higher in PHI patients compared to HD (Fig. 1B), but an even higher level of Gr-MDSC was found in CHI patients (Fig. 1B). We also found that Gr-MDSC expressed CD16 (Fig. 1A) indicating that the identified subset was not immature/promyelocitic MDSC, but Gr-MDSC. We grouped PHI patients according to Fiebig stages and MDSC level was evaluated. A higher frequency of Gr-MDSC was observed in Fiebig stages II/III compared to HD (Fig. 1C), indicating that MDSC play a role soon after infection. Further, Gr-MDSC level was stably high in all the other stages but stage V, where it was comparable to HD (Fig. 1C). No significant differences in MDSC frequency were detected among Fiebig stages.

Figure 4. Role of GM-CSF and TRAIL on MDSC viability



TRAIL is a member of the TNF superfamily, closely related to Fas ligand, and it is able to induce apoptosis in several cell types, including MDSC. Since it has been demonstrated that GM-CSF is a pivotal in modulating MDSC frequency, we wondered if GM-CSF may explain the opposite correlation between TRAIL and Gr-MDSC frequency in PHI and CHI by modulating Gr-MDSC apoptosis.
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 and 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.

Conclusion

In conclusion, we found that Gr-MDSC are expanded during the very first stages of primary HIV infection, 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. Finally, cART was not able to decrease MDSC, suggesting that although HIV suppression, other factors maintain MDSC expansion. Further studies are mandatory in order 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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