



Research Paper

IL18 gene polymorphism and its influence on CD4+ T-cell recovery in HIV-positive patients receiving antiretroviral therapy

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ABSTRACT

Background: Pyroptosis has been reported to be critical in human immunodeficiency virus type 1 (HIV-1) pathogenesis and acquired immunodeficiency syndrome (AIDS) progression. Even after achieving viral suppression to undetectable levels during antiretroviral therapy (ART), exacerbated CD4+ T-cell death by pyroptosis has been suggested as one of the main causes of immunological non-response. Thus, variants in genes of pyroptosis pathway were studied in individuals with poor CD4+ T-cell reconstitution under antiretroviral therapy against HIV-1.

Methods: 248 virologically suppressed ART-treated patients, 126 immunological non-responders (INR) and 122 immunological responders (IR) were recruited. Genotyping was performed using TaqMan probe-based realtime PCR platform. Genotype-guided flow cytometry analysis with general and recent thymic emigrant (RTE) CD4+ T-cells in pyroptosis was performed based on associated polymorphisms.

Results: Both IL18 rs187238 G allele and GG genotype were associated as protection factors against poor CD4+ T-cell recovery (OR = 0.22; 95%CI = 0.50–0.77; $P = .010$ and OR = 0.58; 95%CI = 0.36–0.93; $P = .022$, respectively). It was demonstrated a statistical association between IL18 rs187238 genotypes of ART-treated patients and death by Caspase-1 levels ($P = .020$). The GG genotype showed lower pyroptotic RTE CD4+ T-lymphocytes levels in the ART-treated groups comparing with CC ($P = .029$) and CG ($P = .018$) genotypes, suggesting that the G allele presence may be related to a lower IL-18 production and thus reduced dead CD4+ T-cells levels by Caspase-1.

Conclusion: We observed that IL18 G variant allele and genotype were associated with a better immunological response, which may influence on immunological recovery of HIV-positive patients receiving antiretroviral therapy, and low Caspase-1 activity levels was observed on GG genotype when compared CC genotypes.

1. Introduction

It is widely known that approximately 30% of patients achieving plasma human immunodeficiency virus (HIV) load suppression do not recover their CD4+ T-cell levels during antiretroviral therapy (ART). These ART-treated patients are defined as immunological non-responders (Aiuti and Mezzaroma, 2006; Li et al., 2011). Despite being considered a multifactorial condition, poor CD4+ T-cell reconstitution has been associated with two main mechanisms: reduced CD4+ T-cells production and exacerbated cell death of these lymphocytes (Corbeau

and Reynes, 2011; Gaardbo et al., 2012).

Pyroptosis is inflammatory programmed cell death, mainly mediated by caspase-1, that has emerged as an important mechanism of innate immunity against pathogens (Boucher et al., 2016; Jorgensen and Miao, 2015). The main activation pathway of caspase-1 occurs through inflammasome, a signaling protein complex assembled in response to cell disorders or intracellular pathogens. Several types of inflammasome are able to activate caspase-1 but, specifically in HIV-1 infection, the NLRP3 and IFI16 inflammasomes are the major signaling pathways into cells that recognize this virus in the early stages of its

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replicative cycle and prevent cell infection, called non-permissive cells. Both IFI16 and NLRP3 sensors connect to caspase-1 via ASC, an adapter protein containing a CARD (caspase activation and recruitment domain) region that promote the interaction. Among CARDS variety, studies have demonstrated that CARD8 acts as a negative regulator of the NLRP3 inflammasome, thus dampening NF- κ B, with consequent absence of caspase-1 activation (Ito et al., 2014; Kesavardhana and Kanneganti, 2017; Miao, 2011). Once caspase-1 is activated, the cleavage of the pro-IL-1B and pro-IL-18 molecules occurs, converting them into active proinflammatory cytokines, resulting in an inflammatory process following HIV infection (Kesavardhana and Kanneganti, 2017; Man et al., 2017; Miao, 2011).

In HIV-1 infection, it is believed that pyroptosis presents as a pathogenic vicious cycle, in which inflammatory stimuli are released, recruiting more cells to the sites of infection thus promoting augmented cell death (Doitsh et al., 2014). Hence, the activation of the pyroptosis pathway turns out to be a form of chronic immune activation in lymphoid tissues, contributing to the progression to AIDS (Doitsh and Greene, 2016; Gaiha and Brass, 2014).

In this study, we evaluated SNPs in *CARD8*, *NLRP3*, *IL1B*, *IL18* and *IFI16* genes involved in the pyroptosis pathway, aimed at finding a possible association with poor immune recovery of CD4 + T cells in subjects living with HIV submitted to antiretroviral therapy.

2. Methodology

2.1. Study population

The study population consisted of 248 HIV-positive patients (107 males and 141 females) under ART enrolled at Instituto de Medicina Integral Professor Fernando Figueira (IMIP), Pernambuco state (Northeast Brazil), between 2011 and 2014. The subjects were recruited according to the following inclusion criteria: age over 18 years old, on ART for at least one year with prolonged undetectable viral load (< 50 copies/mL), good adherence to treatment; and exclusion criteria: pregnancy, autoimmune diseases and history of injecting drug use. Sociodemographic and clinical data were collected from medical records: age and body mass at ART start date; time until ART start after HIV infection tests; ART regimens received (2NRTI + PI/r or 2NRTI + NNRTI regimens, as recommended by the Brazilian guidelines available at the time of patient recruitment); pre- and post-treatment viral loads as well as CD4+ T-cell counts; and serological data regarding co-infections (hepatitis B virus – HBV, hepatitis C virus – HCV, syphilis, cytomegalovirus – CMV, toxoplasmosis, human T-cell lymphotropic virus types I and II (HTLV-I/II)). All patients answered standard questionnaires and signed written informed consent, providing blood samples for genetic and immunological analyses. The IMIP Research Ethics Committee approved this study (protocol number: 3629-13)

2.2. Determination of study groups

The ART-treated patients, who had persistently undetectable plasma HIV concentration (< 50 copies/mL) during the first year of therapy, were classified according to gains in CD4+ T cell counts or percentages. Patients that gained < 200 CD4+ T-cells/ μ L compared with pre-treatment count or presented T-cell percentages in relation to total lymphocytes < 30% (if absolute counts were not available) after the first year of ART were classified as immunological non-responders (INR) (adapted from (Li et al., 2011)). All others subjects were defined as immunological responders (IR).

2.3. Selection of single base polymorphisms (SNPs)

The SNPs selection was based on the literature, functional characteristics and minor allele frequency (MAF) > 10% in European,

Amerindian and African populations, which reflect the Brazilian genetic admixture (Coelho et al., 2015). Thus, five SNPs in pyroptosis pathway genes were evaluated: *NLRP3* (rs10754558 C > G, MAF: 0.35) inflammasome activator; *CARD8* (rs2043211 A > T, MAF: 0.32) – molecular adapter; *IL1B* (rs1143634 G > A, MAF: 0.13) and *IL18* (rs187238 C > G, MAF: 0.21) – effector molecules; and *IFI16* (rs6940 A > T, MAF: 0.23) – intracellular DNA sensor. SNPs selection was performed using NCBI dbSNP (<https://www.ncbi.nlm.nih.gov/projects/SNP/>) and 1000 Genomes Project Browser (<http://browser.1000genomes.org>).

2.4. Sampling and DNA extraction

Peripheral blood sample (4 mL) was collected from all ART-treated patients in EDTA tubes for genomic DNA extraction using *mini salting out* protocol (Miller et al., 1988) with “in-house” modifications. DNA quantification and purity were assessed using the Thermo Scientific™ NanoDrop 2000 (ThermoFisher) spectrophotometer, considering absorbance values: 260/280 nm and 260/230 nm ratios.

Genotyping was performed using TaqMan® allele-specific probes: *NLRP3* rs10754558 (C_26052028_10), *CARD8* rs2043211 (C_11708080_1), *IL18* rs187238 (C_2408543_10), *IL1B* rs1143634 (C_9546517_10) and *IFI16* rs6940 (C_7483779_10); on ABI® real-time platform 7500 (Applied Biosystems) using protocols recommended by the manufacturer.

2.5. Genotype-guided flow cytometry analysis

Flow cytometric analysis was performed according to *IL18* rs187238 polymorphism genotypes, the only one associated with immunological non-response. The Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque density gradient technique and washed twice in phosphate-buffered saline (PBS 1 \times), following manufacturer's guidelines (Healthcare, 2007). Cell viability (> 90% in average) was determined by Trypan blue (0.4%) exclusion test. For pyroptosis detection, we employed FAM-FLICA Caspase-1 (FAM-YVAD-FMK) Assay kit following the manufacturer's instructions (ImmunoChemistry Technologies), and PBMCs were also stained with immunofluorescent monoclonal antibodies APC-CD4 and PE-CD31 (BD Biosciences) and analyzed by flow cytometry using BD Accuri C6 cytometer (BD Biosciences). In this analysis, 20,000 events were acquired and gated to detect death by Caspase-1 CD4+ T-cells (CD4 + FLICA-Caspase1+) and dead recent thymic emigrant CD4+ T-cells by Caspase-1 activation (CD4 + CD31 + FLICA-Caspase1+). Acquired data were analyzed using FCS Express 6 Plus software.

2.6. Statistical analysis

Genotypic and allelic frequencies were calculated by direct counting, and χ^2 test was used to verify the conformity with Hardy-Weinberg equilibrium. Sample size analyses were performed in the G*Power® software using post-roc power test. Student's *t*-test was used to compare means of groups for variables that followed a normal distribution (according to Shapiro-Wilk test), and Wilcoxon-Mann-Whitney test for variables that do not follow a normal distribution. Fisher exact test was used to assess whether genetic, sociodemographic and clinical variables were associated with INR status. The variables deemed to have clinical importance or reached a *p*-value \leq .20 during univariate analysis were included in the logistic regression analysis. The statistical significance level (α) was set at 0.05 for all tests. Statistical analyses were performed using R program, version 3.5.0.

Table 1
Allelic and genotype frequencies of polymorphisms among ART-treated groups (immunological non-responders and immunological responders).

Gene (SNP)	INR n (%)	IR n (%)	OR (95%CI)	p-Value ^a	
<i>NLRP3</i> rs10754558 (n = 206/248)	<i>Genotypes</i>				
	CC	39 (38)	40 (39)	Reference	–
	CG	48 (47)	46 (45)	0.93 (0.49–1.77)	0.88
	GG	16 (15)	17 (16)	1.03 (0.42–2.53)	1.00
	<i>Alleles</i>				
	C	126 (61)	126 (61)	1 (0.66–1.51)	1.00
G	80 (39)	80 (39)			
<i>CARD8</i> rs2043211 (n = 181/248)	<i>Genotypes</i>				
	AA	49 (56)	43 (46)	Reference	–
	AT	32 (36)	44 (47)	1.56 (0.81–3.02)	0.16
	TT	7 (8)	6 (7)	0.97 (0.25–3.69)	1.00
	<i>Alleles</i>				
	A	130 (74)	130 (70)	1.21 (0.74–1.98)	0.41
T	46 (26)	56 (30)			
<i>IL1B</i> rs1143634 (n = 195/248)	<i>Genotypes</i>				
	GG	59 (60)	61 (64)	Reference	–
	GA	35 (35)	33 (34)	0.91 (0.48–1.73)	0.88
	AA	5 (5)	2 (2)	0.39 (0.03–2.5)	0.44
	<i>Alleles</i>				
	G	153 (77)	155 (81)	0.81 (0.48–1.36)	0.46
A	45 (23)	37 (19)			
<i>IL18</i> rs187238 (n = 193/248)	<i>Genotypes</i>				
	CC	60 (60)	46 (49.5)	Reference	–
	CG	36 (36)	33 (35.5)	0.84 (0.44–1.61)	0.64
	GG	4 (4)	14 (15)	0.22 (0.50–0.77)	0.010
	<i>Alleles</i>				
	C	156 (78)	125 (67)	0.58 (0.36–0.93)	0.022
G	44 (22)	61 (33)			
<i>IFI16</i> rs6940 (n = 187/248)	<i>Genotypes</i>				
	AA	62 (64)	66 (73)	Reference	–
	AT	32 (33)	21 (23)	0.62 (0.30–1.23)	0.19
	TT	3 (3)	3 (4)	0.94 (0.12–7.28)	1.00
	<i>Alleles</i>				
	A	156 (80)	153 (85)	0.72 (0.40–1.29)	0.27
T	38 (19)	27 (15)			

INR = Immunological non-responders; IR = Immunological Responders; OR = Odds Ratio; SNP = Single Nucleotide Polymorphism.

^a Fisher's Exact Test.

3. Results

3.1. Genotype analysis

A total of 248 virologically suppressed patients living with HIV were recruited for this study. Thus, 126 ART-treated patients (62 males and 64 females) were classified as INR, whereas the remaining 122 subjects (45 males and 77 females) were included in the IR group; the median age at the beginning of therapy in both groups was 32 years (28.5–37.5). Population characterization data are shown in Supplementary Table 1.

Five variants of genes involved in cell death pathway by pyroptosis (*NLRP3*, *CARD8*, *IL1B*, *IL18* and *IFI16*) were genotyped (Table 1). Genotypes distribution was consistent with the Hardy-Weinberg equilibrium for all the variants analyzed. The post-hoc power tests for all genes was > 80%, where the sampled N of the population has shown representative for the analysis.

The *NLRP3* polymorphism (rs10754558) analysis included 206

subjects (INR = 103 and IR = 103 groups). The G allele frequency in the both groups was 39%. Fisher's exact test showed that there was no association of neither G variant allele (OR = 1.00; 95%CI = 0.66–1.51; P = 1.00) nor GG genotype (OR = 1.03; CI-95% = 0.42–2.53; P = 1.00) with immunological recovery failure in the analyzed population. The *CARD8* rs2043211 polymorphism also showed no association with poor CD4 + T-cell reconstitution, either the T allele (OR = 0.97, 95%CI = 0.25–3.69; P = 1.00) or the TT genotype (OR = 1.21, 95%CI = 0.74–1.98, P = .41). The polymorphism frequencies observed in the INR and IR groups were 26% and 30% respectively, being analyzed in 181 individuals (88 immunological non-responders and 93 immunological responders).

The *IL1B* (rs1143634) and *IFI16* (rs6940) polymorphisms were analyzed in 195 and 187 individuals, respectively. The allelic frequency of variant A (rs1143634 G > A) was 23% in the INRs and 19% in the IR group. Association analysis for this polymorphism did not show correlation between the AA genotype (OR = 0.39; 95%CI = 0.03–2.5; P = .44) and A variant allele (OR = 0.81; 95%CI = 0.48–1.36; P = .46) with immunological recovery failure. For *IFI16* gene, the observed T allele (rs6940 A > T) frequencies were 19% in the INR group and 15% in the IR group. The hypothesis that this variant could be associated with immunological recovery failure was not confirmed in our study population: TT genotype (OR = 0.94; 95%CI = 0.12–7.28; P = 1.00) or T variant allele (OR = 0.72; 95%CI = 0.40–1.29; P = .27).

The *IL18* (rs187238 C > G) polymorphism analysis demonstrated a G allele frequency of 22% in the immunological non-responders group and 33% in the immunological responders group, among a total of 193 individuals genotyped (100 in the case group and 93 in the control group). According to Fisher test both the GG genotype (OR = 0.22; 95%CI = 0.50–0.77; P = .010) and the G variant allele (OR = 0.58; 95%CI = 0.36–0.93; P = .022) were statistically associated with immune reconstitution as a protection factor against the occurrence of immunological non-response in the ART-treated groups.

3.2. Genotype-guided immunophenotypic analysis

The genotype-guided analysis consisted of 29 individuals, 13 immunological non-responders (CC = 01; CG = 05; GG = 07) and 16 immunological responders (CC = 02; CG = 04; GG = 10) (Fig. 1).

When comparing the CD4 + /FLICA-Caspase1 + T-cell population, no statistical association (P = .67) was found among the ART-treated groups regarding genotype presence. Despite this, it was observed a lower death CD4 + T-cell levels in the ART-treated individuals carrying the G variant allele.

Regarding recent thymic emigrants (RTE) CD4+ T-cell death by Caspase-1 (CD4 + /CD31 + FLICACaspase1 +) it was demonstrated statistical association between *IL18* genotypes of ART-treated patients and pyroptosis levels (P = .020). An immunological non-responder individual with CC genotype showed higher Caspase-1 RTE CD4 + T-cell levels (99.3%) than individuals carrying GG genotype in the same group (48.1%), as well as comparing with the immunological responders for these genotypes (CC = 53.9%; GG = 50.3%; P = .018). Considering CG and GG genotypes in the ART-treated groups (INR = CG (62.5%) and GG (48.1%) vs IR = CG (32.8%) and GG (50.3%), it was also observed statistically significant difference (P = .029) in relation RTE CD4 + T-cell death by Caspase-1, suggesting that the G allele presence may be related to a lower IL-18 production and thus reduced level of Caspase-1 detection in CD4 + T-cells.

3.3. Logistic regression analysis

Even though some clinical variables have not been associated with immunological recovery failure in univariate tests, they were included in the logistic regression analyses together with associated rs187238 (*IL18*) genotype because of their clinical importance and p-values (Table 2). Others were not included because they showed either not

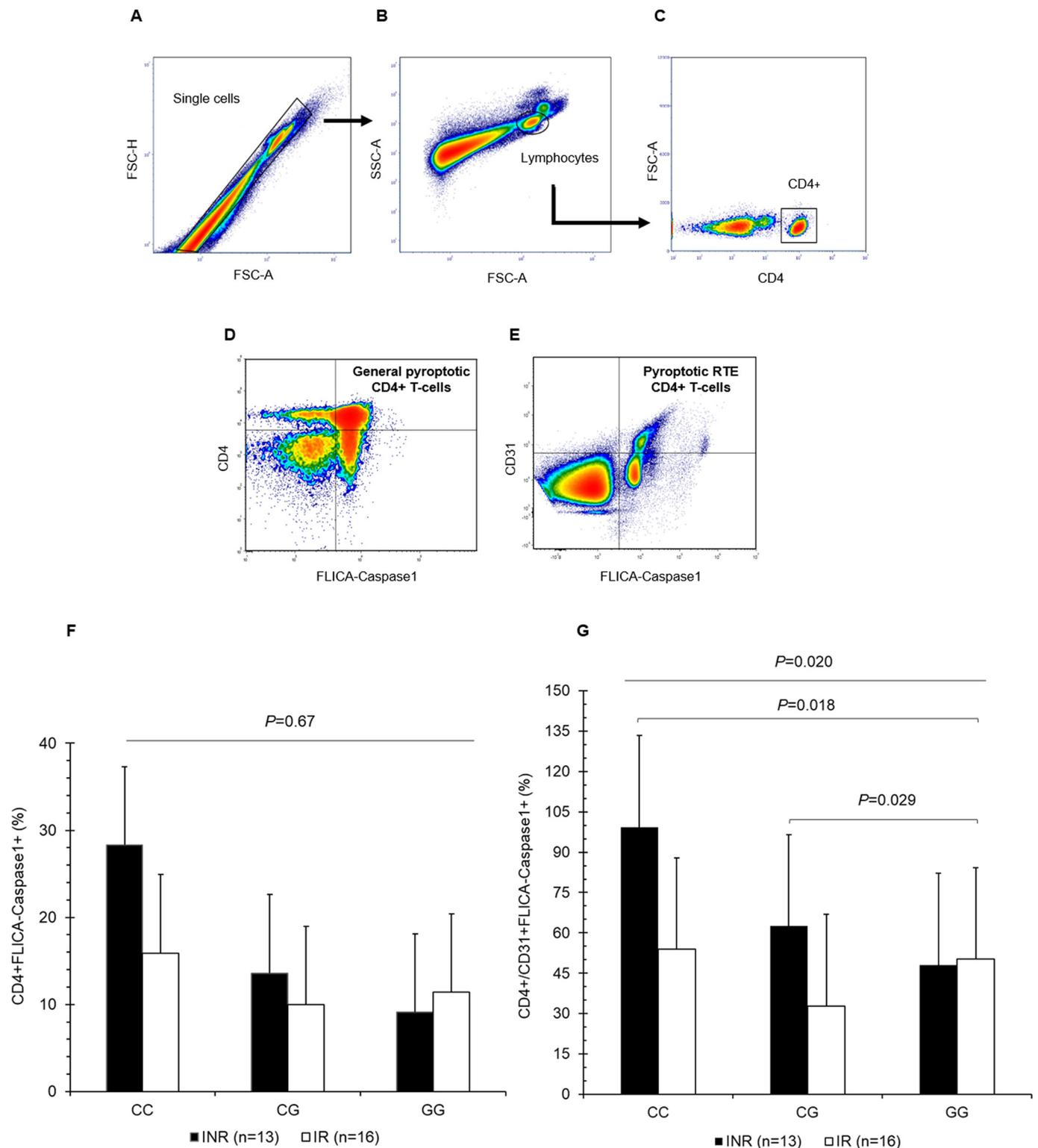


Fig. 1. Pyroptosis levels of CD4 T-cells based on FLICA-Caspase1 activity in ART-treated patients (immunological non-responders – INR and immunological responders – IR). (A) Initial gating was performed to identify single cells. (B) Lymphocytes were selected from singles cells based on forward scatter (FSC) and side scatter (SSC). (C) Cells expressing CD4+ were selected to genotype-guided pyroptosis analyses. (D) Representative flow cytometry plots illustrating the gating strategy for (D) general pyroptotic CD4+ T-lymphocytes (CD4 + FLICA-Caspase1+) and (E) pyroptotic recent thymic emigrants (RTE) CD4+ T-cells (CD4+/CD31 + FLICA-Caspase1+). Pyroptosis levels of general CD4+ T-cells and RTE CD4+ T-cells are shown in (F) and (G) bar plots, respectively. Mean values, standard deviation, and *P* value (according to *t*-test) are shown. FLICA: Fluorescent-Labeled Inhibitors of Caspases.

significant p-values or sparse data, making not possible the logistic regression model calculate the internal parameters. The results of the logistic regression analysis maintained significant association between rs187238 GG genotype and immune reconstitution, as a protection

factor to immunological recovery failure occurrence (OR = 0.157, 95%CI = 0.027–0.921, *P* < .040). The model's internal validation showed that the analysis had good quality and adherence, fitting adequately the data (AUROC = 0.699; Z = -0.452; *P* = .651) and, thus,

Table 2

Variables included in a fitted logistic regression model to explain immunological recovery failure of HIV-positive patients receiving antiretroviral therapy.

Variables	Estimate (β) ^a	OR	95% CI	P
rs187238 (<i>IL18</i>) CC genotype	0.0968	1.102	0.425–2.856	0.842
rs187238 (<i>IL18</i>) CG genotype	0.0113	1.011	0.345–2.966	0.984
rs187238 (<i>IL18</i>) GG genotype	−1.8540	0.157	0.027–0.921	0.040
Male sex	0.7233	2.061	0.848–5.011	0.110
ART regimen change	−0.4697	0.625	0.060–6.486	0.694
IP/r-containing ART	−0.7905	0.454	0.204–1.011	0.053
Pre-treatment CD4+ T-cell count (intercept)	0.0006	1.001	0.998–1.003	0.680
	0.1485	–	–	0.783

ART: antiretroviral therapy; CI: confidence interval; OR: odds ratio; PI/r: ritonavir-“boosted” protease inhibitor.

^a Model's internal validation: AUROC = 0.699; Z = −0.452; P = .651.

being appropriated to do predictions for immunological recovery failure occurrence.

4. Discussion

Evidence shows that only 5% of the cells are permissive to HIV-1 infection, generating new viral particles and culminating in apoptosis. The remaining 95% of cells are non-permissive and provoke an abortive infection through the recognition of the new synthesized HIV-1 cDNA by cytoplasmic DNA sensors, eliciting cell death by pyroptosis, suggested as death by activation of caspase-1 (Doitsh et al., 2010; Doitsh and Greene, 2016).

Functional analysis evidenced that the SNP located at the position −137 of the promoter region of the *IL18* gene alters the binding site of the H4TF-1 nuclear transcription factor. Thus, the G allele variant may decrease the bindings site strength, decreasing *IL18* gene expression (Giedraitis et al., 2001). Increase in the transcriptional level of some genes, among them the *IL18* gene, in the pyroptosis pathway, suggested as Caspase-1 activation cell death in immunological non-responders was observed in some studies, while lower levels of expression were observed in the group of immunologic responders (Bandera et al., 2018). Thus, considering more complete analyzes, the presence of the G allele may present as an influencing aspect that corroborates both studies.

Once interleukin-18 is released after activation via Caspase 1, it recruits cells to the infection site, stimulating the differentiation of naive T cells into Th1 response cells (Arimitsu et al., 2006), which in turn can be infected and the death via pyroptosis process activated, generating a chronic inflammatory cycle as demonstrated by (Doitsh and Greene, 2016). Thus, a decrease in the transcriptional level and IL-18 protein production would entail a functional reduction in recruitment of cells, resulting in the possible protective character of the polymorphism.

We observed higher levels of Caspase-1 activation RTE CD4+ T cells in INRs compared to the IR group. This difference may be related to the immune modulation in response of released cytokines and cellular contents during pyroptosis pathway (Man et al., 2017). After caspase-1 activation, pro-interleukins IL-18 is cleaved into their active form, which modulate inflammatory responses and promote cell activation and migration to the infection site (Boucher et al., 2016). Researches have also demonstrated that IL-18 is able to play functions in synergy with other interleukins inducing *CCR5* expression, which acts as the main co-receptor in the process of HIV-1 infection (Li et al., 2004; Rodriguez-Galan et al., 2005). Although the observed results were methodologically concise, these results may be observed carefully, since the scarce number of individuals with CC genotype.

An increased expression of this gene could be related to higher level of abortive infections in the thymus. As a result, it may indicate stimulate pyroptosis as the T lymphocytes migrate to mature in the

secondary lymphoid organs, as observed in our study according to pyroptotic by Caspase-1 RTE CD4+ T cells, which was higher in the INR group. Another explanation is based on cellular contents released by pyroptosis. Besides pro-inflammatory cytokines, pyroptotic cells also release pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively), stimulating the NLRP3 inflammasome activation and assembly (Guo et al., 2014). In the thymus, lipid molecules from membrane destruction or age-related lipid depositions act as triggers for NLRP3 activation, stimulating cell death by pyroptosis in T lymphocytes that migrate to peripheral blood (Chanpimol et al., 2017; Harijith et al., 2014; Lecossier et al., 2001).

Despite our study we did not find any association, some studies have shown the importance of *IFI16*, *NLRP3*, *CARD8*, and *IL1B* genes to HIV-1 infection and CASP-1 cell death pathway. *IFI16* was described as the main DNA sensor that triggers inflammasome pathways and pyroptosis in HIV-1 infection. It was suggested that HIV-1 has the capacity to increase the expression of NLRP3 inflammasome (Bandera et al., 2018; Pontillo et al., 2010) and *CARD8* protein is suggested as NLRP3 inhibitor, while *IL1 β* is correlated with inflammatory process (Langmia et al., 2016; Pontillo et al., 2013). However, the association of these polymorphisms observed in other studies was not observed in our population. So, new studies should be performed in other polymorphisms and populations, given the functional importance of these genes in pyroptosis pathway.

Thus, based on the analyzed data, the present study demonstrated a possible association of the *IL18* rs187238 polymorphism with immunological recovery failure in ART-treated patients.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.meegid.2019.103997>.

Declaration of Competing Interest

There is no conflict interest on this paper.

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