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BRIEF COMMUNICATION

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Expression of TIGIT, PD-1 and HLA-DR/CD38 markers on CD8-T cells of children and adolescents infected with HIV and uninfected controls

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ABSTRACT

Immune exhaustion and senescence are scarcely studied in HIV-pediatric patients. We studied the circulatory CD8 T cells activation/exhaustion and senescent phenotype of children and adolescents vertically infected with HIV or uninfected controls based on the expression of human leukocyte antigen (HLA-DR), CD38, T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT), programmed death 1 (PD-1) and CD57 by flow cytometry, during approximately one year. Eleven HIV-infected (HI) and nine HIV-uninfected (HU) children/adolescents who received two doses or one dose of meningococcal C conjugate vaccine (MenC), respectively, were involved in this study. Blood samples were collected before the immunization (T0), 1–2 months after the first dose (T1), and 1–2 months after the second dose (T2), which was administered approximately one year after the first one. HI patients not receiving combined antiretroviral therapy (cART) showed a higher frequency of CD8 T cells TIGIT$^+$, PD-1$^+$ or CD57$^+$, as well as a higher frequency of CD8 T cells co-expressing CD38/HLA-DR/TIGIT or CD38/HLA-DR/PD-1 when compared to HI treated or HU individuals, at all times that they were assessed. CD8 T cells co-expressing CD38/DR/TIGIT were inversely correlated with the CD4/CD8 ratio but positively associated with viral load. The co-expression of CD38/DR/TIGIT or CD38/DR/PD-1 on CD8 T cells was also inversely associated with the CD4 T cells expressing co-stimulatory molecules CD127/CD28. The results showed a higher expression of exhaustion/senescence markers on CD8 T cells of untreated HI children/adolescents and its correlations with viral load.

KEYWORDS: HIV-1 infection. CD8 T cells. Immune exhaustion. Senescence. Children.

INTRODUCTION

Human immunodeficiency virus (HIV) infection induces persistent activation of the immune system, leading to its exhaustion, even in the context of viral replication successfully suppressed by combined antiretroviral therapy (cART)[1].

Phenotypically, the exhausted T cells show up-regulation of inhibitory receptors such as programmed death 1 (PD-1), T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT), and T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3)[2]. T cell senescence is another dysfunctional phenotype in HIV-infected patients, characterized by increased expression of CD57 and reduced expression of CD28 associated with loss of proliferative

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capacity[3]. The main feature of this chronic T cell exhaustion and/or senescence phenotype is the impairment of effector functions. Response to vaccines may also be affected[4]. CD8 T cells have many functions to restrain HIV replication, such as cytokine production and cytotoxicity[5].

Tailor et al. reported that untreated HIV perinatally-infected children have a higher frequency of CD8 memory T cells expressing PD-1 than treated or unexposed children, which was reduced after initiation of antiretroviral therapy (ART). The co-expression of other inhibitory receptors on PD-1$^+$ CD8 T cells predicted poor HIV-specific proliferative response[6].

CD57$^+$CD8 T cells fail to proliferate in response to HIV antigens[7]. Higher frequency of effectors memory CD57$^+$CD8 T cells or TIGIT$^+$CD8 T cells was observed in ART-treated patients when compared to healthy controls[7,8]. These data suggest an association between CD57 and/or TIGIT expression with inflammation and a trend of terminal differentiation of memory CD8-T cells in HIV patients.

Immune exhaustion and senescence are scarcely studied in pediatric patients. In this study, we took advantage of a cohort of HIV-infected and non-infected children and adolescents vaccinated with *Neisseria meningitidis* C conjugate vaccine (MenC)[9], to study CD8 T cell activation/exhaustion and the senescent phenotype at different times during a follow-up of approximately one year.

MATERIALS AND METHODS

Study population and blood collection

This study is part of an original investigation approved by Instituto de Puericultura e Pediatria Martagao Gesteira, Universidade Federal do Rio de Janeiro (IPPMG/UFRJ), with Institutional Review Board (IRB Nº 24/09), and the Comissao Nacional de Etica em Pesquisa (CONEP), under Nº 15578. Details of the study design, including ethical guidelines, have been published[9,10].

For this work, we selected eleven HIV-infected (HI) six on cART – and nine HIV-uninfected (HU) children and adolescents based on the availability of stored peripheral mononuclear blood cells (PBMC). The median age of HU individuals was 8.6 (5.3–10.7) years old, and for HI on cART (HI/cART) and HI without cART (HI/no cART) it was 11.4 (8.3–17.2) and 15 (9.3–19) years old, respectively. Among HU individuals, 88.9% were male. In HI/cART and HI/no cART groups, 67% and 40% of participants were male, respectively. At baseline, CD4 count was 958 (523–1,267) and 525 (479–628) cells/μL, nadir CD4 was 13% (1–34) and 22% (16–26), and HIV RNA was <50 and 17,492 (301–65,701) copies/mL for HI/cART and HI/no-cART group, respectively, as previously described[11]. The median length of cART was 5.9 years (5.4–11.9). All HI patients were infected perinatally. The HU volunteers consisted of children/adolescents assisted at IPPMG/UFRJ.

Blood samples were collected before vaccination (T0) and 1–2 months after the first dose (T1) for HI and HU groups. The HI group received a booster dose about one year after the first dose, and blood samples were collected 1–2 months later (T2) (Figure 1A)[9]. PBMC were obtained by density-gradient centrifugation over Histopaque (Sigma, St. Louis, MO, USA) and stored in RPMI medium/20% fetal bovine serum/10% DMSO in liquid nitrogen. Immunological and virological data were obtained from the medical records[10].

Flow cytometry assay

Flow cytometry was performed at the LIM/60, Laboratorio de Imunologia Clinica e Alergia, Universidade de Sao Paulo, Sao Paulo State, Brazil. Briefly, frozen PBMC were thawed in a 37 ºC water bath and cells were counted ($1x10^6$/ tube) and incubated with the following conjugated monoclonal antibodies: (I) Invitrogen (Carlsbad, California, USA): CD3 phycoerythrin (PE)-Texas Red; (II) BD Biosciences Pharmingen (San Diego, California, USA): CD8 Brilliant violet (BV) 605, CD38 allophycocyanin (APC); (III) Biolegend (San Diego, CA, USA): HLA-DR Alexa fluor 700, CD127 PE-Cy5, PD-1 APC-Cy7, CD28 BV 785, CD57 PerCP-Cy5.5; (IV) eBioscience (Carlsbad, CA, USA): TIGIT. Live dead amine aqua dye (Invitrogen, Carlsbad, CA, USA) was used to exclude dead cells. The analyses were performed using FlowJo software (version 10, TreeStar Inc., Ashland, USA), and a representative strategy used to analyze the T cell populations is shown in Figure 2.

Statistical analysis

Statistical analyses were performed using GraphPad-Prism (version 8.0, GraphPad Software, San Diego, CA, USA). The significance was calculated using the non-parametric Mann–Whitney's test. Analyses of correlations were performed using Spearman's rank test. $p < 0.05$ was considered significant.

RESULTS

We have recently described a clear association between circulating exhausted CD4$^+$ T cells with poor meningococcal C conjugate vaccine antibody response in the same HI cohort of this study[11]. Our interest in this

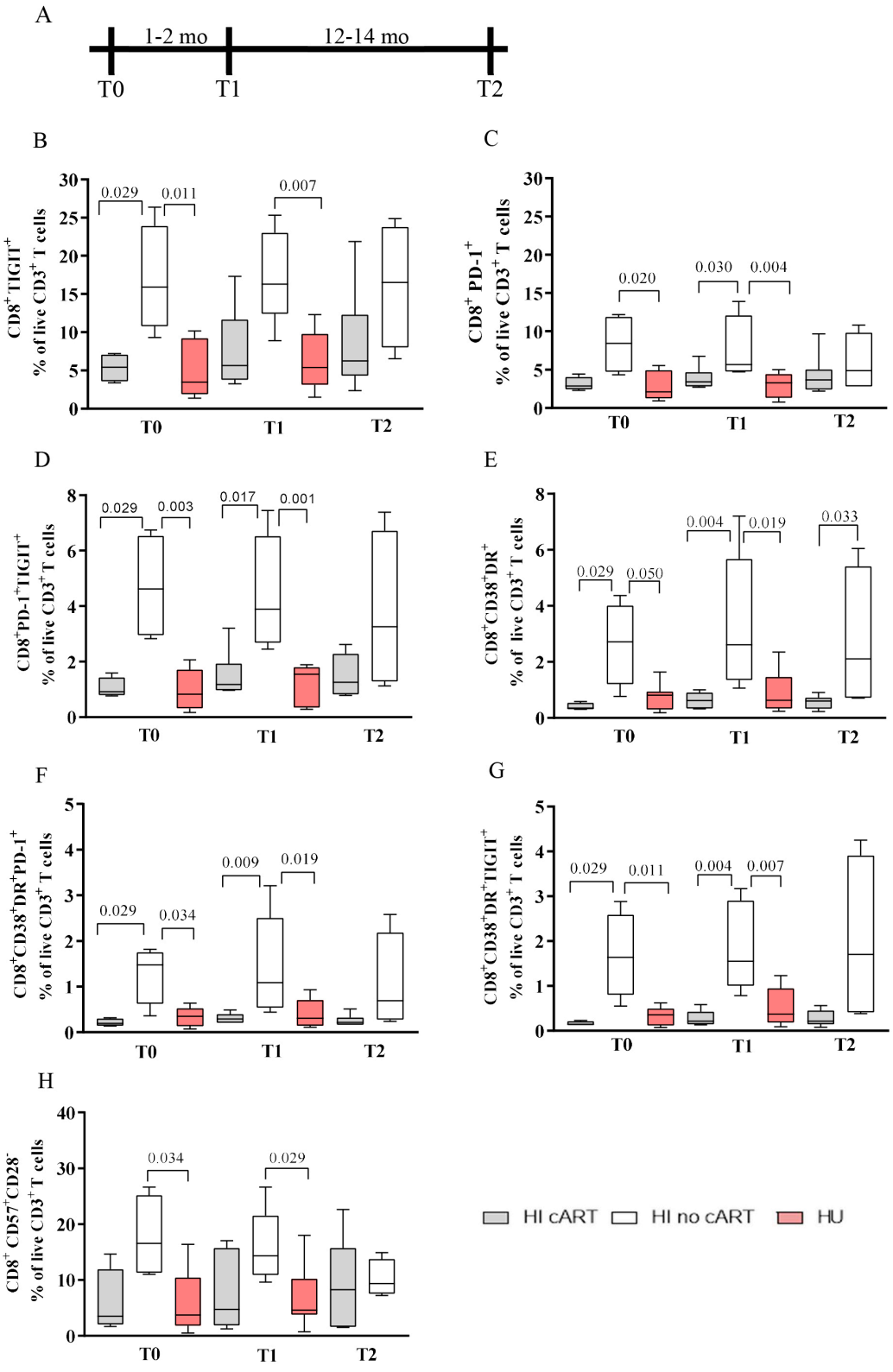

Figure 1 - Increased expression of activation, exhaustion, and senescent markers on CD8-T cells from HIV-untreated patients. Blood samples were collected before immunization (T0), 1–2 months after the first dose (T1), and 1–2 months after a booster dose of MenC (T2) (A). Frequency of $CD8^+$ T cells expressing (B) TIGIT, (C) PD-1, (D) co-expressing PD-1-TIGIT, (E) CD38-DR, (F) CD38-DR-PD-1, (G) CD38-DR-TIGIT, (H) $CD57^+CD28^-$ at different time-points. P-values were calculated using the Mann–Whitney's test. $p < 0.05$ was taken as significant. cART = combined antiretroviral therapy; HI = HIV infected; HU = HIV uninfected; MenC = meningococcal C conjugate vaccine.

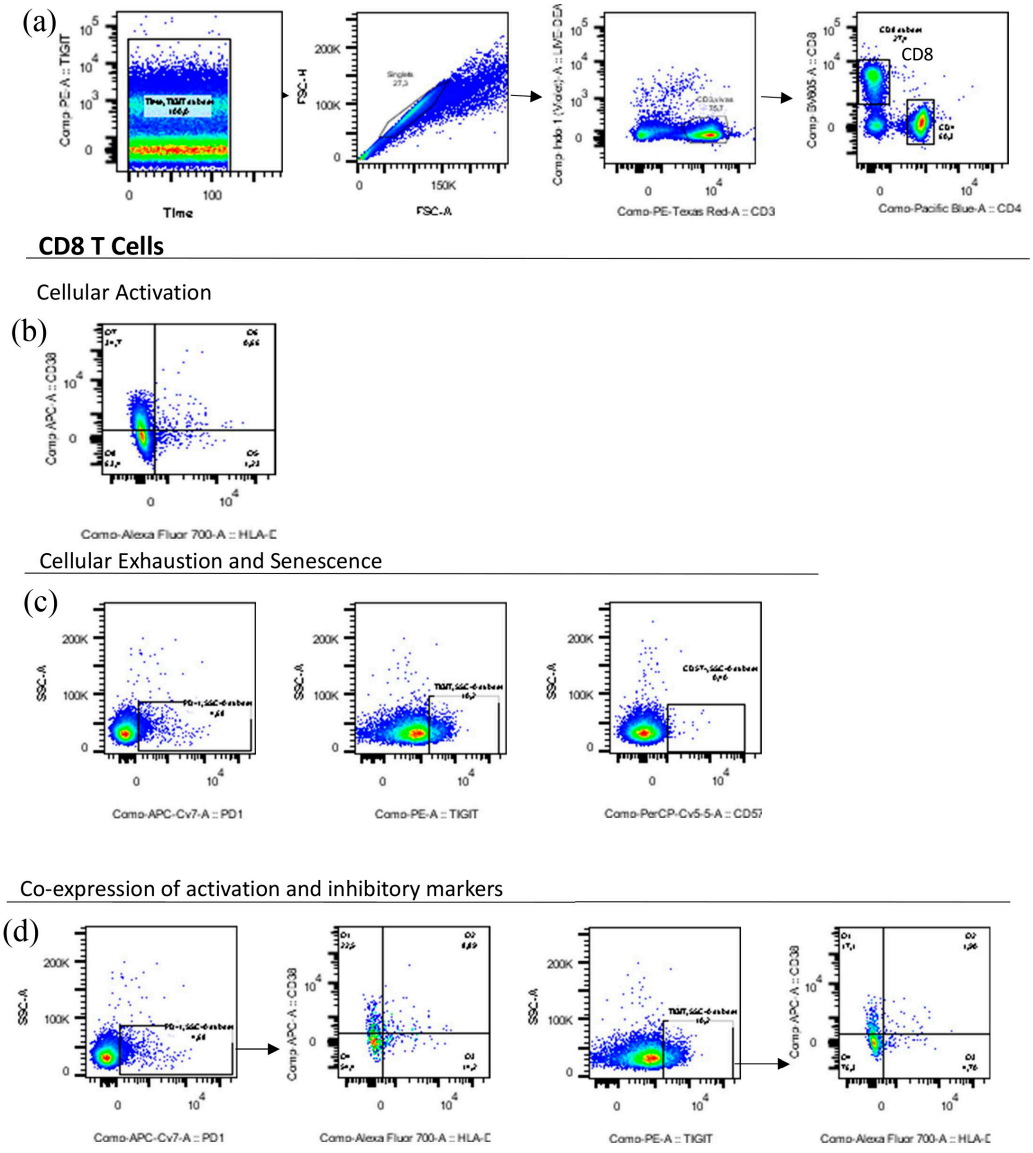

Figure 2 - Strategy of flow cytometry's analysis from one representative experiment with PBMC samples of an HIV-infected patient: (A) Different parameters were chosen first: Time x PE to check laser status; Singlets to exclude the cell doublets; Live/Dead x CD3 to select living cells expressing CD3; and Gate CD8xCD4 to choose $CD8^+$ T cells; (B) Cellular activation was characterized through the expression of HLA-DR and CD38; (C) Cellular exhaustion and senescence were characterized by the expression of PD-1, TIGIT and CD57; (D) Co-expression of activation and inhibitory markers was done by first gating TIGIT or PD-1 $CD8^+$ T cells, and afterwards gating cells positive for CD38 and HLA-DR.

investigation was to analyze the expression of the same activation/exhaustion markers (TIGIT, PD-1, HLA-DR, and CD38) studied before, to characterize CD8 T cells of HI/cART and HI/no cART patients, when compared to HU individuals. CD8 T lymphocytes play a crucial role in controlling HIV replication during acute and chronic infection, and persistent activation of these cells will contribute to diminished effector function and residual viremia[5,12].

Figure 1A shows the time of blood collection according to patient visits at the pediatric clinic. Figure 1B demonstrates a higher percent ($p < 0.05$) of $TIGIT^+CD8$ T cells in blood cells of HI/no cART when compared to HI/cART group (T0) and to HU cohort (T0 and T1). About one year after T0, HI/no cART patients maintained a higher expression of TIGIT on CD8 T cells, but due to the great variability between the individual response and the small sample size, it did not reach statistical significance. HI/cART and HU cohorts showed similar levels of CD8 T cells expressing TIGIT. This last observation was constant for the different analyses described below.

A similar picture as described above can be seen for CD8 T cells expressing PD-1 (Figure 1C). However, we can observe a smaller frequency of $PD\text{-}1^+$ CD8 T cells when

compared to TIGIT$^+$ CD8 T cells, especially for the HI/no cART group at T1 ($p = 0.032$). The biological significance of higher frequency of TIGIT$^+$ CD8 T cells when compared to PD-1$^+$ cells deserves further investigation. As expected, we can see in Figure 1D a significantly higher ($p < 0.05$) co-expression of TIGIT and PD-1 in HI/no cART when compared with the two other cohorts.

Persistent immune activation in chronic HIV infection has been historically translated into higher CD38 and HLA-DR expression on T cells[13,14]. These two markers were consistently expressed in superior frequency on CD8 T cells of HI/no cART patients when compared to HI/cART and HU during the total period of the study ($p < 0.05$, Figure 1E). A similar pattern can be seen for the co-expression of CD38/DR/PD-1 and CD38/DR/TIGIT (Figures 1F and 1G), except for T2. Finally, Figure 1H shows that the CD57 molecule, a senescence marker, is more frequently expressed on CD8 T cells of HI/no cART, reaching statistical significance when compared with the HU group ($p < 0.05$). Then, we asked whether there is any association between the expression of exhaustion markers on CD8 T cells with clinical parameters. Unsurprisingly, Figures 3A and 3B show that the higher expression of activation/exhaustion markers (CD38/DR/TIGIT) was negatively (r = -0.88, $p < 0.001$) associated with CD4/CD8 ratio, but positively (r = 0.91, $p < 0.001$) associated with viral load.

We took advantage of our previous study[11] and analyzed the association between CD8$^+$ T cells and CD4$^+$ T cells co-expressing CD38/DR/TIGIT or CD38/DR/PD-1. A significant association was observed between CD4 and CD8 T cells co-expressing CD38/DR/TIGIT for T0 (r = 0.86, $p < 0.01$, data not shown) and T1 (r = 0.78, $p < 0.01$, Figure 3C). A similar finding was observed for CD38/DR/PD-1 in T0 (Figure 3D). On the other hand, Figures 3E and 3F show negative associations between the frequency of CD8 T cells expressing CD38/DR/TIGIT or CD38/DR/PD-1 and CD4 T cells expressing co-stimulatory molecules (CD127/CD28), indicating that there is a sum of phenotype changes in course.

DISCUSSION

Although immune exhaustion and senescence during HIV infection have been studied in different adult cohorts, little is known in the context of childhood infection. However, it is essential to better understand these phenomena and the degree of immune recovery after cART in pediatric patients, as they are more susceptible to infectious diseases. Thus, we studied the expression of exhaustion/activation and senescence markers on CD8 T cells from HIV-infected (treated or not) and uninfected children and adolescents during a follow-up of approximately one year.

In our cohort, we observed that children/adolescents not receiving cART showed a higher frequency of CD8 T cells expressing exhaustion/activation markers as TIGIT/PD-1/CD38/DR, when compared to treated patients and uninfected controls. The median length of cART was 5.9 years and 5 out of 6 patients on cART were in CDC category C[11] since the Brazilian guidelines for cART initiation in children and adolescents at the time of the onset of this study, in 2011, were restricted to patients in the CDC clinical categories B or C or patients with at least one of the following parameters: CD4 count less than 350 cells/mL, CD4 percentage less than 15%, or viral load >100,000 copies/mL[15]. Therefore, these data suggest that the early onset of cART probably would have contributed to a greater immune reconstitution of those pediatric patients, as described in the literature[16]. In a previous study, we reported no significant differences in the expression of HLA-DR/CD38 molecules on blood CD4$^+$ T cells of HI/cART patients who did or did not develop a protective antibody response after vaccination against meningococcus C[17]. However, when we searched for the co-expression of HLA-DR/CD38 and CCR5, we observed a significant frequency of these phenotypes on CD4 T cells of non-responders to the vaccine. Indeed, there was a negative correlation between the frequency of CD4$^+$DR$^+$CD38$^+$CCR5$^+$ T cells and bactericidal antibodies, indicating an impaired function of the immune cells with persistent immune activation[17]. It is possible that triple expression of activation markers on CD8 T cells may contribute to a more accentuated dysfunctional capacity of these cells.

CONCLUSION

We demonstrated the association between the expression of exhaustion markers on CD8 T cells and clinical parameters, such as viremia and CD4/CD8 ratio. These findings suggest an impairment on CD8 T cell function and clinical deterioration caused by persistent immune activation. Accordingly, HIV vertically infected youth under stable ART showed an increased expression of TIGIT on memory CD8 T cells when compared to healthy controls, and exhibited an inverse correlation between CD8 T cells expressing CD57 or TIGIT with CD4/CD8 ratio[18]. These results suggest that the accumulation of exhausted/inhibitory markers on T lymphocytes may be an important fact associated with the dysfunctionality of these cells. Accordingly, we previously reported the existence of a negative association of exhausted CD4 T cells with blood levels of IL-4, contrasting with a negative link with CXCL-13 levels[11].

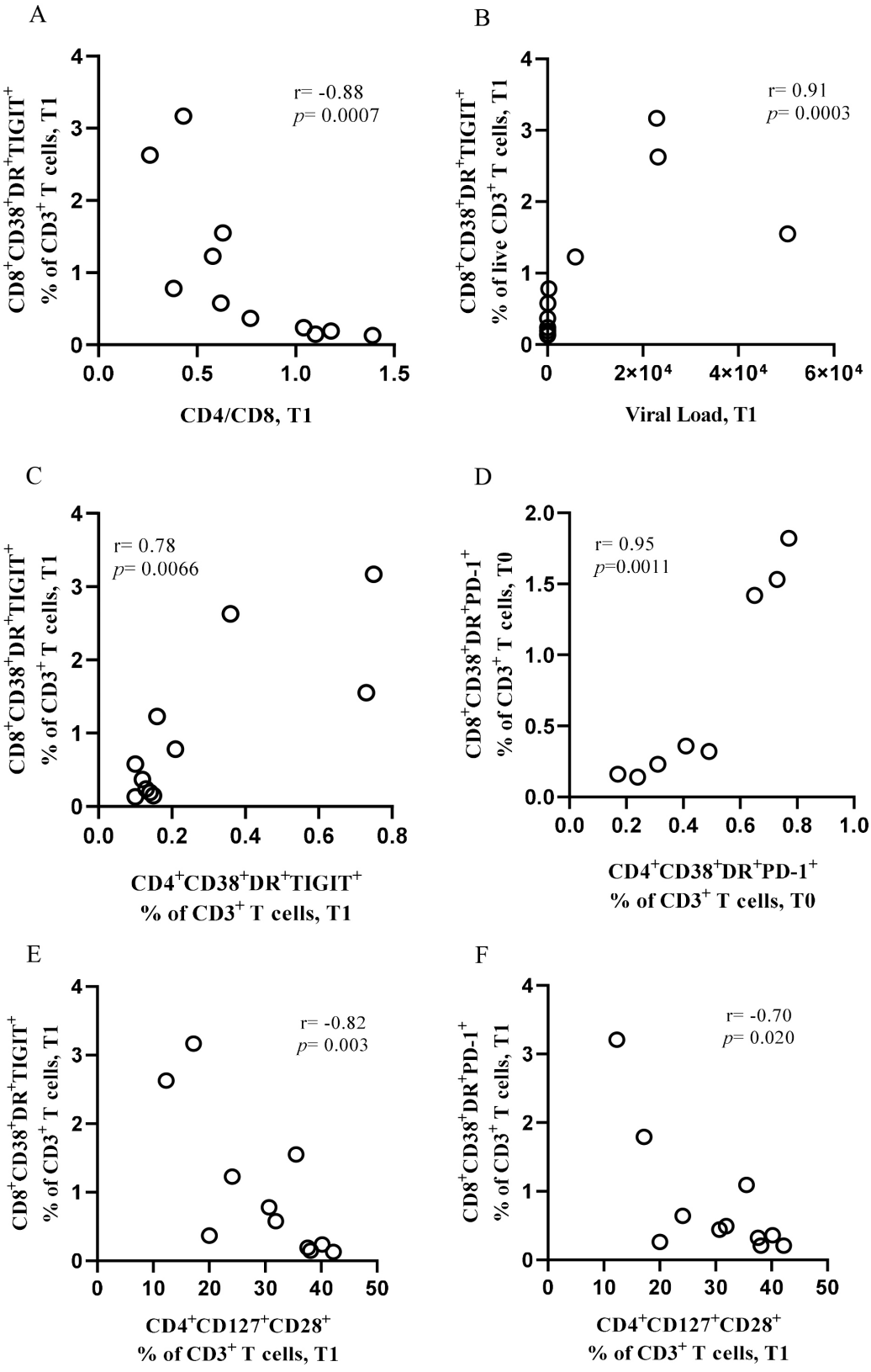

Figure 3 - CD8+ T cells expressing CD38-DR-TIGIT or PD-1 inversely correlated with CD4/CD8 ratio and CD4 T cells expressing stimulatory molecules. Associations of CD8 and CD4 T cells exhausted profile. $CD8^+$ T cells expressing CD38-DR-TIGIT negatively correlated with CD4/CD8 ratio (A) and were positively associated with viral load (B). Positive correlation of CD8 T cells $CD38^+DR^+TIGIT^+$ with CD4 T cells $CD38^+DR^+TIGIT^+$ at T0 (C) and T1 (D). CD8 T cells expressing CD38-DR-TIGIT (E) or PD-1 (F) inversely correlated with $CD4^+$ T cells $CD127^+CD28^+$. Spearman's non-parametric test was used for correlation analyses. $p < 0.05$ was taken as significant.

Data showed a significant expression of exhaustion/senescence markers on CD8 T cells of HI/no cART, which was significantly associated with the viral load and CD4/CD8 ratio, suggesting a diminished biological function of these cells. Further studies are required to investigate the functional status of exhausted CD8 T cells from patients of larger cohorts, such as the ability to produce cytokines and a cytotoxic effect.

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AUTHORS' CONTRIBUTIONS

WFPM, GPS, PRC and DAC performed flow cytometry experiments; EK supervised flow cytometry experiments; ACF, BF, DMB and CBH coordinated the recruitment and immunization of patients, the collection of biological specimens and patient follow-up; LGM, WFPM and GPS designed experiments and analyzed the data; WFPM and LGM wrote the manuscript; LGM, WFPM and GPS made figures; AF and CB provided critical review of data and manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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