Journal of Microbiology, Immunology and Infection 57 (2024) 854—867

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Original Article

The persistence of low CD4/CD8 ratio in chronic HIV-infection, despite ART suppression and normal CD4 levels, is associated with pre-therapy values of inflammation and thymic function

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https://doi.org/10.1016/j.jmii.2024.08.007

Received 7 June 2024; received in revised form 9 August 2024; accepted 14 August 2024
Available online 22 August 2024

KEYWORDS
CD4/CD8 ratio;
HIV-Infection;
Immunological dysfunction;
Nadir-CD4 T-cell;
sj/β-TRECs

Abstract *Background:* Persistence of a low CD4/CD8 ratio is associated with an increased morbimortality in people living with HIV (PLWH) under effective antiretroviral therapy. We aimed to explore the immunological significance of a persistently low CD4/CD8 ratio, even despite normal CD4 levels, and assess whether these features vary from those associated to a low nadir-CD4, another well-established predictor of disease progression.
Methods: CD4-recovered PLWH were classified by CD4/CD8 ratio after three-years of ART (viral suppression, CD4≥500; $R < 0.8$, $n = 24$ and $R > 1.2$, $n = 28$). sj/β-TRECs ratio and inflammatory-related markers were quantified. PBMCs were immunophenotyped by CyTOF and functionally characterized by ELISPOT. Subjects were also reclassified depending on nadir-CD4 ($N \leq 350$/$N > 350$).
Results: $R < 0.8$ showed a differential inflammatory profile compared to $R > 1.2$ (increased β2-microglobulin, D-dimers and IP-10 before ART). $R < 0.8$ presented lower baseline thymic function, being inversely correlated with post-ART inflammation. $R < 0.8$ at follow-up showed most alterations in CD8 subsets (increasing frequency and exhibiting a senescent phenotype [e.g., CD57+, CD95+]) and enhanced T-cell IFNγ/IL-2 secretion. However, comparing $N \leq 350$ to $N > 350$, the main features were altered functional markers in CD4 T-cells, despite no differences in maturational subsets, together with a restricted T-cell cytokine secretion pattern.
Conclusion: Persistence of low CD4/CD8 ratio in successfully-treated PLWH, with normal CD4 counts, is associated with baseline inflammation and low thymic function, and it features post-therapy alterations specific to CD8 T-cells. Differently, subjects recovered from low nadir-CD4 in this setting feature post-therapy alterations on CD4 T-cells. Hence, different mechanisms of disease progression could underlie these biomarkers, potentially requiring different clinical approaches.

Introduction

Immune dysfunction and low-grade chronic inflammation persist in HIV-infected subjects, even after normal CD4 levels due to a successful antiretroviral treatment (ART) and undetectable viremia.[1] These alterations underlay premature immunosenescence and increased risk of age-related comorbidities and death.[2] Absolute CD4 T-cell counts and plasma viral load have been traditionally used for monitoring the response to ART and further clinical progression. However, given the persistence of immunological damage in therapeutically-suppressed HIV-infection, these markers may not properly reflect the recovery of immune function.

In the last years, the CD4/CD8 ratio has been proposed as a more accurate marker of clinical progression than the CD4 counts alone.[3,4] During infection, HIV lowers CD4 T-cells, while CD8 T-cells rise, resulting in a low/inverted CD4/CD8 ratio. This ratio usually improves after ART, although two out of three patients fail to normalize it after eight years.[5] Maintenance of a low CD4/CD8 ratio has been widely associated with comorbidities and mortality.[6—8] Alterations in the host's immune system underlying the persistence of a low CD4/CD8 ratio have been scarcely studied. As far as we know, only the CD8 T-cell compartment has been explored, finding an enrichment of effector phenotypes, with increased expression of activation, exhaustion, and senescence markers (i.e., CD38, HLA-DR, CD57 and PD-1).[8] Furthermore, although suggested as key drivers of the CD4/CD8 ratio inversion, evidence showing associations with proinflammatory markers remains elusive.

Besides, nadir-CD4 T-cell count is also a well-known predictor of disease progression.[9] A low nadir-CD4 has been related to many AIDS-defining and non-AIDS-defining diseases.[10,11] Recently, reservoir size has also been inversely correlated not only with nadir-CD4 but also with CD4/CD8 ratio at the time of ART initiation.[12]

Undoubtedly, both the CD4/CD8 ratio and nadir-CD4 are relevant markers of clinical progression, but to date, their physiopathological meaning remains largely uncovered, especially in the setting of normal CD4 levels. Here, we aimed to deep into the remaining immunological alterations underlying a low CD4/CD8 T-cell ratio despite normal CD4 T-cell counts under successful ART. As a secondary outcome, we aimed to explore whether such alterations could differ from those imprinted by having had a low nadir-CD4 T-cell count.

Methods

Subjects and study design

Patients were selected from the Spanish CoRIS Cohort and their respective samples (peripheral blood mononuclear

cells —PBMC- and plasma) were kindly provided by its HIV BioBank[13] where biological samples were processed frozen immediately. CoRIS participants provided their written informed consent prior to enrolling in the cohort. CoRIS cohort was approved by the Research Ethic Committee of Gregorio Marañón Hospital. A flowchart for patient selection and exclusion criteria is represented in Fig. 1. Briefly, from a total of 15509 available patients, 123 met the eligible criteria; we finally analyzed samples from those whose CD4/CD8 T-cell ratio was <0.8 ($R < 0.8$, n = 24) or >1.2 ($R > 1.2$, n = 28) after 3 years under suppressive ART and normalized CD4. For secondary analyses, we reclassified all the patients with analyzed samples (n = 52) according to nadir-CD4 value ≤ 350 ($N \leq 350$, n = 24) and >350 ($N > 350$, n = 28). Clinical and demographic data were obtained from CoRIS. Most assays were assessed at the follow-up timepoint (3—3.5 years after ART initiation), except for soluble plasma biomarkers that were also measured at baseline (0—6 months before ART) and for thymic function, which was only determined at baseline.

Soluble biomarkers

Soluble biomarkers were determined by standardized techniques at the Biochemistry Service of Virgen del Rocío University Hospital: high-sensitivity C reactive protein (hsCRP), β2-microglobulin, homocysteine, lactate dehydrogenase (LDH), D-dimers, and ferritin. Soluble cytokines related to inflammation were analyzed in plasma using the ProcartaPlex™ Human Inflammation Panel 20-plex (Invitrogen, ThermoFisher), following manufacturer's instructions (see Supplementary Methods for details).

Immunophenotyping by mass cytometry

Samples from 25 patients were randomly selected from both study groups (R < 0.8, n = 13 and R > 1.2, n = 12) using *Random Case Selection* in SPSS software to ensure unbiased representation and PBMCs were deeply immunophenotyped by mass cytometry (CyTOF) using a Maxpar Direct Immune Profiling Assay (x25 samples assay kit, Fluidigm) following manufacturer's instructions. Additional in-house labeled antibodies were also used. Detailed information on the staining protocol, antibodies and the conjugation of in-house labeled antibodies can be found in Supplementary Material. A minimum of 300,000 events per sample were acquired using a CyTOF2-Helios mass cytometer (Fluidigm). This panel allowed us to identify populations of innate and adaptive immunity, as well as additional function-related markers. Gating strategy was applied as manufacturer's instructions, while the additional markers were gated as shown in Supplementary Figure 1.

ELISpot assay

IFNγ and IL-2 production was quantified by ELISPOT assay (Human IFNγ and IL-2 ELISpot kits, Mabtech) (detailed in Supplementary Methods). Briefly, DNase-treated PBMCs were stimulated with peptide pools for CD4 (CEFTA), CD8 (CEF) or HIV-1-specific cells (consensus-B Gag) in anti-IFNγ or anti-IL-2 antibody pre-coated plates. CEFTA and CEF were used as positive controls of viral responses. Stimulation was carried out during 48 h at 37 °C and 5%CO_2. Cells were then removed, and plates were treated following manufacturer's instructions. Spots were analyzed with Astor ELISpot Reader (Mabtech).

sj/β-TRECs ratio quantification

Thymic output was estimated based on the sj/β TRECs ratio measured by ddPCR, as detailed elsewhere.[14] Briefly, DNA was extracted from 500 mL of whole blood by using Omega BIO-TEK-E.Z.N.A blood DNA kit. Then, sj/β TREC ratio was determined from 150 ng of DNA in a single reaction using QX200 system (BIORAD). Analysis was performed in the Quantasoft 1.7.1 software.

Data analysis

Quantitative variables are expressed as median and interquartile range. Cross-sectional comparisons between groups were performed using nonparametric Mann-Whitney *U* test, while longitudinal comparisons, using Wilcoxon rank-sum test. Categorical variables were recorded as the number of cases and percentages, with comparisons among groups using $\chi 2$ or Fischer's exact test. Correlations were assessed using Spearman's rho correlation coefficient. A *p*-value<0.05 was considered statistically significant. Analysis was performed using Statistical Package for Social Sciences software (SPSS 25; IBM) and atypical values were detected by Dixon's Q test using *Outliers* package in R. Dot plots and heatmaps were created using the *ggplot 2* package in R. For mass cytometry data, conventional gating and dimensionality reduction analysis were performed using FCS Express 7 (DeNovo Software). Pie charts were plotted and analyzed using Pestle v1.6.2 and Spice v6.1.

Results

Characteristics of the study cohort

CD4/CD8 ratio groups did not differ in any demographic or clinical characteristics, but R < 0.8 presented lower nadir-CD4, baseline and follow-up CD4 counts than R > 1.2 ($p < 0.001$; Table 1). Consequently, 75% of subjects in R < 0.8 were also in N ≤ 350 when reclassified by nadir-CD4. Despite such overlap, baseline CD8 counts were only different between groups when classified by CD4/CD8 ratio, and differences in follow-up CD8 counts and baseline CD4/CD8 ratio were also more evident in this comparison. Besides, N ≤ 350 showed trends to be younger and to have lower diagnosis to treatment time than N > 350. Additionally, no differences were found between groups when comparing opportunistic infections, or active hepatitis C infection before ART initiation (Table 1).

R < 0.8 exhibited a proinflammatory profile mainly before ART

While R < 0.8 showed higher levels of several inflammation-related parameters compared to R > 1.2, N ≤ 350 tend to

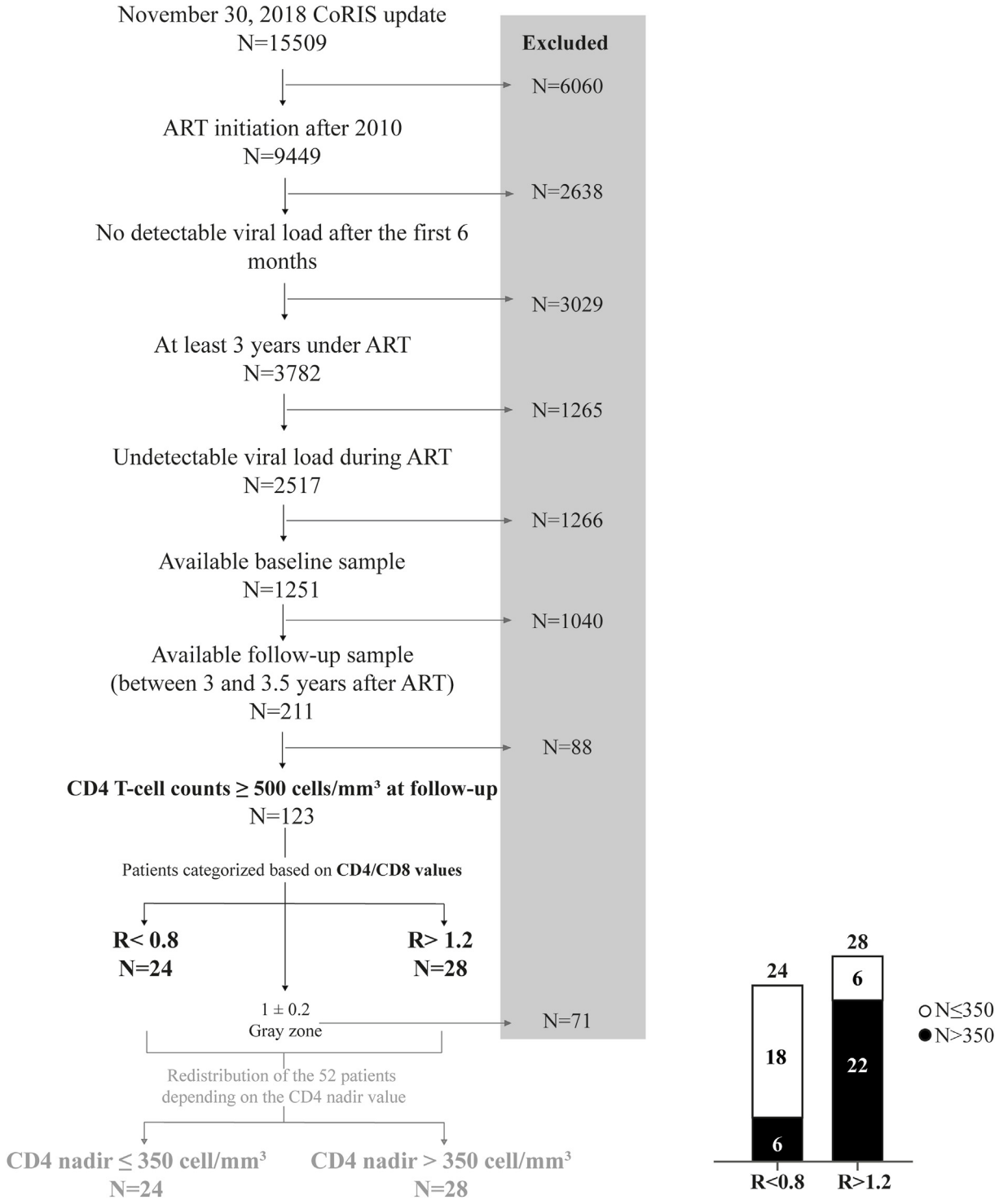

Figure 1. Flow chart and inclusion criteria. We selected patients from the November 30, 2018 updated CoRIS database. Briefly, exclusion criteria were a) no ART or ART initiation before 2010, b) detectable viral load after the first 6 months of ART, c) less than 3 years under ART, d) detectable viral load at any time-point of the first 3 years of ART (excluding the first 6 months), e) no baseline sample, f) no follow-up sample (between 3 and 3.5 years after ART initiation) and g) CD4 T-cell counts under 500 cells/mm^3. From the 15509 patients with available data, only 123 met the inclusion criteria. We only selected those whose CD4/CD8 T-cell ratio was <0.8 or >1.2, corresponding to 1 ± 0.2 CD4/CD8 ratio values from the eligible patients, to avoid mixed phenotypes. Final selected patients were then reclassified for a secondary analysis according to their nadir-CD4 in ≤ 350 or >350 cell/mm^3, since 350 cells/mm^3 is a critical threshold for AIDS diagnosis, for monitoring treatment interruptions and also for identification of late-presentation.

Table 1 Characteristics of the study subjects classified according to their follow-up CD4/CD8 ratio values or their nadir-CD4 values.

	Entire cohort n = 52	CD4/CD8 ratio			Nadir-CD4		
		R < 0.8 n = 24	R > 1.2 n = 28	*p*-value	N ≤ 350 n = 24	N > 350 n = 28	*p*-value
Parameters at follow up							
Age (years)	39 [33–46]	39 [33–50]	39 [31–44]	0.335	37 [29–44]	42 [35–47]	*0.078*
Male, n (%)	52 (100)	24 (100)	28 (100)	1	24 (100)	28 (100)	1
Origin, n (%)				0.162			0.572
Spain	34 (65.4)	19 (79.2)	15 (53.6)		14 (58.3)	20 (71.4)	
Europe	14 (26.9)	3 (12.5)	11 (39.3)		8 (33.3)	7 (25.0)	
South America	4 (7.7)	2 (8.4)	2 (7.1)		2 (14.3)	1 (3.6)	
Mode of infection, n (%)				0.887			0.727
IDU	3 (5.8)	2 (8.3)	1 (3.6)		1 (4.2)	2 (7.1)	
Heterosexual	6 (11.5)	3 (12.5)	3 (10.7)		4 (16.7)	2 (7.1)	
Homo/Bisexual	41 (78.8)	18 (75)	23 (82.1)		18 (75)	23 (82.1)	
Other/Unknown	2 (3.8)	1 (4.2)	1 (3.6)		1 (4.2)	1 (3.6)	
ART duration (months)	39 [37–40]	40 [37–41]	39 [37–39]	*0.095*	39 [37–40]	39 [38–40]	0.576
ART composition, n (%)				0.122			0.122
2NRTI + 1NNRTI	36 (69.2)	15 (62.5)	24 (85.7)		17 (70.8)	20 (71.3)	
2NRTI + 1PI	1 (1.9)	6 (25)	2 (7.1)		0	1 (3.6)	
2NRTI + 1II	10 (19.2)	3 (12.5)	1 (3.6)		5 (20.9)	5 (17.8)	
Other	5 (9.6)	0	1 (3.6)		2 (8.3)	2 (7.3)	
CD4 (cell/mm^3)	779 [610–1015]	663 [560–749]	904 [753–1107]	**<0.0001**	643 [553–855]	893 [738–1092]	**<0.0001**
CD8 (cell/mm^3)	741 [606–1077]	985 [886–1346]	607 [525–695]	**<0.0001**	918 [696–1234]	677 [578–865]	**0.018**
CD4/CD8 ratio	1.23 [0.65–1.58]	0.63 [0.58–0.73]	1.53 [1.33–1.78]	**<0.0001**	0.67 [0.58–1.18]	1.36 [1.21–1.68]	**<0.0001**
Diagnosis to treatment time (months)	4 [2–18]	5.5 [1.3–15.5]	4 [2–21]	0.876	3.5 [0.3–12.8]	5.0 [2.0–21.0]	*0.070*
Time to reach viral load undetectability (months)	3 [2–5]	3.5 [2–5]	3 [2–5]	0.896	3 [2–5]	3 [2–5]	0.730
Parameters before ART initiation							
Log viral load (copies/mL)	4.69 [4.27–5.07]	4.50 [4.15–5.02]	4.68 [3.79–5.20]	0.664	4.69 [4.14–5.17]	4.71 [4.29–4.99]	0.710
Nadir CD4 (cell/mm^3)	362 [285–475]	296 [250–352]	447 [356–607]	**<0.0001**	275 [224–304]	466 [397–612]	**<0.0001**
CD4 (cell/mm^3)	388 [303–574]	317 [261–377]	494 [399–632]	**<0.0001**	303 [230–361]	479 [391–623]	**<0.0001**
CD8 (cell/mm^3)	808 [602–1205]	949 [756–1463]	738 [593–808]	*0.066*	903 [628–1463]	794 [593–1025]	0.578
CD4/CD8 ratio	0.50 [0.29–0.80]	0.38 [0.24–0.48]	0.78 [0.51–1.01]	**0.001**	0.36 [0.24–0.58]	0.68 [0.45–0.82]	**0.031**
AIDS, n (%)	1 (1.9)	1 (4.2)	0	0.275	1 (4.2)	0	0.462
OI, n (%)							
PCP	1 (1.9)	1 (4.2)	0	0.275	1 (4.2)	0	0.275
Candidiasis (esophageal, bronchial, trachea, or lungs)	1 (1.9)	1 (4.2)	0	0.275	1 (4.2)	0	0.275
HCV RNA, n (%)				0.273			0.536
Positive	1 (1.9)	1 (4.2)	0		1 (4.2)	0	

Negative	46 (88.5)	22 (91.7)	24 (85.7)	21 (87.5)	25 (89.3)
Unknown	5 (9.5)	1 (4.2)	4 (14.3)	2 (8.3)	3 (10.7)

Follow-up parameters are shown at the top of the table, while parameters before ART initiation are shown at the bottom.
Patients included in this study were diagnosed with HIV infection from November 2007 to May 2015.
Quantitative variables expressed as median [IQR]; statistical analyses between groups were performed using a non-parametric Mann-Whitney test. Qualitative variables expressed as number of cases (%); statistical analyses were performed using $\chi 2$ or Fischer's exact tests. No differences between groups were found in educational level.
Statistical significances ($p < 0.05$) are highlighted in bold, while trends ($0.1 < p < 0.05$) are shown in italics.
Abbreviations: IDU, injectable drugs user; ART, antiretroviral treatment; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; IP, protease inhibitors; INSTI, integrase strand transfer inhibitors; AIDS, Acquired immunodeficiency syndrome; OI, Opportunistic infections; PCP, *Pneumocystis carinii* pneumonia; HCV, Hepatitis C virus.

have lower values compared to N > 350 (Fig. 2; Supplementary Tables 2 and 3). Notably, baseline differences were more evident when comparing by CD4/CD8 ratio. Thus, R < 0.8 had significantly higher baseline levels of β2M, D-dimers and IP-10 than R > 1.2.

Longitudinally, β2M and DD decreased in both, R < 0.8 and R > 1.2; IP-10 and hsCRP levels trended downward in R < 0.8, while IL-6, IL-10, IL-13 and ferritin decreased in R > 1.2 (Supplementary Tables 2 and **3)**. E-selectin increased during ART in both groups, while ICAM-1 increased only in R < 0.8 ($p = 0.052$). When comparing by nadir-CD4, N ≤ 350 showed reductions in β2M, DD, and IP-10, and increases in E-selectin and ICAM-1 during ART (all $p < 0.050$). N > 350 showed decreases in β2M, DD, ferritin, IP-10, IL-13 but increases in P-selectin and E-selectin.

Lastly, at follow-up, R < 0.8 had significantly higher levels of β2M and a trend toward higher hsCRP levels compared to R > 1.2. Differently, N ≤ 350 showed lower levels of IL-8 and a trend toward lower ferritin levels compared to N > 350.

CD8 T-cells, rather than CD4, were altered in R < 0.8

Immune populations were characterized in 25 randomly selected samples from R < 0.8 (n = 13) and R > 1.2 (n = 12) groups. We created a global immune cell profile of our study groups using a dimensionality reduction approach (tSNE, Fig. 3A left). No evident distinctive cell clusters specific to any of the groups were found, however, differences in frequencies in CD4 and CD8 T-cell populations were patent (Fig. 3A right). Exploring all the different subsets described in the Methods section, we found differences among several adaptive populations (Fig. 3B). Total CD4 T-cell frequency was lower in R < 0.8 ($p < 0.001$), but without differences among CD4 maturational subsets. In R < 0.8, total CD8 T-cells were higher ($p < 0.0001$), exhibiting a lower Naïve population, while a trend to higher Effector Memory population was observed. Interestingly, a significantly higher frequency of double-positive CD4+CD8+ T-cells and a trend to higher Th1 subset was found in R < 0.8. Besides, patients with N ≤ 350 (n = 13), compared to N > 350 (n = 12), also exhibited lower frequencies of total CD4 T-cells and higher of CD8 T-cells, but without differences among maturational subsets.

Regarding innate populations, R < 0.8 exhibited higher frequency of total dendritic cells (DC), total myeloid-derived suppressor cells (MDSCs) and lower frequency of myeloid DC (mDC) compared to R > 1.2 ($p < 0.030$; Fig. 3C). Differently, N ≤ 350 showed higher frequency of plasmacytoid DC (pDC) than N > 350 ($p < 0.035$). Finally, in both comparisons, a trend to higher frequency of the MAIT/iNKT subset was observed in both problem groups compared to their respective comparison groups.

Regarding functional biomarkers, tSNE showed differences in the expression of Glut 1 and α4β7 integrin among populations when comparing R < 0.8 and R > 1.2 (Supplementary Figure 2). Detailed analysis with a two-dimensionality gating strategy (Fig. 4 and Supplementary Table 4) confirmed higher frequencies of α4β7+Naïve CD4 T-cells and α4β7+Treg, as well as Glut1+Th2 cells and

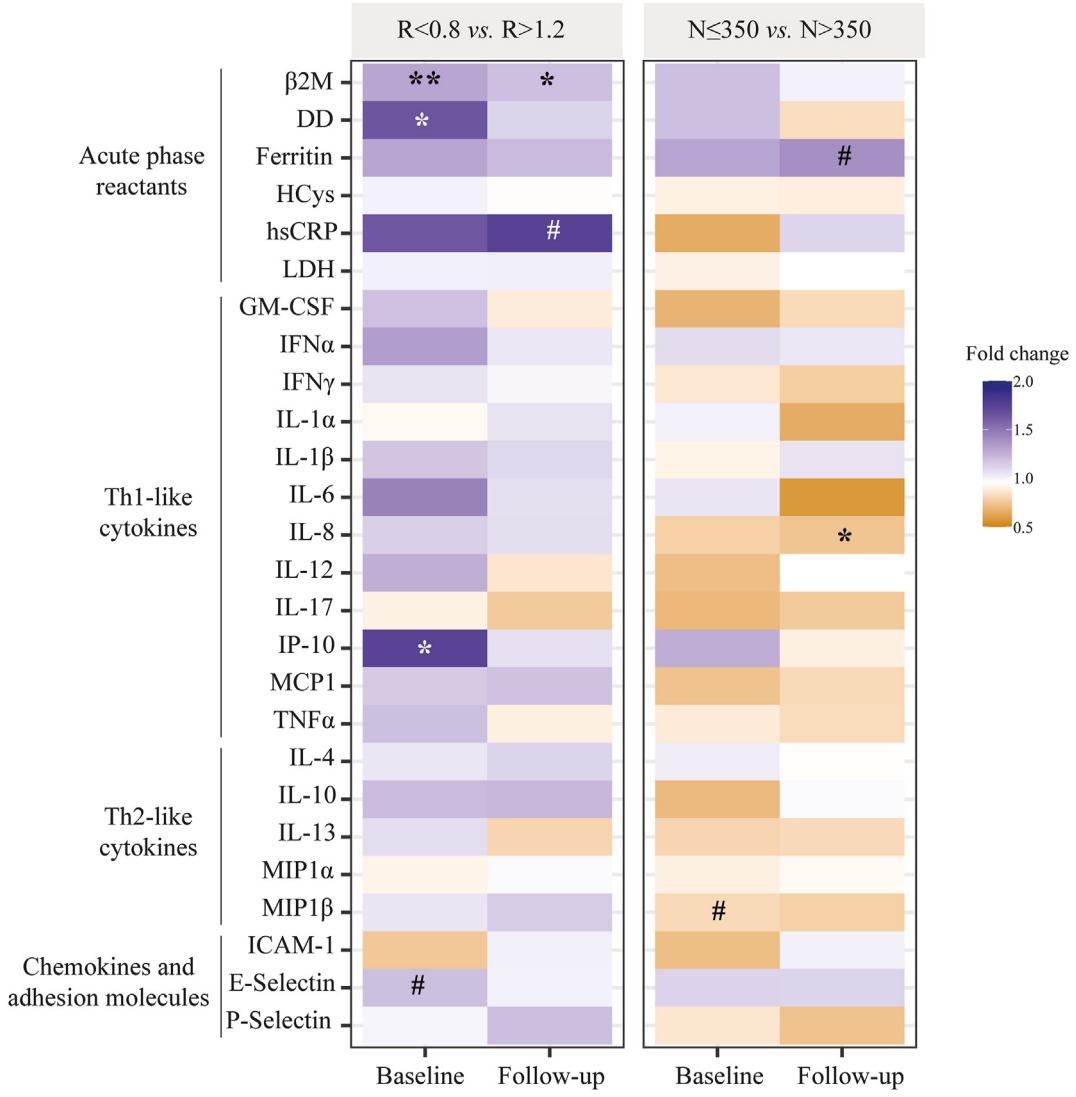

Figure 2. Inflammatory profile. Differences between groups in soluble inflammation-related markers represented by the color legend as a fold change (R < 0.8/R > 1.2 or N ≤ 350/N > 350). Mann-Whitney tests were performed to compare the distribution of the variables between groups R < 0.8 *vs*. R > 1.2 or N ≤ 350 *vs*. N > 350; when statistically significant, *p*-values are indicated as * ($p < 0.05$) or ** ($p < 0.01$). *P*-values between 0.1 and 0.05 are indicated as #. Sample size: R < 0.8, n = 24 and R > 1.2, n = 28; N ≤ 350, n = 24 and N > 350, n = 28. Abbreviations: β2M, β2-microglobulin; DD, D-dimers; HCys, homocysteine; hsCRP, high sensitivity C reactive protein; LDH, lactate dehydrogenase; GM-CSF, Granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP-10, interferon-γ-inducible protein 10; MCP1, monocyte chemoattractant protein-1; TNF, tumor necrosis factor; MIP-1, macrophage inflammatory protein-1; ICAM-1, intercellular adhesion molecule-1.

CD57+Treg and CD57 + Th2 cells in R < 0.8 (all $p < 0.030$). CD98+Central Memory CD4 T-cells were lower in R < 0.8. Moreover, R < 0.8 showed higher levels of CD95 + CD8 T-cells and CD57+Naïve CD8 T-cells. However, R < 0.8 exhibited lower levels of PD1+ and activated (CD38+HLA-DR+) Central Memory CD8 T-cells.

Differently, when comparing by nadir-CD4 (Supplementary Table 5), N ≤ 350 showed significantly higher frequencies of α4β7+ and Glut 1+TemRA CD4 T-cells. The frequencies of CD57+Central Memory CD4, CD57 + Th2 and CD57+Treg were also significantly higher in N ≤ 350. Furthermore, activated (CD38+HLA-DR+) and CD95+Central Memory CD8 T-cells, as well as CD95+Effector Memory CD8 were lower in N ≤ 350.

Enhanced cytokine secretion in R < 0.8

Patients with R < 0.8 and R > 1.2 exhibited different patterns of IFNγ and IL-2 secretion (Fig. 5A, up). A higher proportion of R < 0.8 presented positive response against all three stimuli compared to R > 1.2 for both cytokines and, for IFNγ, none of the R < 0.8 exhibited negative response to any of the three stimuli whereas 14% of R > 1.2 showed null-response. For IL-2 the proportion of null-response was also lower in R < 0.8.

The proportion of subjects in N ≤ 350 with an IFNγ-positive response against any of the three stimuli was slightly lower compared to N > 350 (Fig. 5A, down-left). Besides, higher proportion of N ≤ 350 did not secrete

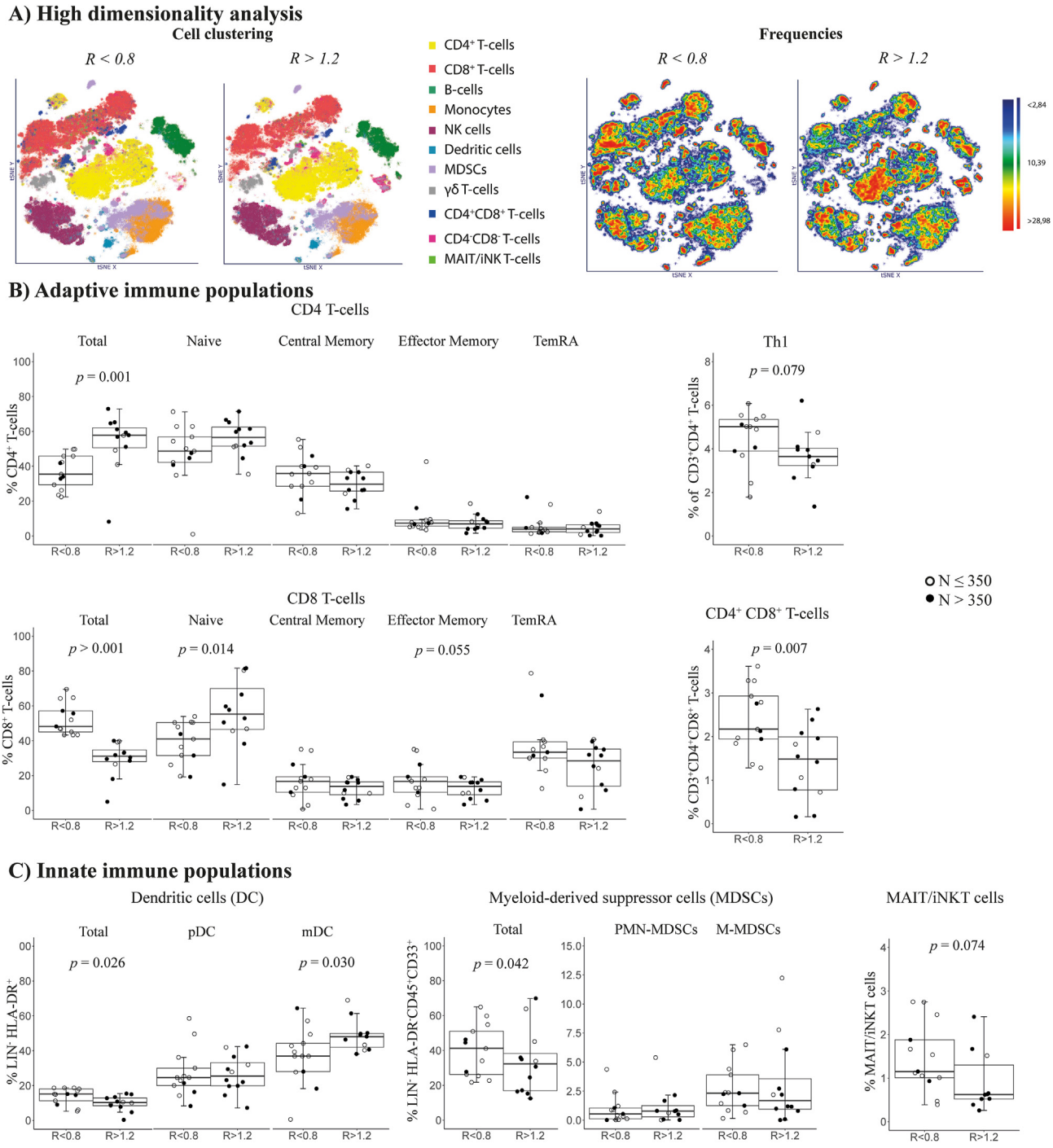

Figure 3. Phenotypical characterization of innate and adaptive immune populations in HIV-infected subjects according to CD4/CD8 ratio. Characterization of immune populations using a high dimensionality analysis (tSNE dimensionality reduction) including all populations (A) or a conventional two dimensionality gating strategy, including adaptive (B) or innate (C) populations. Color dots indicate the nadir-CD4 value (blue, N $\leq$ 350 and orange, N $>$ 350). *P*-values represent statistical differences between R $<$ 0.8 and R $>$ 1.2 groups. Only subsets showing significant and trend to significant differences are represented ($p < 0.1$). Sample size: R $<$ 0.8, n = 13 and R $>$ 1.2, n = 12; N $\leq$ 350, n = 13 and N $>$ 350, n = 12. Dimensionality reduction (tSNE) analysis was performed with the default configuration (Iteration: 1000, Perplexity: 80, Amount of approximation: 0.30) and a minimum of 100,000 cells and 42 markers were used to generate representative tSNE maps. Populations were then annotated according to the expression of lineage-specific differentiation markers.

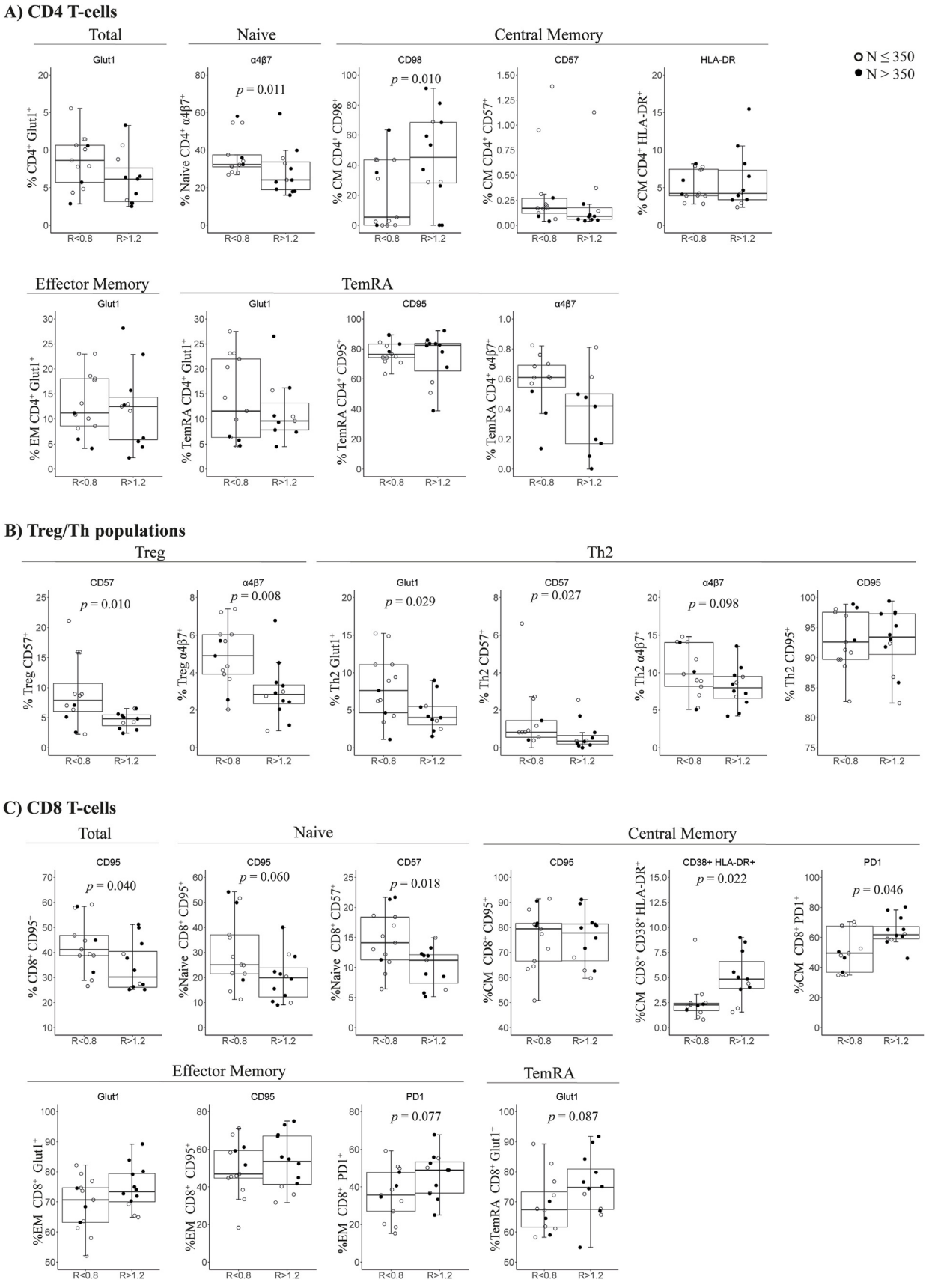

Figure 4. Functional characterization of immune cell subsets in HIV-infected subjects according to CD4/CD8 ratio. Expression of cellular markers related to gut-homing, metabolism, activation, exhaustion, senescence and apoptosis susceptibility.

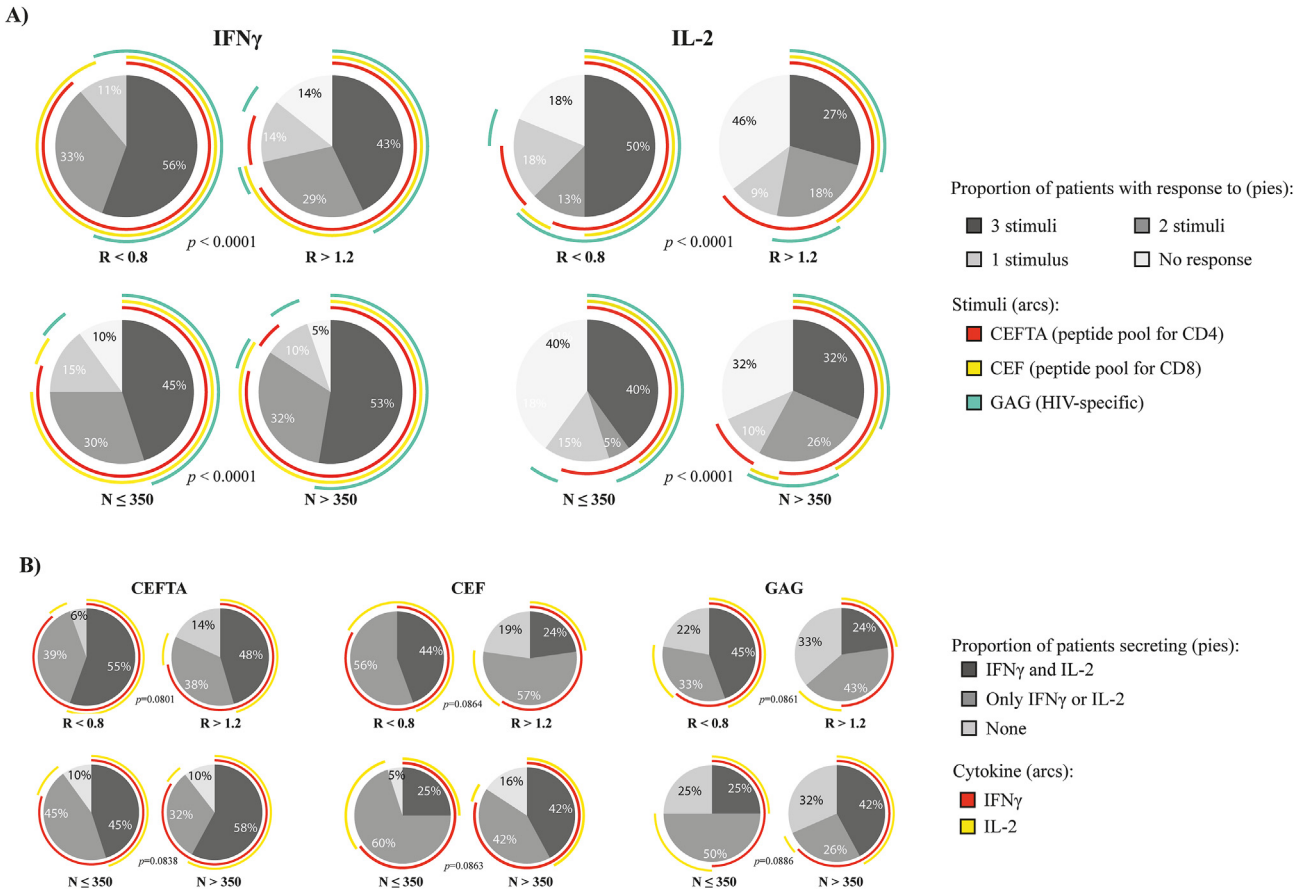

Figure 5. Cytokine secretion pattern in response to specific stimulation. A) Proportion of subjects with positive response to CEFTA, CEF or GAG by secreting IFNγ or IL-2 according to CD4/CD8 ratio (up) or nadir-CD4 (down); pies represent the proportion of subjects with positive response to three, two, or one stimuli, or none response, while arcs represent the specific stimuli; B) Proportion of patients secreting IFNγ and/or IL-2 in response to CEFTA, CEF and Gag according to CD4/CD8 ratio (up) or nadir-CD4 (down); pies represent the proportion of subjects producing the two cytokines, only one of them or none, while arcs represent the specific cytokine. Sample size: R < 0.8, n = 18 and R > 1.2, n = 22; N ≤ 350, n = 20 and N > 350, n = 20.

IFNγ in response to any of the three stimuli compared to N > 350. Differently, the proportion of IL-2 response to all three stimuli was higher in N ≤ 350 (Fig. 5A, down-right) although the proportion of N ≤ 350 exhibiting null-response was higher.

The combined IFNγ/IL-2 secretion patterns in response to each stimulus were also compared (Fig. 5B), resulting in trends in all cases ($p < 0.09$). Curiously, the proportion of patients simultaneously secreting IFNγ and IL-2 in all three stimuli was higher in R < 0.8 compared to R > 1.2, whereas the contrary was observed comparing N ≤ 350 to N > 350. Nevertheless, groups did not show any differences regarding Spot Forming Units (SFU) to any stimuli as well as other related parameters including Total Spot Volume, Relative Spot Volume, and SFU normalization by CD4 counts (Supplementary Figures 3 and 4, for CD4/CD8 and nadir-CD4 comparisons, respectively).

Lower baseline thymic function was associated with inflammation

R < 0.8 group showed a trend to lower thymic function compared to R > 1.2 ($p = 0.092$; Fig. 6A) while no difference was found between groups when comparing by nadir-CD4 ($p = 0.622$). Besides, the β-TRECs were undetectable in 13 samples, preventing us from determining the sj/β-TREC ratio in these subjects. Such indetermination rate was higher among subjects from the R < 0.8 group than from the R > 1.2 group, though not statistically significant [33% (8/24) *vs.* 18% (5/28) (χ^2, $p = 0.199$)]. Sj/β-TRECs ratio was negatively associated with several inflammatory markers at baseline (DD and IL-8, both $p < 0.03$; Fig. 6B), as well as with follow-up P-selectin ($p = 0.023$). Trends were also found at this timepoint with ICAM-1 and E-selectin ($p < 0.1$; Fig. 6C).

Color dots indicate the nadir-CD4 value (blue, N ≤ 350 and orange, N > 350). *P*-values represent statistical differences between R < 0.8 and R > 1.2 groups. Only subsets showing significant and trend to significant differences are represented ($p < 0.1$). Sample size: R < 0.8, n = 13 and R > 1.2, n = 12; N ≤ 350, n = 13 and N > 350, n = 12.

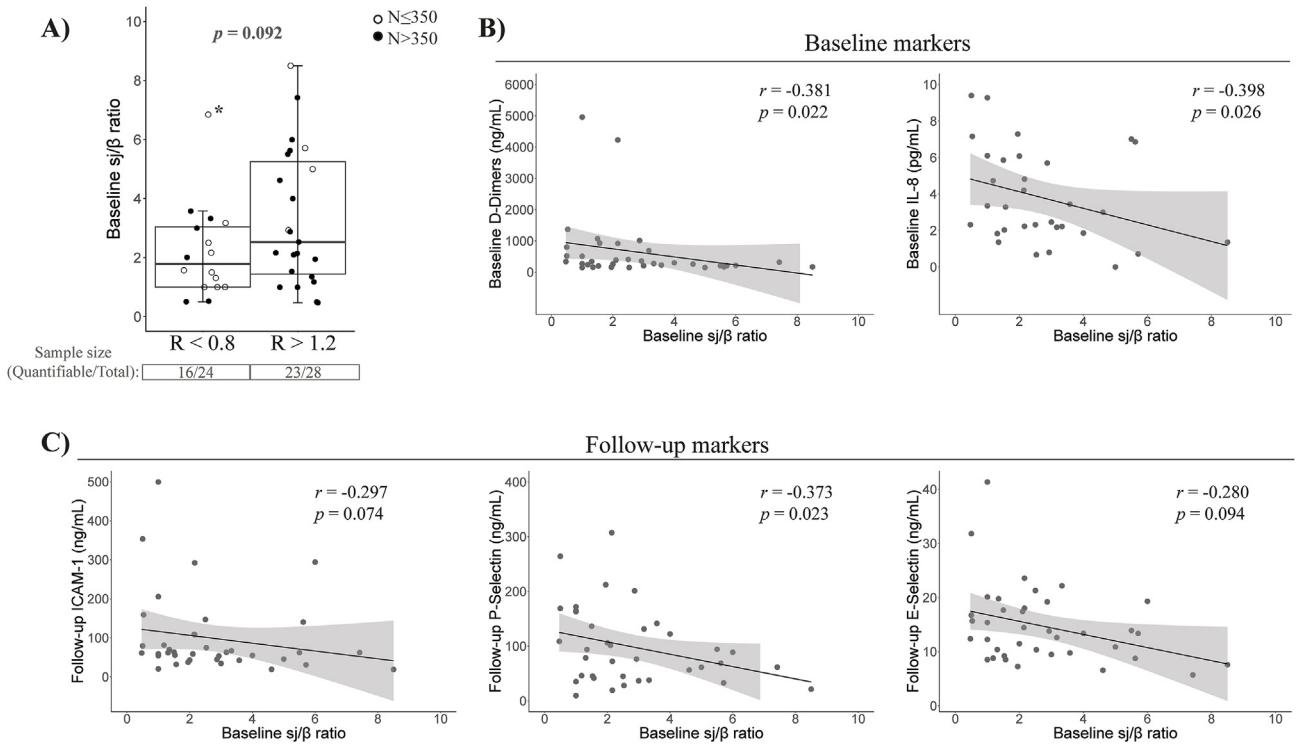

Figure 6. Baseline thymic function estimated by the sj/β TRECs ratio. A) Comparisons of sj/β TRECs ratio between patients classified according to CD4/CD8 ratio. *Outlier value ($p = 0.181$ for direct comparison between $R < 0.8$ and $R > 1.2$; *$p = 0.092$ after excluding the outlier value). For the comparison between $N \leq 350$ and $N > 350$, $p = 0.622$. Color dots indicate the nadir-CD4 value (blue, $N \leq 350$ and orange, $N > 350$). Associations with B) soluble inflammatory markers at baseline (D-Dimers and IL-8) or C) after 3 years under ART (ICAM-1, sTfR, P-Selectin and E-Selectin). Sample size (indicated as quantifiable/total measurements): $R < 0.8$, n = 16/24 and $R > 1.2$, n = 23/28; $N \leq 350$, n = 14/24 and $N > 350$, n = 25/28.

Discussion

PLWH keeping low CD4/CD8 ratio despite suppressive ART and normal CD4 counts still have higher morbi-mortality risk, but underlying immune alterations remain largely unexplored. We report that a low CD4/CD8 ratio is associated to preexisting and persistent inflammation and lower baseline thymic function. Furthermore, we found predominant alterations in CD8 T-cell phenotypes, rather than in CD4 T-cells, together with an enhanced capability of IFNγ and IL-2 secretion after T-cell stimulation.

Implications of a low CD4/CD8 ratio despite ART have been widely studied and even associated with clinical progression.[15–17] However, previous studies did not consider CD4 normalization, failing to distinguish whether a low CD4/CD8 ratio or merely CD4 counts cause immunological damage. This strict selection criterion (CD4≥500 cell/mm^3) has limited the sample size and the inclusion of patients with very low CD4/CD8 ratios. This is the reason why we chose to compare groups using the threshold of 1 ± 0.2, also allowing us to exclude patients with intermediate CD4/CD8 ratios (closer to 1, probably an ambiguous zone). Several publications have previously used a CD4/CD8<0.8 as threshold for ratio inversion,[18–20] as well as CD4/CD8 ≥ 1.2 as the definition for "ratio normalization" in PLWH.[21,22]

Our data show the persistence of lower CD4/CD8 T-cell ratio associated with a pro-inflammatory environment, with higher baseline levels of β2M, D-dimers and IP-10 and the persistence of elevated β2-microglobulin and hsCRP levels after ART. A lower CD4/CD8 ratio has been previously associated with hsCRP.[8] Interestingly, we observed an increase in ICAM-1 during ART only in lower CD4/CD8 ratio patients. ICAM-1 was found elevated in ART-naïve HIV-subjects compared to ART-treated, and uninfected subjects, and was associated with AIDS-related death,[23,24] cardiovascular, autoimmune diseases and cancer.[25–27] Moreover, ICAM-1 promotes cytokine signaling and CD8 T-cell differentiation during antigenic stimulation,[28] likely contributing to the inversion of the CD4/CD8 ratio. Additionally, IP-10 is elevated during HIV-infection and associated with CD4 T-cell counts, viral load and disease progression.[29] Finally, patients with lower CD4/CD8 ratio seemed to have poor resolution of the pro-inflammatory status which could be linked to their higher risk of cardiovascular progression.[30,31] Based on our previous findings highlighting the key role of the baseline thymic function in maintaining peripheral CD4/CD8 ratio after ART,[32] we sought additional factors linked to baseline thymic function. Interestingly, several inflammatory markers were negatively associated. Given the stablished connection between inflammatory status and thymopoiesis,[33] it is plausible that inflammation could hinder the restoration of the CD4/CD8 ratio by limiting thymic output.

At a cellular level, our data unexpectedly report slight changes in CD4 T-cell maturational subsets or T-helper

populations in patients with lower CD4/CD8 ratio. However, the increased Effector Memory CD8 T-cells, DCs and MDSCs in this group suggest higher antigenic presentation and a need for suppressor cells to manage such activation. Given the inverse association between the CD4/CD8 ratio and viral reservoir,[12] we speculate a larger reservoir contributing to increased antigenic exposure. Moreover, CD4+CD8+ T-cells, known for high activation and effector memory phenotype,[34] along with higher IFNγ and IL-2 T-cell secretion in response to antigens, may contribute to this activated environment.[35] However, we cannot exclude the impact of CMV co-infection, higher proportions of senescent (CD57 + CD28-) CD4+ T-cells or viral fitness.[36]

In terms of functionality, lower CD4/CD8 ratio patients presented higher frequencies of Treg and α4β7+ Naïve CD4 T-cells, likely due to increased intestinal homing, the main location of HIV reservoir, which is also associated with higher susceptibility to HIV-infection.[37] Predominantly expressed markers in the CD8 T-cell subset suggest an enrichment of senescent phenotypes, consistent with previous studies.[8]

Despite the challenge of studying the isolated impacts of a persistently low CD4/CD8 ratio or having recovered from low nadir-CD4, due to their interdependence, we found distinct immune settings depending on the main discriminator used. Notably, CD8 counts, at baseline and also at follow-up, clearly emerged as the primary difference between these discriminators. Thus, our approach determined that recovery from a low nadir-CD4 affects more specifically CD4 T-cells, rather than CD8, and impacts the functional ability of both, CD4 and CD8, after stimulation. Additionally, CD4/CD8 ratio and nadir-CD4 commonly showed no alterations in the maturational CD4 subsets. However, a persistently low CD4/CD8 ratio was associated with higher levels of total DCs and MDSCs, while a low nadir-CD4 was linked to pDCs and MAIT/iNKT subsets.

Persistent type-I IFN production by innate cells is related to immune activation, decreased CD4 counts, dysregulated T-cell maturation in the thymus and disease progression.[38,39] Despite not finding increased levels of soluble IFNα, other type-I IFNs not included in our panel (e.g. IFNβ) might be altered. Additionally, patients with lower nadir-CD4 showed an increased frequency of Glut 1+, α4β7+ integrin, and a decrease of CD95+TemRA CD4 T-cells, suggesting a metabolically active phenotype. Indeed, TemRA cells have significant cytotoxic activity in viral infections (e.g., cytomegalovirus).[40,41]

Furthermore, the proportion of patients secreting IFNγ in response to different antigens was lower. Conversely, Central Memory, Treg and Th2 cells presented a higher frequency of CD57-expressing cells, an exhaustion marker. However, CD57 expression in CD4 T-cell subsets is also related to cytolytic activity, being this population dramatically increased during acute or untreated chronic HIV-infection.[42] Moreover, the proportion of patients secreting IL-2 was higher in lower nadir-CD4; IL-2 induces a cytolytic phenotype in *in vitro* stimulated-CD4 cells and regulates granzyme-B and perforin expression both *in vitro* and *in vivo*.[43] Altogether, these cells could have a deleterious overactivation, similar to that proposed for pDCs during chronic infection.

This approach has identified persistent alterations in chronic, successfully-treated HIV-infection, with normal CD4, but maintaining low CD4/CD8 ratio. We found that residual inflammation and low thymic function are associated with a persistent a low CD4/CD8 ratio. These alterations differ from those in patients who have recovered from a low CD4 nadir, even despite overlapping comparison groups. How these phenotypical and functional alterations specifically affect disease progression remains unknown. Validating these immune signatures in larger cohorts and exploring their relation to non-AIDS-related diseases, possibly requiring complementary therapeutic approaches to ART, seems worthwhile.

Funding

This work was supported by grants from the Fondo de Investigación Sanitaria [FIS; PI18/01216, PI20/00326 and PI21/00357] and the Ministerio de Ciencia, Innovación y Universidades [CNS2023/1444725] which are co-funded by Fondos Europeos para el Desarrollo Regional (FEDER) "A way to make Europe"; the Instituto de Salud Carlos III [FI19/00298 to V.G-R; CD19/00143 to A.B-R, CM19/00051 to I.O-M and CP19/00146 to A.R]; the Consejería de Transformación Económica, Industria, Conocimiento y Universidades [DOC_01646 to MM.P-B] and the Consejería de Salud y Familias of Junta de Andalucía through the "Nicolás Monardes" [RC-0006-2021 to YM.P]. The HIV BioBank is supported by Instituto de Salud Carlos III (PT20/00138) and Networking Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN (CB22/01/00041). CoRIS cohort are supported by CIBER - Consorcio Centro de Investigación Biomédica en Red- (CB21/13/00091), Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación and Unión Europea — NextGenerationEU, A.R and J.P are also supported by CIBER-INF (CB21/13/00020). The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

CRediT authorship contribution statement

Vanesa Garrido-Rodríguez: Writing — review & editing, Writing — original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ángel Bulnes-Ramos:** Writing — review & editing, Methodology, Formal analysis, Data curation. **Israel Olivas-Martínez:** Writing — review & editing, Methodology, Investigation, Formal analysis, Data curation. **María del Mar Pozo-Balado:** Writing — review & editing, Methodology, Investigation. **Ana Isabel Álvarez-Ríos:** Writing — review & editing, Methodology. **Félix Gutiérrez:** Writing — review & editing, Resources. **Rebeca Izquierdo:** Writing — review & editing, Resources. **Federico García:** Writing — review & editing, Resources. **Juan Manuel Tiraboschi:** Writing — review & editing, Resources. **Francisco Vera-Méndez:** Writing — review & editing, Resources. **Joaquim Peraire:** Writing — review & editing, Resources, Investigation. **Anna Rull:** Writing — review & editing, Resources, Investigation. **Yolanda María Pacheco:** Writing — review & editing, Writing — original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This study would not have been possible without the collaboration of all patients, medical and nursing staff and data managers who have taken part in the Project. We want to particularly acknowledge the patients in this study for their participation, to the HIV BioBank and the collaborating centers for the generous gifts of the clinical samples used in this study. We also thank the Cytometry Unit at the GENYO (Granada, Spain) for their technical assistance in mass cytometry.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.08.007.