

# Increased Mitochondrial Mass Related To Heightened Pyroptosis of CD4<sup>+</sup>T Cells In HIV-1 Infection

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**Research**

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# Abstract

**Background:** In HIV-1 infection, over 90% CD4<sup>+</sup>T cells die of caspase-1 mediated pyroptosis. What governs the increased susceptibility of CD4<sup>+</sup>T cells to pyroptosis is poorly understood.

**Method:** Blood samples were obtained from 31 ART-naive HIV-infected patients, 29 ART-exposed HIV-infected patients, and 21 healthy control donors. Plasma levels of IL-18 and IL-1 $\beta$ , activated caspase-1, mitochondrial mass (MM) and mitochondrial fusion/fission genes of CD4<sup>+</sup>T subsets were measured.

**Results:** Significantly higher IL-18 level of plasma and MM level of CD4<sup>+</sup>T cells were found in HIV-infected patients than that in healthy controls, and the MM<sup>high</sup> phenotype manifested more sensitivity to caspase-1 mediated pyroptosis. Moreover, the increased MM was more pronounced in the early differentiated and inactivated CD4<sup>+</sup>T cells. However, higher MM was not intrinsically linked to T cell differentiation disorder or excessive activation of the CD4<sup>+</sup>T cells. Mechanistically, the increased mitochondrial mass was significantly correlated with an elevated expression level of the mitochondrial fusion gene-mitofusin1.

**Conclusion:** MM increase associates with heightened sensitivity of CD4<sup>+</sup>T cells to pyroptosis even in early differentiated and unactivated CD4<sup>+</sup>T cells in patients with HIV-1 infection, regardless of whether the patients are on HAART or not. These new revelations uncovered a previously unappreciated challenge to immune reconstitution with antiretroviral therapy.

## Introduction

In HIV infected individuals, progressive loss of CD4<sup>+</sup>T cells causes AIDS<sup>[1, 2]</sup>. After nearly four decades of extensive studies, various mechanisms related to CD4<sup>+</sup>T cell exhaustion have been proposed<sup>[3-5]</sup>. Among them, apoptosis and pyroptosis have been suggested to be the two main modes of CD4<sup>+</sup>T cell death, especially the latter which contributes to over 90% depletion of CD4<sup>+</sup>T cells during HIV disease progression<sup>[6, 7]</sup>. What governs this sensitivity to pyroptosis remains poorly understood.

Some studies have indicated a role of caspase-1 in mediating pyroptosis, even in abortive HIV infection<sup>[6, 8, 9]</sup>. Activated caspase-1 can precipitate mitochondrial disassembly and inhibit mitophagy to further amplify mitochondrial damage, suggesting a link between mitochondria and the pyroptosis process<sup>[10, 11]</sup>. We recently found an elevated mitochondrial reactive oxygen species (ROS) production, hyperpolarization of mitochondrial membrane potential and increased mitochondrial mass(MM)in CD4<sup>+</sup>T are concomitant with the progressive loss of CD4<sup>+</sup>T cells during HIV infection. Moreover, the MM was most significant increase in HIV + patients whose CD4<sup>+</sup>T cell counts are below 200/ul, indicating a possible relationship between the increased MM and CD4<sup>+</sup>T cell exhaustion<sup>[12]</sup>. Whether mitochondria change and pyroptosis act together to cause CD4<sup>+</sup>T cell depletion in the context of HIV infection may be biologically important.

HIV infection leads to chronic activation of the adaptive immune system, increased production of pro- and anti-inflammatory cytokines that potentially sensitized CD4<sup>+</sup>T cells to pyroptosis<sup>[13-17]</sup>. CD4<sup>+</sup>T cells exhibit a skewed maturation from naive (CD45RA<sup>+</sup>CCR7<sup>+</sup>) and central memory (CD45RA<sup>-</sup>CCR7<sup>+</sup>) towards the effector memory cells (CD45RA<sup>-</sup>CCR7<sup>-</sup>), this fact raised a question of whether the increased activation and skewed maturation prime CD4<sup>+</sup>T cells to pyroptosis or pyroptosis is a predictive factor of this process<sup>[15, 16, 18]</sup>. Successful highly active antiretroviral therapy (HAART) can result in normal CD4<sup>+</sup>T cell counts and undetectable HIV viremia in most HIV infected individuals<sup>[19, 20]</sup>. When patients stop taking HAART, however, their CD4<sup>+</sup>T cell counts are usually rapidly dropped to pretreatment levels<sup>[21, 22]</sup>. Therefore, it is of medical importance to carefully study the mechanisms of CD4<sup>+</sup>T cell exhaustion in patients with successful virological control. In this study, we examined whether there is a link between mitochondrial change and the sensitivity of CD4<sup>+</sup>T cells to pyroptosis using cells from HIV-1 infected patients who were either HAART naive or HAART treated and having undetectable viral load. Our data demonstrated that mitochondrial network contributes to susceptibility of CD4<sup>+</sup>T cells to pyroptosis, especially in CD4<sup>+</sup>T cells that are either at early differentiation stages and inactivated, regardless of whether the patients were under HAART or not. Furthermore, increased mitochondrial fusion gene level characterizes naive CD4<sup>+</sup>T cells in HIV-infected individuals, and the increased mitochondrial mass correlates with the expression of mitochondrial fusion gene-mitofusin1.

## Subjects And Methods

### Patients and control subjects

Patients from the Outpatient Clinic of Beijing Ditan Hospital, Capital Medical University were enrolled into this study. Written informed consent was obtained from each subject. Patients with chronic diseases, neoplasms, immune inflammatory diseases, other non-HIV-related diseases and metabolic complications were excluded from this study. A total of 60 HIV-infected man-sex-man (MSM) patients were included, 31 were ART naive at the time of enrolment, 29 have been on ART regimens for more than one year and have undetectable viremia. Twenty-one healthy HIV negative, age-adjusted MSM volunteers were included as controls (Table 1).

Table 1  
Clinical characteristics of the individuals included in the present study.

	Healthy controls	HIV-infected no-ART	HIV-infected ART-exposed
numbers	21	31	29
Sex(males/females)	21/21	31/31	29/29
age	28(21–41)	28(19–43)	30.5(21–45)
CD4T cells/ul	NA	306(17–956)	576(209–1280)
CD8T cells/ul	NA	821(115–2257)	820(418–2234)
VL(copies/ml)	–	22245(658-807126)	TND
ART regimen	–	–	TDF + 3TC + EFV
years of ART	–	–	1–2
Sexual preference	male homosexuality	male homosexuality	male homosexuality

### Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Ficoll-Hypaque (Amersham Biosciences, Amersham, Buckinghamshire, United Kingdom) from 20ml venous blood collected in EDTA tubes. Freshly isolated obtained PBMCs at  $1 \times 10^6$  per tube were stained as previously described<sup>[12]</sup>. The following monoclonal antibodies were used for T cell immunophenotyping: CD3-APC, CD4-APC-CY7, CD45RA-PE-CY7, CCR7-PERCP-CY5.5, HLADR-V500 (BD Biosciences, San Jose, CA). After antibody staining, the cells were incubated with 200 uL of 100 nM MitoLite TM Orange FM at 37°C for 15 minutes, washed twice with phosphate buffered saline. After surface staining with antibodies and mitochondrial staining with MitoLite, Caspase 1-FITC was used for intracellular staining. The isotype control of FITC, PE, V500 and PERCP-CY5.5 was performed for each experiment to determine gates of them.

### Isolation of naive CD4<sup>+</sup> T cells.

Naive CD4<sup>+</sup>T cells were enriched by MACS negative selection using the naive CD4<sup>+</sup>T isolation kit (AutoMACS; Miltenyi Biotec). The purity of isolated cells > 95% based on assay with CD4-APC-CY7 and CD45RA-PE-CY7 staining and FACS analysis.

### RNA Isolation and mitochondrial related mRNA detection by qRT-PCR

Total RNA from naive CD4<sup>+</sup>T cells was extracted using QIAamp RNA Mini Kit (Qiagen). The transcript levels of mitofusin1(mfn1), mitofusin2 (Mfn2), Optic atrophy 1 (OPA1) and dynamin-related protein1 (Drp1) were measured using a quantitative real-time reverse transcription PCR (qRT-PCR) assay, beta-

globin was used as an internal control gene. All the probes and primers used were purchased from Thermo Fisher.

## **IL-18 and IL-1 $\beta$ measurement**

Plasma levels of IL-18 and IL-1 $\beta$  were quantified by the Luminex xMAP technology of R&D Systems Luminex Assays (ZLXSAHM-06).

## **Plasma HIV-1 viral load and CD4<sup>+</sup>T cell count**

Plasma HIV-1 RNA levels and CD4<sup>+</sup>T cell counts were measured as described in previous studies twice per year<sup>[23]</sup>, and both measurements were included in the National Quality Assurance Programs.

## **Statistical analysis**

Group data were expressed as median and interquartile range (IQR) or mean and Standard Deviation when appropriate. For data that cannot be assumed to have a normal distribution, comparisons among several groups were made using a one-way Kruskal-Wallis test ( $p < 0.05$  was considered statistically significant). If the Kruskal-Wallis test indicated significance, the Mann-Whitney test was used for post hoc analysis for comparisons between two groups, with Bonferroni corrections for multiple comparisons, for which  $p < 0.05 \times 2/k$  ( $k-1$ ) was considered statistically significant ( $k$  refers to the number of groups). The correlation between the level of mitochondrial mass and pyroptosis of CD4<sup>+</sup>T subsets was analyzed with the Pearson correlation test. The above-mentioned analyses were performed using either Prism5.0 (GraphPad, La Jolla, CA, USA) or SPSS 13.0 (College Station, TX, USA) software.

# **Results**

## **1. CD4<sup>+</sup>T cells display higher level of MM in HIV infected patients**

To examine the possible involvement of mitochondria in pyroptosis of CD4<sup>+</sup>T cells in HIV infection, we first measured the MM of freshly isolated PBMC from HIV + patients and healthy donors using a mitochondrial specific dye (MitoLite TM Orange FM), which binds mitochondrial membrane independently of the membrane potential and gives an index of mitochondrial mass based on staining intensity<sup>[12, 24]</sup>. When gated on the CD4<sup>+</sup>T cells, two distinct populations can be observed: one exhibiting higher MM and the other with lower MM (Fig. 1A). The percentage of CD4<sup>+</sup>T from HIV-patients with high MM (78.0%  $\pm$  5.9%,  $n = 31$ ) was found to be significantly higher compared with that from healthy donors (71.8  $\pm$  7.0%,  $n = 21$ ,  $P = 0.0023$ ) (Fig. 1B). Furthermore, there is a greatly increased mean fluorescence intensity (MFI) of MM, an index of the MM per cell, in CD4<sup>+</sup>T from HIV-patients (MFI = 203.4  $\pm$  28.0) compared with that from healthy donors (MFI = 171.2  $\pm$  23.3,  $P = 0.0002$ ).

No difference in MM<sup>High</sup> was found between CD4<sup>+</sup>T cells from patients with ( $n = 29$ ) and without ( $n = 31$ ) ART treatment (76.7% $\pm$ 4.7% VS 78.0%  $\pm$  5.9% ). Treated HIV-patients had significantly higher levels of

MM<sup>High</sup> CD4<sup>+</sup>T cells (76.7%±4.7%, n = 31) than uninfected healthy donors (71.8 ± 7.0%,n = 21, P = 0.014), and increased MFI of MM (MFI = 192.2.4 ± 21.0 VS MFI = 171.2 ± 23.3, P = 0.0044) (Fig. 1B). Our data revealed that a significant change in mitochondrial mass characterizes CD4<sup>+</sup>T cells in HIV-1 infected patients with or without ART.

## 2. MM<sup>high</sup>CD4<sup>+</sup>T cells from HIV infected patients have a tendency towards pyroptosis

Next, we explored whether CD4<sup>+</sup>T cells from HIV-infected patients exhibiting MM<sup>high</sup> phenotype are more sensitive to pyroptosis compared to those that were MM<sup>low</sup>. Firstly, measurement of CD4<sup>+</sup>T cell pyroptosis by using caspase-1 staining revealed that the ratio of CD4<sup>+</sup>T cells expressing activated caspase-1 was significant higher in HIV-1 infected patients (n = 31, 44.8 ± 15.1) than in healthy controls (n = 21, 32.7 ± 14.2, P = 0.0081). No difference in caspase-1 was found between CD4<sup>+</sup>T cells from patients with (n = 29) or without ART treatment (47.3 ± 15.2 vs 44.8 ± 15.1, P = 0.6359); Treated HIV-patients had significantly higher levels of caspase-1(+) CD4<sup>+</sup>T cells (n = 31; 47.3 ± 15.2) compared to healthy donors (n = 21; 32.7 ± 14.2, P = 0.0022). In addition, the expression of IL-18 was significant higher in HIV-1 infected patients (n = 29 ) than in healthy controls (n = 25) (474.4 ± 36.0 vs 267.1 ± 22.34, P < 0.0001), and the expression level decreased to the normal level after the viremia successful suppression (n = 24, 312.7 ± 18.9 vs 267.1 ± 22.34, P = 0.1276). There was on statistically significantly difference in the expression of IL-1β among three groups (Fig. 2A).

Moreover, CD4<sup>+</sup>T cells undergone caspase-1 activated pyroptosis have a higher MFI MM compare to caspase-1(-) CD4<sup>+</sup>T cells in the three groups (healthy controls: n = 21, 26.4 ± 13.4 VS 17.8 ± 9.4, P = 0.0277; untreated HIV-patients: n = 31, 43.3 ± 19.1 VS 30.1 ± 15.5, P = 0.0023; treated HIV-patients: n = 29, 41.2 ± 15.1 VS 28.6 ± 15.1, P = 0.0024). The difference of MFI MM between caspase-1(-) CD4<sup>+</sup>T cells and caspase-1(+) CD4<sup>+</sup>T cells was more significant in HIV + patients than in healthy controls (P = 0.0277 VS P = 0.0023). MM<sup>high</sup> CD4<sup>+</sup>T cells were found to be more sensitive to caspase-1 activated pyroptosis (40.6% ±13.3%) compared to MM<sup>low</sup> cells (28.0%±12.1% ) in untreated HIV-infected patients (Fig. 2B).

Furthermore, significant correlation was found between the MFI of MM and caspase-1(+) percentage in CD4<sup>+</sup>T cells from HIV-patients regardless of whether they were on ART or not (n = 60, r = 0.7067 P < 0.0001). Significant correlation was also observed between the percentage of MM<sup>high</sup> and caspase-1 in CD4<sup>+</sup>T cells from two HIV + groups (r = 0.6921 P < 0.0001) (Fig. 2C). Therefore, our data clearly demonstrate that increased MM is linked to sensitivity of CD4<sup>+</sup>T cells to pyroptosis, and MM<sup>high</sup> CD4<sup>+</sup>T cells from HIV + groups are more sensitive to pyroptosis than that from healthy controls.

## 3. Over expression of HLA-DR alone does not explain increased MM in CD4<sup>+</sup>T cells from HIV + individuals

Next, we investigated whether the MM of CD4<sup>+</sup>T cells are associated with their activation status (surface expression of the HLA-DR marker). A significantly increased percentage of HLA-DR + cells was found in CD4<sup>+</sup>T cells from untreated HIV + patients compared to those from healthy donors (24.4%±17.2% VS 7.9%

$\pm 3.3\%$ ,  $P < 0.0001$ ). Although a significantly decreased percentage of HLA-DR<sup>+</sup> cells was found in CD4<sup>+</sup>T cells from treated HIV<sup>+</sup> patients compared to untreated HIV<sup>+</sup> patients ( $12.2\% \pm 4.9\%$  VS  $24.4\% \pm 17.2\%$ ,  $P = 0.0017$ ), treated HIV<sup>+</sup> patients still had significantly higher percentage of HLA-DR<sup>+</sup> cells compared with healthy donors ( $P = 0.0018$ ) (Fig. 3A). MM was then measured in HLA-DR<sup>+</sup> and HLA-DR<sup>-</sup> CD4<sup>+</sup> T cells. HLA-DR<sup>+</sup>CD4<sup>+</sup>T cells tend to have a higher MM<sup>high</sup> percentage compared to HLA-DR<sup>-</sup>CD4<sup>+</sup>T cells in the three groups (Healthy controls:  $n = 21$ ,  $78.8 \pm 6.0$  VS  $71.1 \pm 7.1$ ,  $P = 0.0018$ ; Untreated HIV<sup>+</sup> patients:  $n = 31$ ,  $82.2 \pm 5.4$  VS  $76.0 \pm 4.8$ ,  $P = 0.0004$ ; Treated HIV<sup>+</sup> patients:  $n = 29$ ,  $82.0 \pm 4.1$  VS  $76.0 \pm 4.8$ ,  $P < 0.0001$ ). However, the change of MM level was significant only in HLA-DR<sup>-</sup>CD4<sup>+</sup>T cells but not in HLA-DR<sup>+</sup>CD4<sup>+</sup>T cells in the context of HIV infection (Fig. 3B). We found a significant inverse correlation between HLA-DR<sup>+</sup> percentage and MM<sup>high</sup> percentage in CD4<sup>+</sup> T cells from HIV infected patients regardless of whether they were on ART or not ( $r = -0.3379$ ,  $P = 0.0005$ ). No correlation was found between HLA-DR<sup>+</sup> percentage and caspase-1<sup>+</sup> percentage in CD4<sup>+</sup>T cells from HIV infected patients (Fig. 3C). These results demonstrate that the increased HLA-DR<sup>+</sup> percentage had a low negative effect on the increased MM in CD4<sup>+</sup>T cells, but it cannot influence the linkage between increased MM and pyroptosis sensitivity of CD4<sup>+</sup>T cells in HIV-1 patients.

#### **4. Increased mitochondrial mass (MM) in CD4<sup>+</sup>T cells is mainly caused by HIV infection but not the disordered differentiation of CD4<sup>+</sup>T cells**

Above data have shown that an increased MM is linked to pyroptosis sensitivity of CD4<sup>+</sup>T cells, however, it is unknown whether MM is independent of differentiation levels of CD4<sup>+</sup>T cells. In HIV-1 infection, CD4<sup>+</sup>T cells exhibit a skewed maturation from naive (CD45RA<sup>+</sup>CCR7<sup>+</sup>) and central memory (CD45RA<sup>-</sup>CCR7<sup>+</sup>) toward the effector memory (CD45RA<sup>-</sup>CCR7<sup>-</sup>) compartment [15, 16]. Therefore, the observed differences in MM between caspase-1(+) CD4<sup>+</sup>T cells and caspase-1(-) CD4<sup>+</sup>T cells, at least in part, could be accounted for by their different maturation levels. No significant change was observed in the percentages of naive (CD45RA<sup>+</sup>CCR7<sup>+</sup>) CD4<sup>+</sup>T cells, central memory (CD45RA<sup>-</sup>CCR7<sup>+</sup>), and effector memory (CD45RA<sup>-</sup>CCR7<sup>-</sup>) CD4<sup>+</sup>T cells from HIV<sup>+</sup> patients compared to healthy donors (Fig. 4A). This is in agreement with previously published data that a significant change can only be found in AIDS patients<sup>[25]</sup>. Higher percentages of MM<sup>high</sup> were found in naive CD4<sup>+</sup>T cells and central memory CD4<sup>+</sup>T cells from untreated HIV<sup>+</sup> patients compared with those from healthy controls (naive CD4<sup>+</sup>T:  $76.1\% \pm 7.2\%$  VS  $68.0\% \pm 6.2\%$ ,  $P = 0.0005$ ; CM CD4<sup>+</sup>T:  $80.0\% \pm 6.4\%$  VS  $73.0\% \pm 7.2\%$ ,  $P = 0.0028$ ). Comparable percentages of MM<sup>high</sup> effector memory CD4<sup>+</sup>T cells was found in HIV<sup>+</sup> patients and healthy controls ( $79.5\% \pm 5.8\%$  VS  $74.5\% \pm 7.5\%$ ,  $P = 0.0165$ ), but a non-significant trend was found between naive CD4<sup>+</sup>T cells percentages and MM<sup>high</sup> percentage of CD4<sup>+</sup>T cells (Fig. 4B). No correlation was found either between MM of CD4<sup>+</sup>T cells and central memory CD4<sup>+</sup>T percentage or between MM of CD4<sup>+</sup>T cells and effector memory CD4<sup>+</sup>T cells' percentage (Fig. 4C). Although increased MM is most evident in naive CD4<sup>+</sup>T cells, the MM<sup>high</sup> percentages in three CD4<sup>+</sup>T subsets from HIV<sup>+</sup> patients were all higher than

those from healthy controls. Thus, higher mitochondrial mass (MM) in CD4<sup>+</sup>T cell is mainly associated with HIV infection but not the disordered differentiation of CD4<sup>+</sup>T cells.

## 5. Increased mitochondrial fusion characterizes naive CD4<sup>+</sup>T cells from HIV-1 infection patients

Based on the above data, HIV-1 infection leads to increased MM of CD4<sup>+</sup>T cells and caspase-1 activated pyroptosis of CD4<sup>+</sup>T cells, especially those CD4<sup>+</sup>T cells of early differentiation status. Thus, we next examined whether mitochondrial fission and fusion balance is altered in naive CD4<sup>+</sup>T cells, if so, how it contributes to the increased MM of CD4<sup>+</sup>T cells in HIV-1 infection. Mitochondrial fission is mediated by a dynamin family member Drp1 (dynamin-related protein 1). Mitochondrial fusion between mitochondrial outer membranes is mediated by Mfn1 (mitofusin1) and Mfn2 (mitofusin2), whereas fusion between mitochondrial inner membranes is mediated by Opa1 (optic atrophy protein 1)<sup>[26–28]</sup>. After isolation of the naive CD4<sup>+</sup>T cells (CD45RA<sup>+</sup>CD45RO<sup>-</sup>) from HIV + patients and healthy controls, we measured the expression level of Drp1 mRNA, Mfn1 mRNA, Mfn2 mRNA, and Opa1 mRNA.

A significantly increased expression of Mfn1, Mfn2 and Opa1 in naive CD4<sup>+</sup>T cells was found in HIV + patients compared to those in healthy controls (Mfn1: P = 0.0017; Mfn2: P = 0.0217; Opa1: P = 0.0006), suggesting an increased mitochondrial fusion of naive CD4<sup>+</sup>T cells in HIV infection. We did not detect out Drp1 expression in CD4<sup>+</sup>T cells from either HIV + patients or healthy controls. No differences in Mfn1, Mfn2 and Opa1 expression were found between naive CD4<sup>+</sup>T cells from patients under antiretroviral treatment (n = 13) and those from untreated patients (n = 14). Treated HIV + patients still had significantly higher levels of Mfn1, Mfn2 and Opa1 expression compared to healthy donors (Mfn1: P = 0.0124; Mfn2: P = 0.0299; Opa1: P = 0.0166) (Fig. 5A). Our data revealed that an increased mitochondrial fusion characterizes naive CD4<sup>+</sup>T cells from HIV-1 infected patients regardless of whether or not they were on ART regimens.

## 6. Over expression of Mfn1 is related to an increased mitochondrial mass of naive CD4<sup>+</sup>T cells in patients with HIV-1 infection

A significant correlation was found between the MFI of MM and Mfn1 expression in naive CD4<sup>+</sup>T cells obtained from HIV-infected patients regardless of being on ART or not (n = 28, r = 0.4343 P = 0.0051) (Fig. 5C). In contrast, no significant correlation was observed between the percentages of MM and Mfn2 (OPA1) expression levels. Our data indicate that an increased mitochondrial fusion contributes the increased MM, in association with an elevated expression of Mfn1 gene. We did not find a correlation between caspase-1 levels and mfn1 expression (data not shown).

## Discussion

Recently, more and more studies have demonstrated that mitochondria act as an important role in the differentiation, activation, and deathly pathway of immunity cells<sup>[29–31]</sup>. However, whether mitochondrial

toxicity was the mechanism of the exhaust of CD4<sup>+</sup>T cells has not yet received enough attention in the context of HIV infection. Based on our previous findings that mitochondrial mass(MM)in CD4<sup>+</sup>T cells gradually increases in association with the progressive loss of CD4<sup>+</sup>T cells during HIV infection<sup>[12]</sup>, and that pyroptosis accounts for 90% of CD4<sup>+</sup>T cells' death<sup>[6]</sup>, we hypothesized that the increased MM in CD4<sup>+</sup>T cells may be related to their susceptibility to pyroptosis, and tested the hypothesis by using MitoLite TM Orange FM as an index of MM<sup>[32]</sup> and caspase-1 staining for pyroptosis. To our knowledge, this is the first time that the relationship between MM and pyroptosis in CD4<sup>+</sup>T cells from HIV infected patients has been investigated. We found that MM was significantly increased in caspase-1 activated CD4<sup>+</sup>T cells from HIV infected patients, indicating that the MM<sup>high</sup> phenotype specifically characterizes pyroptosis of CD4<sup>+</sup>T cells. The MM<sup>high</sup> phenotype of CD4<sup>+</sup>T cells could not be attributed to disordered CD4<sup>+</sup>T cell differentiation status, as similar levels of MM were found in CD45RA<sup>+</sup>CCR7<sup>+</sup>, CD45RA<sup>-</sup>CCR7<sup>+</sup>, and CD45RA<sup>-</sup>CCR7<sup>-</sup> in CD4<sup>+</sup>T cells from untreated HIV-infected patients, indicating that this mitochondrial parameter is independent of the disordered differentiation status often presents in HIV infection. Meanwhile, over activation cannot explain the relationship between an increased MM and a susceptibility to pyroptosis as we did not find significant correlation between HLA-DR expression and MM level, or the percentage of caspase-1 expression.

In this study, we have demonstrated that MM<sup>high</sup> CD4<sup>+</sup>T cells are sensitive to pyroptosis, directly linking mitochondria to the pyroptosis sensitivity of CD4<sup>+</sup>T cells. This phenomenon should be more significant in immunological non-responders as the highest MM was observed in patients whose CD4<sup>+</sup>T cell below 200/ul <sup>[12]</sup>. Due to the small sample size, we did not analyze our current data based on CD4 cell number strata. As our previous large-scale study has clearly confirmed the presence of mitochondrial toxicity in CD4<sup>+</sup>T cells in HIV-infected patients<sup>[12]</sup>.

Based on previous study, activated caspase-1 precipitating mitochondrial disassembly and inhibiting mitophagy to amplify mitochondrial damage, we hypothesize that the increased MM, as a result of either larger or more organelles, intensifies the damage in mitochondria, further enhances pyroptosis of CD4<sup>+</sup>T cells in HIV infection. We cannot distinguish whether high MM corresponds to larger organelles or higher numbers by the flow-cytometry-based assay, as both would result in higher levels of staining. Therefore, the expression of mitochondrial fusion and fission genes was measured, because excess mitochondrial fusion leads to larger mitochondria and heightened mitochondrial fission leads to higher numbers <sup>[26, 33]</sup>. Increased mitochondrial fusion genes (mfn1, mfn2, Opr1) was found in naive CD4<sup>+</sup>T cells from HIV individuals, compared to that in healthy controls, indicating excessive mitochondrial fusion may exist in CD4<sup>+</sup>T cells during HIV infection. This may be one of the mechanisms responsible for the increased MM related pyroptosis, because excess mitochondrial fusion contributes to an accumulation of damaged mitochondria. More importantly, we found a significant correlation between mfn1 gene expression and MM levels, indicating mfn1 gene may play an important role in MM accumulation in CD4<sup>+</sup>T cells. Although no significant correlation was found between caspase-1 and mfn1 expression, we cannot rule out an over expression of mfn1 may be related to the susceptibility to pyroptosis in CD4<sup>+</sup>T cells. Further

study is needed to explore how HIV infection influences the expression of mfn1 gene, and whether the mfn1 gene encodes an important signal molecule in the pathway of pyroptosis in CD4<sup>+</sup>T cells. We did not detect the expression of mitochondrial fission gene- drp1 in peripheral blood CD4<sup>+</sup>T cells from both HIV infected individuals and healthy donors. Mitochondrial fusion can maximize oxidative capacity in response to toxic stress and mitigate environmental damage through the exchange of proteins and lipids with other mitochondria [26, 28, 33]. However, when a certain threshold of damage is reached, increased fusion may lead to cell death because of lacking renewal [33–35]. Thus, further study needed to explore how the increased MM affects caspase-1 activated pyroptosis of CD4<sup>+</sup>T cells in HIV-1 infection, and if the excessive fusion has effect on it.

As the mitochondrial toxicity of HAART drugs will be obviously appeared in patients treated for more than two years<sup>[12]</sup>, patients who were treated for 1–2 years were recruited to represent the effects of chronic HIV infection in this study. Successful HAART can restore CD4<sup>+</sup>T cell counts and lead to undetectable HIV viremia in most HIV infected individuals [36, 37]. Unfortunately, non–AIDS-defining events such as cardiovascular disease (CVD) and non–AIDS-defining malignancies are still more prevalent in HAART-treated human immunodeficiency virus (HIV)–infected adults than in uninfected adults [38–40], persist immune activation and chronic inflammation often indentified as the mechanism of this phenomenon [41–44]. In this study, we further found the obvious pathological change (increased of MM and pyroptosis sensitivity) of CD4<sup>+</sup>T cells from HAART-treated patients whose viremia was effectively suppressed, which provide us a new pathway to explain the clinical phenomenon. As antiviral therapy alone is not sufficient to restore all immunological functions in HIV-infected patients, paying attention to improve the mitochondrial function and ameliorate the susceptibility to pyroptosis of CD4<sup>+</sup>T cells may provide new therapeutic targets.

## Conclusion

This study suggests that MM increase associates with heightened sensitivity of CD4<sup>+</sup>T cells to pyroptosis even in early differentiated and unactivated CD4<sup>+</sup>T cells in patients with HIV-1 infection, regardless of whether the patients are on HAART or not. These new revelations uncovered a previously unappreciated challenge to immune reconstitution with antiretroviral therapy. Further studies of the detail role of mitochondrial disturbance on the exhaust of CD4<sup>+</sup>T cells should be performed.

## Abbreviations

HIV, human immunodeficiency virus; HAART, highly active antiretroviral therapy; ART, antiretroviral therapy; MM, mitochondrial mass; PBMC, peripheral blood mononuclear cells; CD, cluster of differentiation; MSM, man-sex-man; MACS: magnisort cell separation; FACS: fluorescence activating cell sorter; HLA-DR, human leukocyte antigen-DR; IL: interleukin; CM, central memory; EM, effect memory; Drp1, dynamin-related protein1; Mfn, mitofusin; Opa1, optic atrophy protein 1; CVD, cardiovascular disease.

# Declarations

## Ethics approval and consent to participate

Written informed consent was obtained from each subject. The local human research subject review board approved the study. The study was carried out in accordance with the tenets of the Declaration of Helsinki.

## Consent to publish

Not applicable

## Availability of data and materials

Not applicable

## Competing interests

The authors have no conflicts of interest to declare.

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## Author's Contributions

F.Y., C.M., J.X., L.L., and H.Z. contributed to recruiting the patients; FY contributed to collecting and processing samples; C.M., J.X., L.L., and H.Z. collected clinical data; F.Y., S.S., X.Z., X.X., S.Y., and Y.T. contributed to performing laboratory-based data collection. L.W., F.Z designed the experiments; F.Z. was responsible for analyzing the data; FY, L.W., F.Z., XJ. wrote the manuscript.

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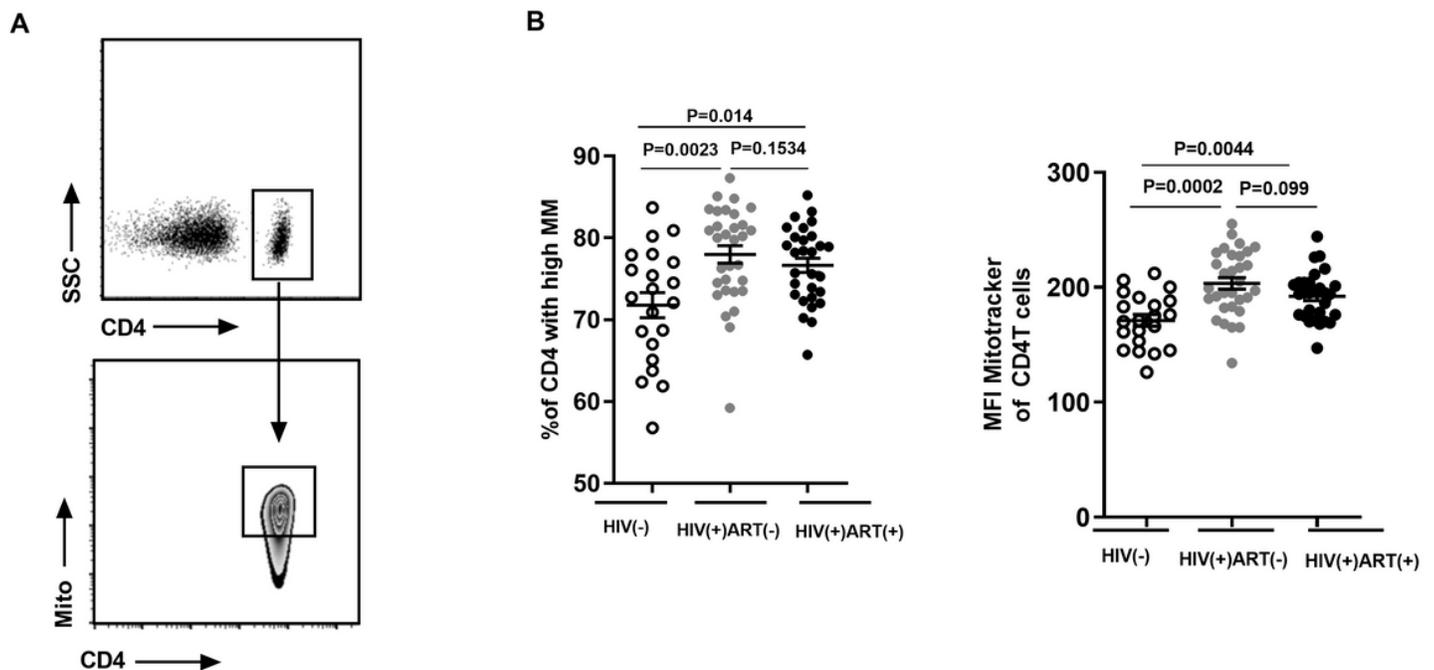
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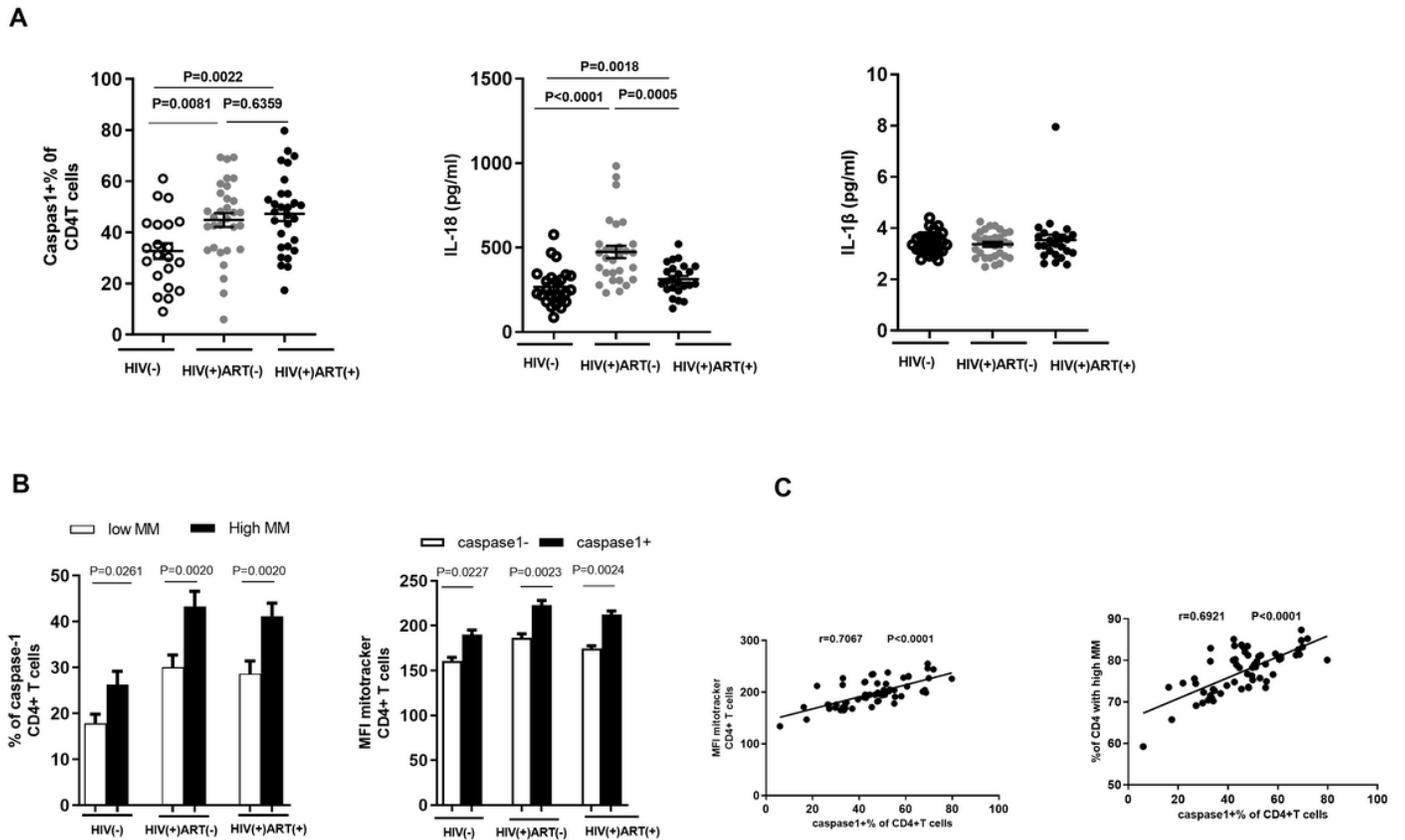
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## Figures



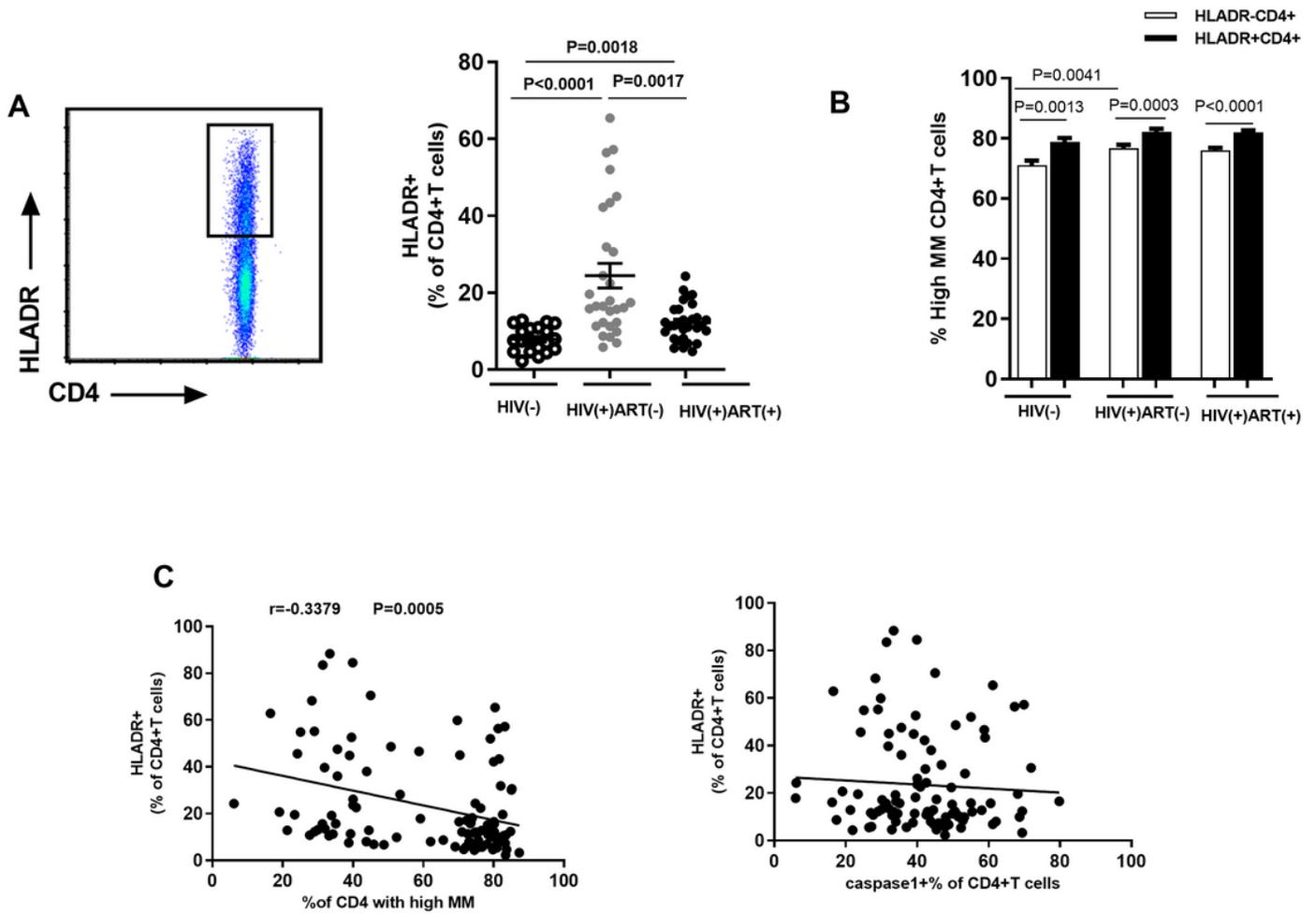
**Figure 1**

CD4+T cells express higher MM in HIV infected patients compared with ones in Healthy donors. (A) The gated CD4T cells were divided into two groups: one exhibit high MM, the other exhibit low MM. (B) The frequency of MMhigh CD4+T cells subsets and the mean fluorescence intensity of MM in CD4T cells were evaluated in peripheral blood from healthy donors (n=21), ART naive HIV-infected patients (n=31), ART exposed HIV- infected patients (n=29).



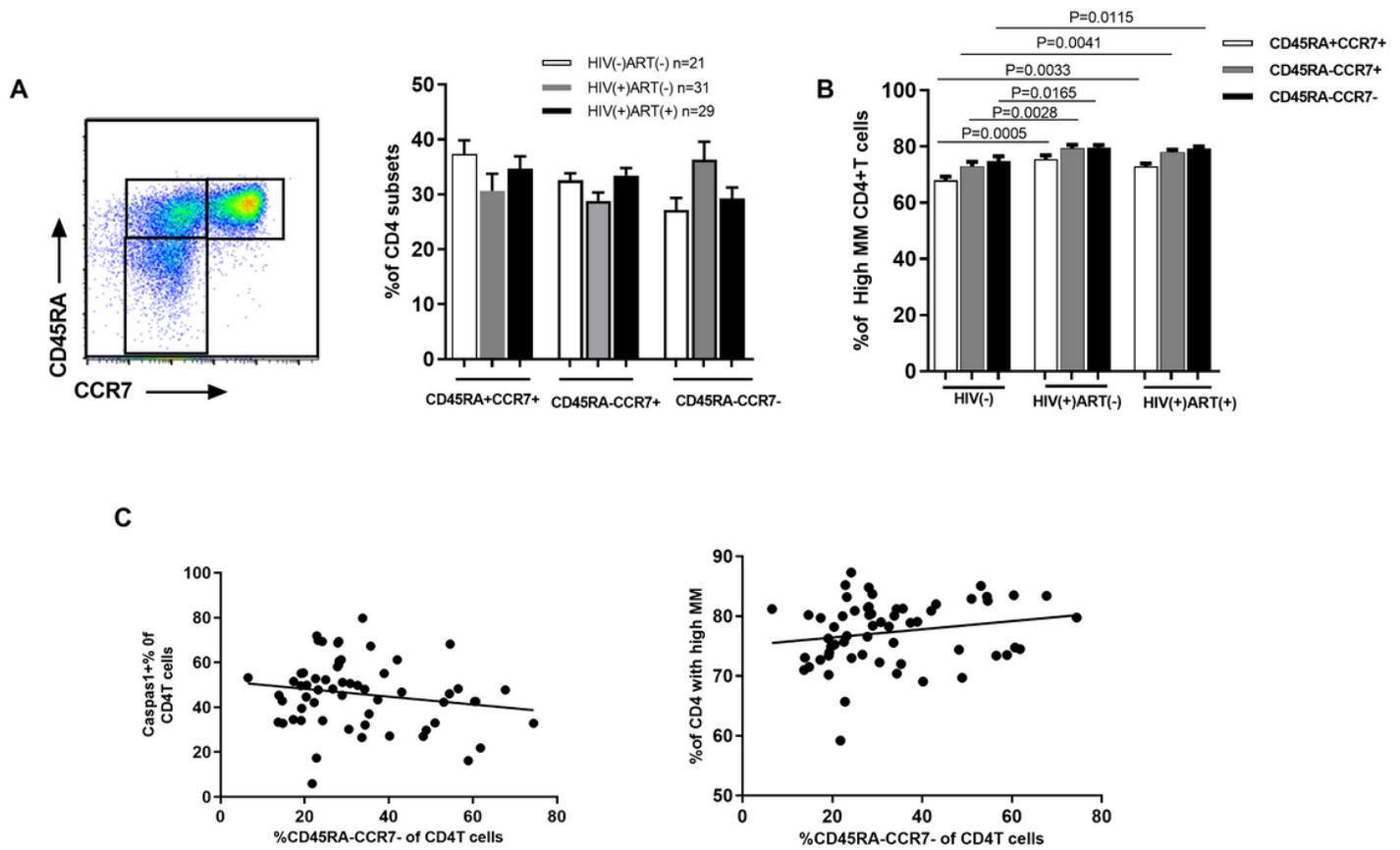
**Figure 2**

MMhigh CD4+T cells from HIV patients are trend to propyptosis. (A) Percentages of caspase-1 (+) in CD4+T cells from healthy donors, HIV (+) ART (-) patients, and HIV (+) ART (+) patients. IL-18 and IL-1 $\beta$  in plasma from healthy donors, ART naive HIV-infected patients, and ART exposed HIV- infected patients. (B) Pyroptosis was measured in MMhigh and MMlow CD4+T cells by caspase-1 staining. Pooled data showing the percentage (%) of caspase-1+ CD4+T cells in MM high and MM low CD4+T cells and the MFI of MM in caspase-1(+) CD4+T cells and caspase-1(-) CD4+T cells. (C) The correlation between the frequency of MMhigh CD4+T cells and the percentage of caspase-1 (+) CD4+T from HIV-infected patients regardless of under ART (n=60); The correlation between the MFI of MM in CD4+T cells and caspase-1 expression level in the context of HIV infection.



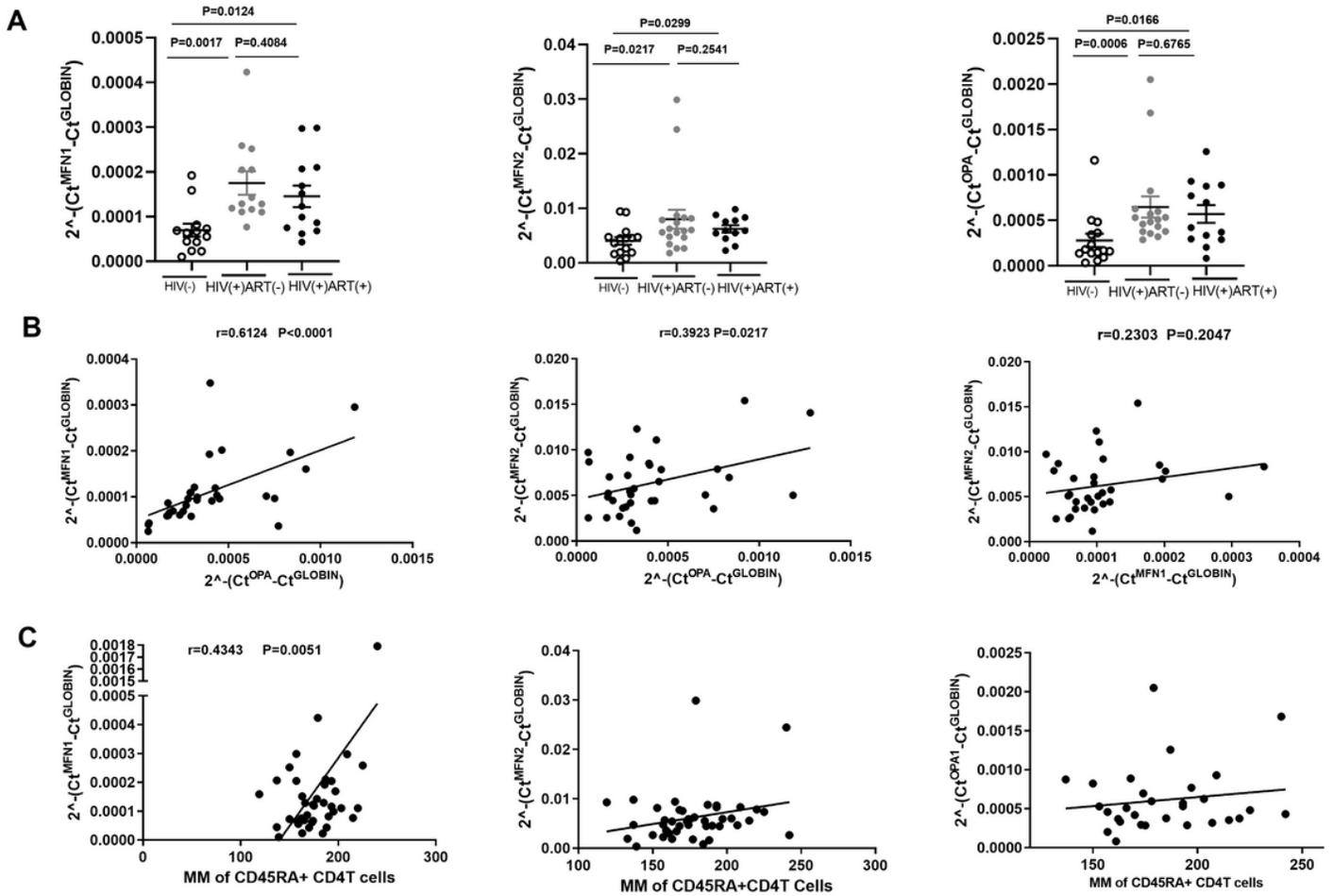
**Figure 3**

HLADR expression, although a negative correlation of MM, does not explain the pyroptosis of CD4+T cells. (A) Respectively flow cytometry showing HLADR expression gating in CD4+T cells. The frequency of HLADR (+) CD4+T subsets in peripheral blood from healthy donors, untreated HIV infected individuals and treated HIV infected individuals. (B) MMhigh percentages were measured in HLADR (+) and HLADR (-) CD4+T cells in peripheral blood from healthy donors, untreated HIV infected individuals and treated HIV infected individuals. (C) The correlation between the MFI of MM in CD4+T cells and HLADR expression level in the context of HIV infection ( $r = -0.3317$ ,  $p = 0.0005$ ). The correlation between the frequency of HLADR expression level and the percentage of caspase-1 (+) CD4+T from HIV-infected patients regardless of under ART ( $n = 60$ ).



**Figure 4**

MM expression level in CD4+T cells of different differentiated status. (A) Respectively flow cytometry showing naive (CD45RA+CCR7+), central memory (CD45RA-CCR7+) and effector memory (CD45RA-CCR7-) subsets gating strategy. The frequency of naive, central memory, and effector memory CD4+T subsets in peripheral blood CD4+T cells from healthy donors, untreated HIV infected individuals and treated HIV infected individuals. (B) MMhigh percentage was measured in naive, central memory and effector memory CD4+T subsets in peripheral blood from healthy donors, untreated HIV infected individuals and treated HIV infected individuals. (C) The correlation between the MFI of MM in CD4+T cells and the percentages of effector memory (CD45RA-CCR7-) CD4+T subsets in the context of HIV infection. The correlation between the frequency of effector memory (CD45RA-CCR7-) CD4+T subsets and the percentage of caspase-1 (+) CD4+T from HIV-infected patients regardless of under ART (n=60).



**Figure 5**

Increased mitochondrial fusion characterizes naive CD4+T cells from HIV-1 infection patients, and Mfn1 related to the increased MM. (A) A significantly increased Mfn1, Mfn2 and Opa1 expression of naive CD4+T cells was found from two HIV+ patients groups compared with ones from Healthy controls. (B) The correlation among the mRNA expression of three mitochondrial fusion genes (Mfn1, Mfn2, and Opa1). (C) Significant correlation was found between the MFI of MM and Mfn1 expression in naive CD4+T cells from HIV-patients regardless of whether under ART (n=28), but not found in Mfn2 and Opa1.