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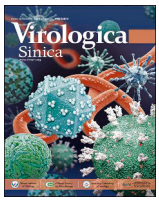

Research article

# The expression of CD39 on NK cells relates to poor HIV-1 suppression in treatment-naïve HIV-1-infected individuals: Association with elevated IL-10 secretion and TIGIT co-expression

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ABSTRACT

CD39 exerts an inhibitory effect on tumour progression by impairing the cytotoxic capacity of natural killer (NK) cells against cancer cells. However, the impact of CD39 expression on the non-cytolytic functions of NK cells in treatment-naïve human immunodeficiency virus type 1 (HIV-1)-infected individuals remains poorly understood. In this study, thirty-four individuals with acute HIV-1 infection (AHI), thirty-eight with chronic HIV-1 infection (CHI), and twenty-four HIV-1-negative healthy controls (HC) were enrolled to explore the role of CD39 expression on NK cells in HIV-1 suppression at different infection stages. Flow cytometry was employed to analyze the immune phenotype and functional characteristics of NK cells. We found that CD39 expression on NK cells was significantly upregulated following HIV-1 infection, and its positive rate was positively associated with HIV-1 viral load in both AHI and CHI individuals. Compared with $CD39^-$ NK cells, $CD39^+$ NK cells exhibited reduced activation; in AHI individuals, the activation level of $CD39^+$ NK cells was positively associated with HIV-1 viral load but inversely correlated with $CD4^+$ T-cell counts. In CHI individuals, the interleukin-10 (IL-10)-producing capacity of total NK cells, $CD39^+$ NK cells, and $CD39^-$ NK cells was enhanced and positively correlated with HIV-1 viral load. Additionally, across the AHI and CHI groups, the overall IL-10-secreting ability of NK cells was positively correlated with the frequency of $CD39^+$ NK cells. In both AHI and CHI individuals, $CD39^+$ NK cells showed lower T-cell immunoglobulin and ITIM domain (TIGIT) expression than $CD39^-$ NK cells, while the $CD39^+TIGIT^+$ NK cell subset displayed significantly stronger IL-10-secreting capacity. POM-1, an inhibitor of CD39 ectonucleotidase activity, could enhance IL-10 secretion by NK cells in both HIV-1-infected individuals and the majority of healthy controls, but attenuate interferon-γ (IFN-γ) secretion by NK cells in HIV-1-infected individuals. In contrast, the CD39-blocking antibody A1 reduced IFN-γ secretion without affecting IL-10 secretion by NK cells in both HIV-1-infected individuals and healthy controls. Our findings reveal a novel $CD39^+$ NK cell-associated mechanism that contributes to ineffective HIV-1 control, and suggest that CD39, alone or combined with TIGIT, may serve as a promising target to restore antiviral NK cell function in treatment-naïve individuals living with HIV-1.

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## INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) infection remains a major global public health challenge, characterized by progressive impairment of the host immune system, primarily driven by the depletion and dysfunction of $CD4^+$ T cells, as well as persistent viral replication that evades immune clearance.

Natural killer (NK) cells, a key component of the innate immune system, play a pivotal role in the early defense against viral infections, including HIV-1 (Björkström et al., 2022). These cells exert antiviral effects through multiple mechanisms: direct cytotoxicity against virus-infected cells via the release of perforin and granzyme, and modulation of adaptive immune responses through the secretion of cytokines such as interleukin-10 (IL-10) and interferon-γ (IFN-γ) (Flórez-Álvarez et al., 2018). However, in HIV-1 infection, particularly during the acute (AHI) and chronic (CHI) phases, NK cells exhibit marked functional dysregulation, including reduced cytotoxicity, impaired cytokine production, and altered surface receptor expression (Alter et al., 2005). Extracellular adenosine triphosphate (eATP) is a danger signal released by damaged and dying cells, and acts as an immunostimulatory signal that promotes inflammation. CD39 converts eATP, a pro-inflammatory damage-associated molecular pattern (DAMP), into adenosine monophosphate (AMP). Subsequent conversion of AMP to adenosine by CD73 (ecto-5′-nucleotidase) generates an immunosuppressive microenvironment, as adenosine signals through the A2A receptor to inhibit the activation and function of T cells and NK cells (Häusler et al., 2011; Seshadri et al., 2019).

CD39 is markedly upregulated in NK cells from cancerous tissues compared with those in the peripheral blood of patients with oesophageal squamous cell carcinoma, and CD39 expression in tumour-infiltrating NK cells predicts poor prognosis (Zheng et al., 2020). The lytic activity of NK cells against ovarian cancer cells is enhanced by blockade of CD39 (Häusler et al., 2011). CD39 expression in NK cells is significantly increased in trauma patients with subsequent infection (Seshadri et al., 2019). During HIV-1 infection, CD39 is significantly upregulated, with higher expression in untreated individuals than in treated individuals (Qian et al., 2022). CD39 expression on NK cells in circulating blood is also upregulated during chronic HBV infection, and $CD39^+$ NK cells from individuals with chronic hepatitis and hepatocellular carcinoma exhibit higher cytotoxicity and capacity to produce IFN-γ and TNF-α (Kang et al., 2023). CD39 has immunosuppressive effects on several immune cell types, including NK cells, via the generation of adenosine. Recent studies have reported upregulated CD39 expression on immune cells during HIV-1 infection, but its specific role in regulating NK cell function, particularly in the context of viral control in treatment-naïve individuals, remains poorly defined.

IL-10, which was first identified for its ability to inhibit the activation and effector functions of T cells, monocytes, and macrophages (Moore et al., 2001; Saxton et al., 2021), is an immunoregulatory cytokine with both anti-inflammatory and pro-inflammatory properties and is frequently dysregulated during viral infections (Saxton et al., 2021). In addition to $CD4^+$ and $CD8^+$ T cells and B cells, macrophages, monocytes, dendritic cells (DCs), neutrophils, mast cells, eosinophils, and NK cells can secrete IL-10 in response to different stimuli (Rutz and Ouyang, 2016; Ouyang and O'Garra, 2019). IL-10 production by $CD39^+$ NK cells from HIV-1-infected individuals has also been reported (Qian et al., 2022). IL-10 is upregulated in multiple cell types during viremic HIV-1 infection and reversibly inhibits the proliferative capacity and cytokine secretion of HIV-specific T cells (Brockman et al., 2009).

In addition to CD39, T cell immunoreceptor with Ig and ITIM domains (TIGIT), an inhibitory receptor expressed on NK cells and T cells, has been implicated in NK cell dysfunction during chronic viral infections, including HIV-1 infection (Vendrame et al., 2020; Sánchez-Cerrillo et al., 2025; Wang et al., 2025). TIGIT binds to its ligands (e.g., CD155) on target cells, delivering inhibitory signals that reduce NK cell cytotoxicity and cytokine production (Kyrysyuk and Wucherpfennig, 2023). Emerging studies suggest cross-talk between CD39 and TIGIT in regulating immune cell function (Brauneck F. et al., 2021; Brauneck E. et al., 2025), but their combined roles in modulating NK cell IL-10 secretion and HIV-1 control remain unclear.

In this study, we hypothesized that CD39 expression on NK cells enhances IL-10 secretion, thereby contributing to NK cell dysfunction and poor HIV-1 suppression in treatment-naïve individuals. To test this hypothesis, we systematically analyzed CD39 expression on NK cells from treatment-naïve HIV-1-infected individuals (including AHI and CHI subgroups) and healthy controls (HC). We further investigated the association between CD39 expression, NK cell IL-10 secretion, and clinical markers of HIV-1 disease (viral load and $CD4^+$ T-cell count). Additionally, we explored the potential interplay between CD39 and TIGIT in regulating IL-10 secretion by NK cells. Lastly, the CD39-blocking agents were employed to further investigate their effects on IL-10 and IFN-γ secretion by NK cells. Our findings aim to clarify the role of $CD39^+$ NK cells in HIV-1 pathogenesis and identify novel targets for improving antiviral immunity in HIV-1-infected individuals.

## RESULTS

### The clinical parameters of the participants in this study

Thirty-four acute HIV-1-infected individuals and thirty-eight chronic HIV-1-infected individuals from the Beijing Primary Infection Monitoring & Observation (PRIMO) clinical cohort were recruited for this study. The basic information and clinical parameters are provided in Table 1. There was no significant difference between the AHI individuals and the CHI individuals in terms of these parameters, including age, ratio of $CD4^+/CD8^+$ T-cell count, and HIV-1 viral load, except for the $CD4^+$ T-cell count and $CD8^+$ T-cell count.

### Upregulation of $CD39^+$ NK cells in untreated HIV-1-infected individuals and its association with HIV-1 viremia

The expression of CD39 in NK cells is determined by gating representative plots derived from flow cytometry (Fig. 1A). In HIV-1-infected individuals at the acute and chronic infection stages, CD39 expression

**Table 1**
Basic information and clinical parameters of the subjects.

| | Healthy controls (n = 24) | HIV-1-infected individuals | | |
|---|---|---|---|---|
| | | AHI (1∼3 M) (n = 34) | CHI (9 M∼3Y) (n = 38) | *P*-value |
| Age (Y) | 33 (25–62) | 34 (20–58) | 35 (21–60) | *ns* |
| $CD4^+$ T-cell counts (cells/μL) | *ND* | 544 (155–1084) | 449 (1170–947) | 0.043 |
| $CD8^+$ T-cell counts (cells/μL) | *ND* | 1363 (457–3220) | 1061 (417–2564) | 0.047 |
| CD4/CD8 ratio | *ND* | 0.49 (0.08–1.71) | 0.49 (0.12–1.42) | *ns* |
| Viral load (Log10, copies/mL) | *NA* | 4.32 (2.23–6.45) | 4.63 (2.54–6.41) | *ns* |
| Antiretroviral therapy | *NA* | *ND* | *ND* | |

Data in Table 1 is showed in mean (range); AHI: acute HIV-1 infection; CHI: chronic HIV-1 infection; *ND*: not done; *NA*: not applicable; *ns*: not significant; *M*: month; *Y*: year.

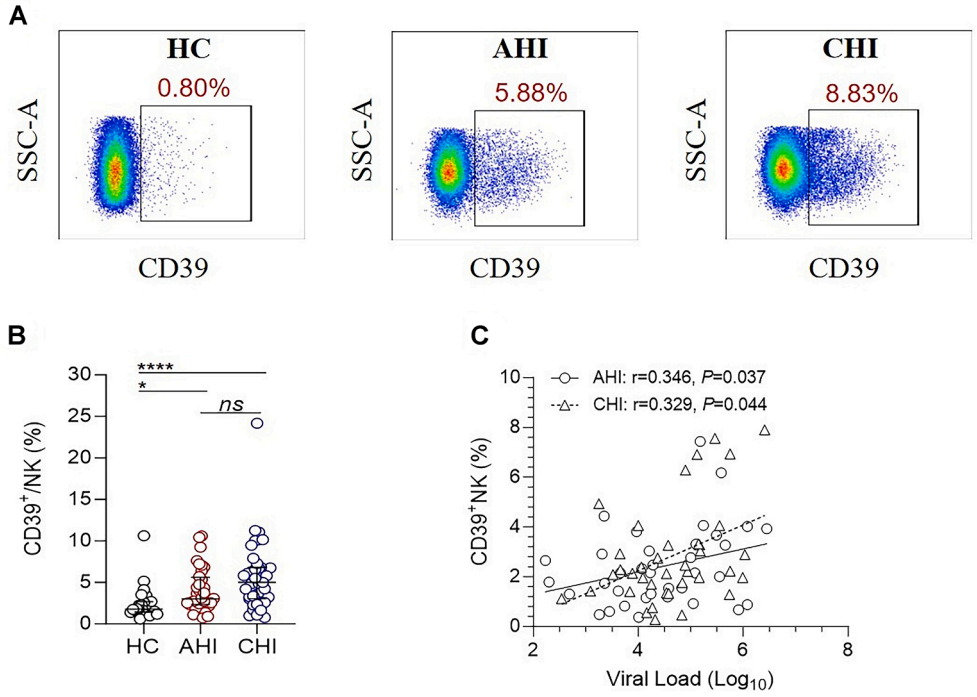


**Fig. 1.** CD39 upregulation on NK cells following HIV-1 infection. **A** Representative flow cytometry plots of CD39 expression in NK cells from healthy controls (HC), acute HIV-1 infection (AHI) and chronic HIV-1 infection (CHI) individuals. **B** Proportion of CD39 expression in NK cells. HC: healthy controls with negative HIV-1 antibodies; AHI: 1–3 months after HIV-1 infection without receiving ART; CHI: 9 months to 3 years after HIV-1 infection without receiving ART. **C** Positive correlation between the proportion of $CD39^+$ NK cells and the HIV-1 viral load in AHI and CHI individuals. Data are presented as the median and interquartile range. Statistical analyses were performed as described in Materials and Methods.*$P < 0.05$; ****$P < 0.0001$; *ns*, not significant.

on NK cells was significantly elevated. Notably, there was no statistically significant difference in $CD39^+$ NK cell frequency between the AHI and CHI groups, indicating that CD39 upregulation on NK cells is induced early after HIV-1 infection and persists through the chronic phase in the absence of treatment (Fig. 1B). Correlation analysis further revealed that the frequency of $CD39^+$ NK cells was significantly positively correlated with HIV-1 viral load in both AHI and CHI individuals (Fig. 1C). To validate whether persistent viral replication drives CD39 upregulation, we analyzed an additional cohort of HIV-1-infected individuals who achieved durable viral suppression through effective antiretroviral therapy (ART). As shown in Supplementary Fig. S1, the proportion of $CD39^+$ NK cells in ART-treated individuals was not significantly different from that in HC. These results indicate that the sustained presence of HIV-1 replication is critical for maintaining the upregulation of $CD39^+$ NK cells.

## Diminished activation of $CD39^+$ NK cells relative to $CD39^-$ NK cells and its inverse correlation with $CD4^+$ T-cell counts in AHI individuals

CD69 is a well-recognized early activation marker for NK cells, whose expression correlates with NK cell cytotoxicity and effector function (Dons'koi et al., 2011). We therefore used CD69 expression to assess NK cell activation, with representative flow cytometry plots displayed in Fig. 2A. As shown in Fig. 2B, compared with HC, CD69 expression on total NK cells was significantly upregulated in the CHI group, while the AHI group exhibited a trend toward increased CD69 expression that did not reach statistical significance ($P = 0.053$, Fig. 2B).

When stratifying NK cells by CD39 expression, we observed a striking functional dichotomy: in both AHI and CHI groups, the activation activity of $CD39^+$ NK cells was significantly lower than that of $CD39^-$ NK cells. Moreover, HIV-1 infection selectively enhanced the activation of $CD39^-$ NK cells, as evidenced by a higher frequency of $CD69^+$ cells. This frequency showed a trend toward significance in AHI individuals and was significantly higher in CHI individuals compared to HC. In contrast, the activation of $CD39^+$ NK cells remained unchanged relative to HC (Fig. 2C). Correlation analyses further revealed that the activation level of $CD39^+$ NK cells was positively associated with HIV-1 viral load (Fig. 2D) and significantly inversely correlated with the $CD4^+$ T-cell count (Fig. 2E) in AHI individuals but not in CHI individuals. These findings suggest that although $CD39^+$ NK cells are relatively less activated, their activated subset correlates with both increased viral replication and reduced $CD4^+$ T-cell counts specifically in the acute phase, implying a potential role of activated $CD39^+$ NK cells in $CD4^+$ T cells depletion during early HIV-1 infection.

## Enhanced IL-10-producing ability of $CD39^+$ NK cells correlated positively with the HIV-1 viral load in chronic HIV-1 infection

To explore whether CD39-expressing NK cells release IL-10, a cytokine with established immunosuppressive effects that may impair anti-HIV-1 immunity, we quantified IL-10 secretion by total NK cells and $CD39^+$ NK cell subsets using flow cytometry. Representative gating strategies for identifying IL-10-secreting NK cells (including $CD39^+$ and $CD39^-$ subsets) are provided in Fig. 3A. Compared with HC, the frequency of IL-10-secreting total NK cells was significantly higher in both AHI and CHI individuals (Fig. 3B). This finding confirms that HIV-1 infection induces a systemic increase in IL-10-secreting NK cells, regardless of acute or chronic stage. In both HC and HIV-1-infected individuals, the proportion of $CD39^+IL\text{-}10^+$ NK cells was lower than that of $CD39^-IL\text{-}10^+$ NK cells. However, relative to HC, the frequencies of $CD39^+IL\text{-}10^+$ NK cells and $CD39^-IL\text{-}10^+$ NK cells were significantly

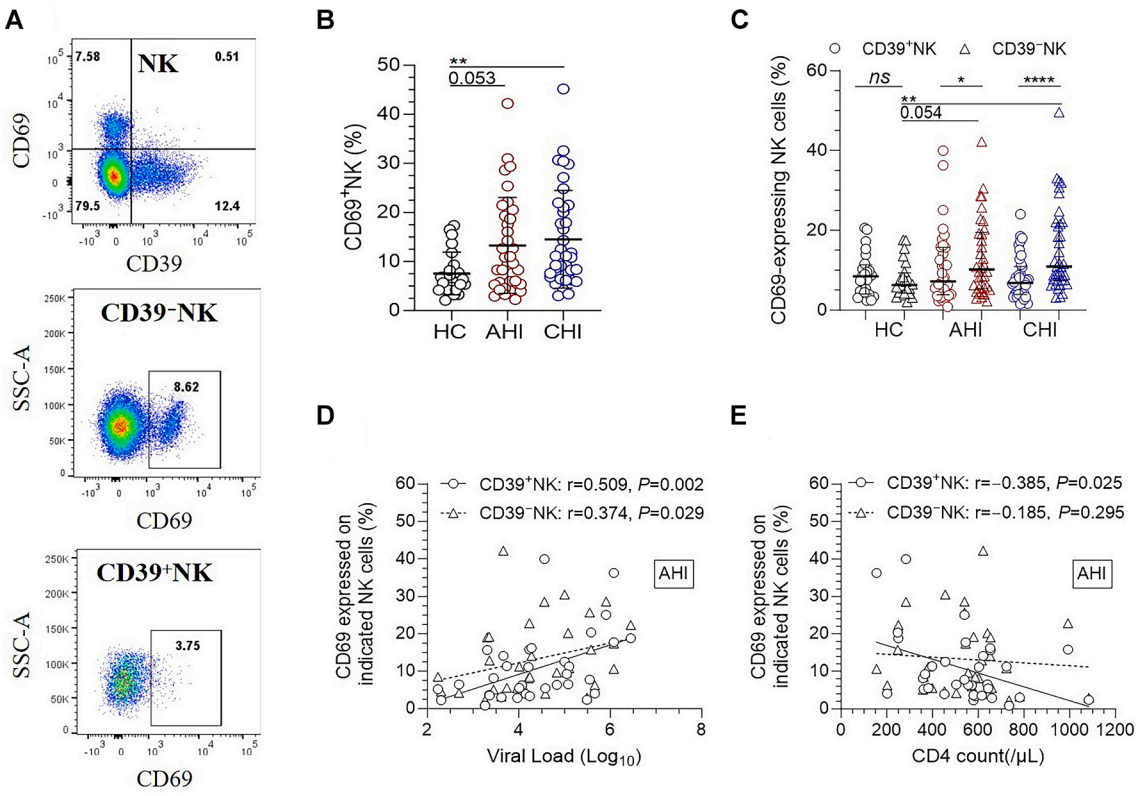


**Fig. 2.** Activation of CD39-expressing NK cells in HIV-1-infected individuals. **A** Flow cytometry example diagrams of CD69 expression on total NK cells, $CD39^+$ NK cells and $CD39^-$ NK cells. **B** Percentage of CD69 expressed at the surface of NK cells from healthy controls (HC, n = 24), acute HIV-1 infection (AHI, n = 34) and chronic HIV-1 infection (CHI, n = 38). HC: healthy controls with negative HIV-1 antibodies; AHI: 1–3 months after HIV-1 infection without receiving ART; CHI: 9 months to 3 years after HIV-1 infection without receiving ART. **C** Comparison of the activation ability of $CD39^+$ NK cells and $CD39^-$ NK cells. **D** Positive correlation between the activation level of $CD39^+$ NK cells, $CD39^-$ NK cells and the HIV-1 viral load in AHI individuals. **E** Inverse correlation between the ability of $CD39^+$ NK cells and $CD4^+$ T-cell counts in AHI individuals. Data are presented as the median and interquartile range. Statistical analyses were performed as described in Materials and Methods. $^*P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$; *ns*, not significant.

elevated in both AHI and CHI groups (Fig. 3C), indicating that HIV-1 infection promotes the expansion of this double-positive subset.

Notably, across HC and HIV-1-infected individuals, the proportion of IL-10-secreting cells within $CD39^+$ NK cells was significantly higher than that within $CD39^-$ NK cells (Fig. 3D). This confirms that $CD39^+$ NK cells have an inherent advantage in IL-10 secretion, regardless of infection status. Moreover, HIV-1 infection further amplified IL-10 production in both subsets. Compared with HC, the frequency of IL-10-secreting cells among $CD39^+$ and $CD39^-$ NK cells was markedly elevated in AHI and CHI groups (Fig. 3D), indicating a systemic enhancement of NK cell IL-10-secretion that is potentially driven by HIV-1. In CHI individuals, the IL-10-secreting capacity of total NK cells, as well as $CD39^+$ and $CD39^-$ NK subsets, was significantly positively correlated with plasma HIV-1 viral load (Fig. 3E and F). Additionally, across AHI and CHI groups, the overall IL-10-secreting capacity of NK cells showed a positive correlation with the frequency of $CD39^+$ NK cells (Fig. 3G). These results demonstrate that $CD39^+$ NK cells are functional IL-10 producers. Their capacity to secrete IL-10, an inherent trait amplified by HIV-1 infection, is closely linked to HIV-1 progression, particularly viral load in the chronic stage.

## Lower TIGIT expression on $CD39^+$ NK cells, with enhanced IL-10 secretion by $CD39^+TIGIT^+$ NK cells

We previously demonstrated that TIGIT expression impairs the cytotoxic function and the IFN-γ secretion of NK cells (Zhang et al., 2021); however, whether TIGIT modulates the IL-10-producing capacity of $CD39^+$ NK cells remained unclear. In the present study, TIGIT expression in total NK, $CD39^+$ NK and $CD39^-$ NK cells was analyzed via flow cytometry (Fig. 4A). Across all groups, the frequency of $CD39^+TIGIT^+$ NK cells was lower than that of $CD39^-TIGIT^+$ NK cells (Fig. 4B). When comparing HIV-1-infected individuals with HCs, TIGIT expression was significantly upregulated in total NK cells, $CD39^+$ NK cells and $CD39^-$ NK cells from the CHI individuals, but not in those from the AHI individuals (Supplementary Fig. S2A, Fig. 4C). Notably, TIGIT expression levels in total NK cells, $CD39^+$ NK cells, and $CD39^-$ NK cells were positively correlated with HIV-1 viral load in AHI individuals (Supplementary Fig. S2B, Fig. 4D), whereas no such correlation was observed in CHI individuals. The frequency of NK cells coexpressing CD39 and TIGIT was significantly increased starting from the acute phase of HIV-1 infection (Fig. 4B), and this frequency was positively associated with HIV-1 viral load in AHI individuals and exhibited a trend toward a positive association in CHI individuals (Fig. 4E).

On the other hand, the number of $TIGIT^+IL\text{-}10^+$ NK cells was lower than that of $TIGIT^-IL\text{-}10^+$ NK cells across all groups (Supplementary Fig. S2C). Nevertheless, a higher proportion of $TIGIT^+$ NK cells relative to $TIGIT^-$ NK cells was capable of producing IL-10, and the IL-10-secreting capacity of both $TIGIT^+$ and $TIGIT^-$ NK cells increased significantly from the acute phase onward (Fig. 4F). Additionally, $CD39^+TIGIT^+$ NK cells exhibited a stronger ability to secrete IL-10 than $CD39^-TIGIT^+$ NK cells, and this secretory capacity of $CD39^+TIGIT^+$ NK cells was significantly enhanced in both AHI and CHI individuals compared with HC (Fig. 4G). These outcomes demonstrate that while $CD39^+$ NK cells consistently exhibit lower TIGIT expression compared to $CD39^-$ NK cells across study groups, the $CD39^+TIGIT^+$ NK cell subset possesses a markedly stronger IL-10 secreting capacity, which is further enhanced in the context of HIV-1 infection.

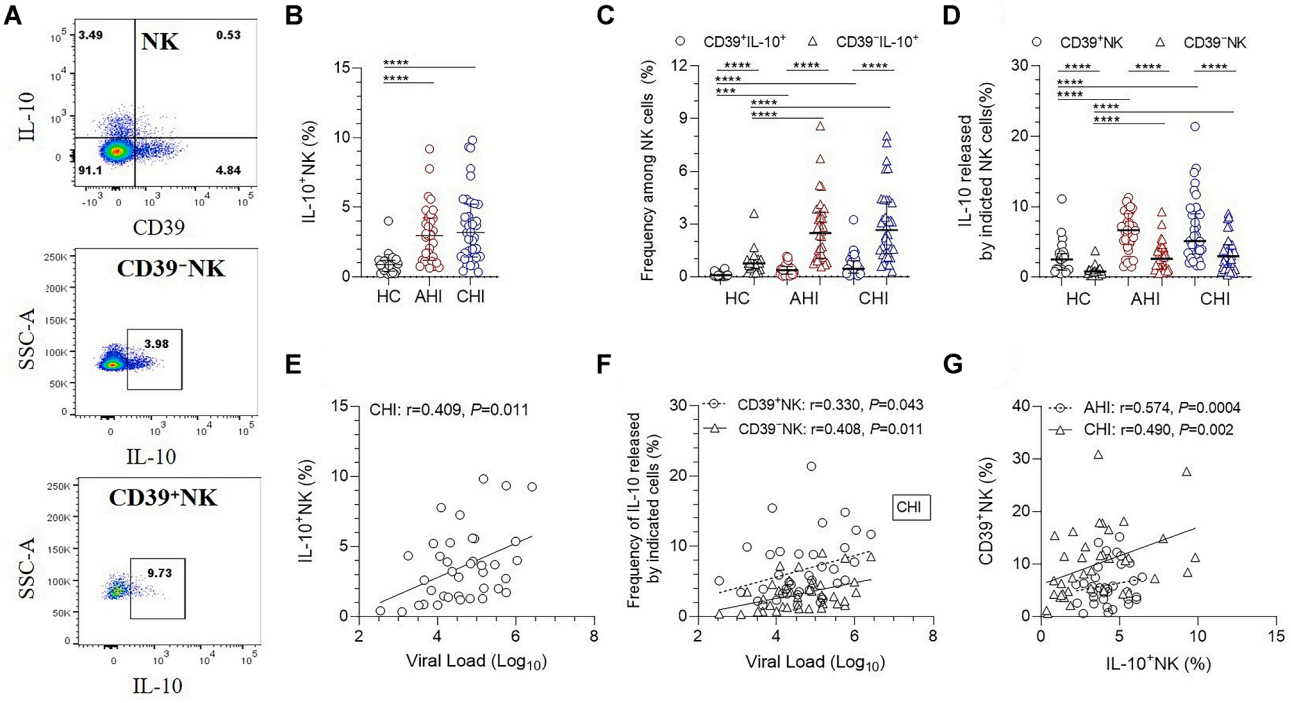


**Fig. 3.** The ability of CD39$^+$ NK cells to release IL-10 in HIV-1-infected individuals. **A** Flow cytometry example diagrams of IL-10 release by total NK cells, CD39$^+$ NK cells and CD39$^-$ NK cells. **B** The level of total NK cells to release IL-10 after HIV-1 infection. **C** Comparison of the percentage of CD39$^+$ IL-10$^+$ cells and CD39$^-$ IL-10$^+$ cells among total NK cells in healthy controls (HC, n = 24), acute HIV-1 infection (AHI, n = 34) and chronic HIV-1 infection individuals (CHI, n = 38). HC: healthy controls with negative HIV-1 antibodies. AHI: 1–3 months after HIV-1 infection without receiving ART. CHI: 9 months to 3 years after HIV-1 infection without receiving ART. **D** Comparison of the ability of CD39$^+$ NK cells and CD39$^-$ NK cells to release IL-10 from HC, AHI, and CHI individuals. **E, F** Positive correlation of the ability to produce IL-10 by total NK cells (**E**), CD39$^+$ NK cells, and CD39$^-$ NK cells (**F**) with HIV-1 viral load in CHI individuals. **G** Positive correlation between the proportion of CD39$^+$ NK cells and the proportion of IL-10$^+$ NK cells in AHI and CHI individuals. Data are presented as the median and interquartile range. Statistical analyses were performed as described in Materials and Methods. ***$P < 0.001$; ****$P < 0.0001$.

## CD39-targeting agents of POM-1 and A1 differentially modulate NK cell to secrete IL-10 and IFN-γ

POM-1 has been reported to have antitumor effects (Jiang et al., 2025). To investigate whether CD39 expression modulates NK cell production of IL-10 and IFN-γ, we employed two CD39-targeting agents with distinct mechanisms of action: the A1 antibody (a CD39 molecule blocker) and POM-1 (sodium polyoxotungstate, an inhibitor of CD39 ectonucleotidase activity). The secretion of IL-10 and IFN-γ by NK cells was then subsequently evaluated following treatment with these agents, with representative flow cytometry plots provided in Fig. 5A. In treatment-naive HIV-1-infected individuals, POM-1 significantly enhanced the IL-10-secreting capacity of NK cells relative to the no-blocking-reagent control group (Fig. 5B). Consistently, POM-1 also increased this IL-10-secreting ability in the majority of healthy subjects (Fig. 5C). In contrast, the A1 antibody failed to promote IL-10 secretion by NK cells in either HIV-1-infected individuals or healthy subjects (Fig. 5B and C). Further analysis of the fold change in the percentage of IL-10-producing NK cells confirmed that POM-1 was significantly more effective than A1 at driving NK cell-derived IL-10 secretion in HIV-1-infected individuals (Fig. 5D).

Conversely, the A1 antibody attenuated IFN-γ production by NK cells in both treatment-naive HIV-1-infected individuals and healthy subjects (Fig. 5E and F). POM-1 also reduced NK cell IFN-γ-secreting capacity, but this inhibitory effect was restricted to HIV-1-infected individuals and not observed in healthy controls (Fig. 5E and F). When analyzing the fold change in the percentage of IFN-γ-producing NK cells, A1 exhibited greater potency than POM-1 in inhibiting NK cell IFN-γ production in healthy subjects (Fig. 5G). Additionally, in HIV-1-infected individuals, the mean fluorescence intensity (MFI) of IFN-γ secreted by NK cells was significantly lower in the presence of A1 and POM-1 compared to the no-blocking-reagent group (Fig. 5H). In healthy controls, A1 treatment resulted in a significant reduction in IFN-γ MFI, whereas POM-1 showed a non-significant trend toward decreasing IFN-γ MFI (Fig. 5I). These findings indicate that POM-1 and A1 exert distinct effects on NK cell secretion of IL-10 and IFN-γ, highlighting the functional diversity of CD39-targeting strategies in modulating NK cell immune responses.

## DISCUSSION

Upon HIV invasion, numerous immune cells, including T lymphocytes, B lymphocytes and NK cells, undergo cell death, either as a consequence of immune system-mediated attack or direct viral cytotoxicity. This cell death, often involving necrosis of stressed or dying immune cells, leads to the release of adenosine triphosphate (ATP) into the extracellular space. As the rate-limiting enzyme in the adenosine metabolic pathway, CD39 acts as a critical “immunological switch”: it catalyzes the conversion of extracellular ATP (eATP) into adenosine monophosphate (AMP), which is further converted to adenosine by CD73. This process culminates in the accumulation of immunosuppressive adenosine, a key mediator of immune evasion.

CD39 is highly expressed in various human tumours types, and its upregulation is a well-documented mechanism enabling tumours to escape antitumor immune responses (Guo et al., 2022; Liu et al., 2022). While extensive research has focused on CD39's role in regulating immunity within the tumor microenvironment, its function in modulating the innate immune response to HIV-1 infection remains largely unexplored. In the present study, we observed a consistent upregulation of CD39 expression on NK cells following HIV-1 infection, with this elevation persisting into the chronic phase of infection.

Furthermore, in both AHI and CHI individuals who had not received ART, the frequency of CD39$^+$ NK cells was positively correlated with plasma HIV-1 viral load: higher viral loads were associated with a

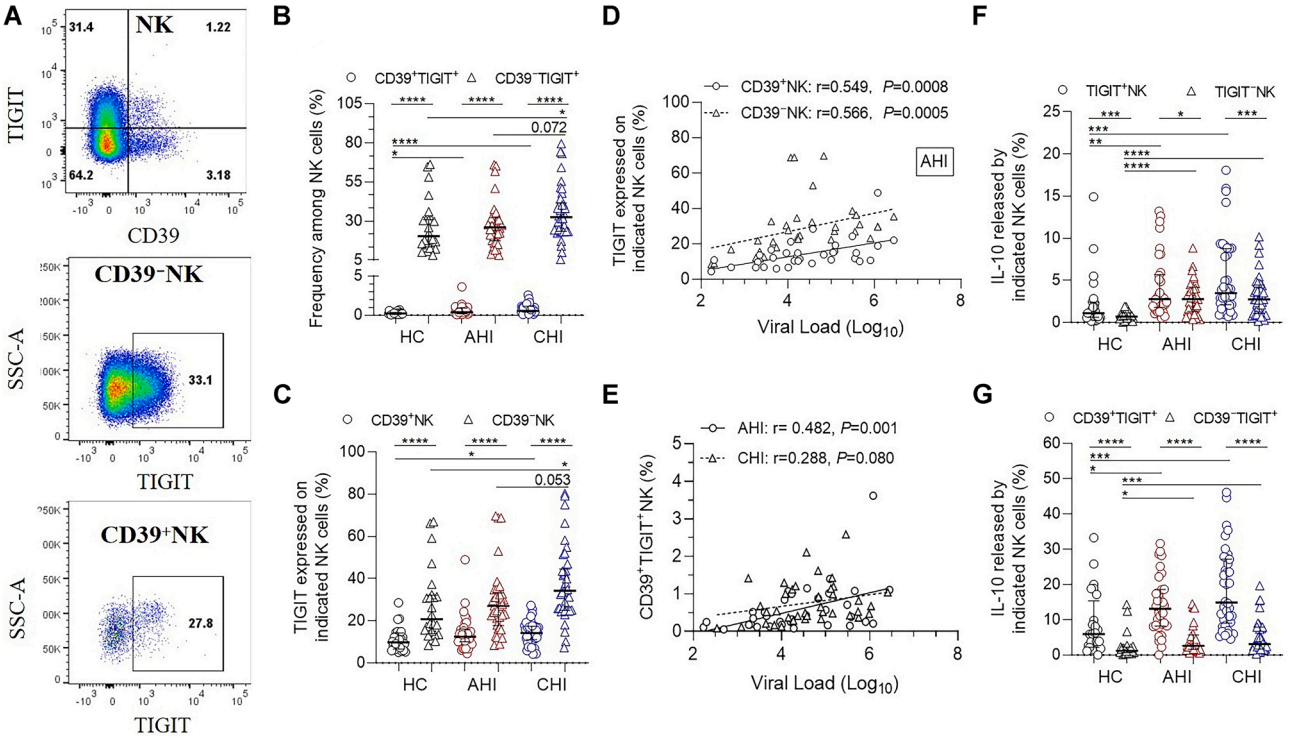


**Fig. 4.** TIGIT expression in CD39$^+$ NK cells and the ability of CD39$^+$ TIGIT$^+$ NK cells to secrete IL-10. **A** Flow cytometry example diagrams of TIGIT expressed in total NK cells, CD39$^+$ NK cells and CD39$^-$ NK cells. **B** Comparison of the frequencies of CD39$^+$TIGIT$^+$ and CD39$^-$TIGIT$^+$ subset within NK cells. HC (n = 24): healthy controls with negative HIV-1 antibodies. Acute HIV-1 infection individuals (AHI, n = 34): 1–3 months after HIV-1 infection without receiving ART. Chronic HIV-1 infection individuals (CHI, n = 38): 9 months to 3 years after HIV-1 infection without receiving ART. **C** Percentage of TIGIT expressed on CD39$^+$ NK and CD39$^-$ NK cells in HC, AHI and CHI individuals. **D** Positive correlation of the proportions of TIGIT expressed on CD39$^+$NK cells and CD39$^-$ NK cells with the HIV-1 viral load in AHI individuals. **E** Positive correlation of the frequency of CD39$^+$TIGIT$^+$ NK cells with HIV-1 viral load in HIV-1 individuals. **F** Comparison of the ability to secrete IL-10 by TIGIT$^+$ NK cells to TIGIT$^-$ NK cells. **G** Comparison of the ability to secrete IL-10 by CD39$^+$TIGIT$^+$ NK cells and CD39$^-$TIGIT$^+$ NK cells. Data are presented as the median and interquartile range. Statistical analyses were performed as described in Materials and Methods. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

greater number of CD39-expressing NK cells. Additionally, the proportion of CD39$^+$ NK cells in treated HIV-1-infected individuals was similar to that in healthy controls. These findings collectively demonstrate that HIV-1 infection drives CD39 upregulation on NK cells, and inadequate viral suppression, characterized by high viral loads, exacerbates this phenomenon. A potential mechanism underlying CD39 upregulation involves the elevated plasma levels of IL-6 and IL-15 observed during both acute and chronic HIV-1 infection (Huang et al., 2016). Prior studies have shown that IL-6 induces CD39 expression on tumor-infiltrating NK cells (Zheng et al., 2020), while IL-15 promotes the generation of CD39$^+$ NK cells (Kang et al., 2023). Thus, these proinflammatory cytokines may act as key drivers of CD39 upregulation on NK cells in the context of HIV-1 infection.

During the host response to viral infection, the transmembrane CD69 protein is highly upregulated in all immune cells, serving as a marker of early activation and tissue retention. In line with this, we observed a higher frequency of CD69$^+$ NK cells in the peripheral blood of AHI and CHI individuals compared to HC. However, a striking functional dichotomy emerged when NK cells were stratified by CD39 expression: HIV-1 infection selectively enhanced the activation of CD39$^-$ NK cells, whereas CD39$^+$ NK cells showed no increase in activation relative to HC. Furthermore, in both AHI and CHI groups, the activation of CD39$^+$ NK cells was significantly lower than that of CD39$^-$ NK cells. These results indicate that CD39 expression is associated with reduced NK cell activation ability. In AHI individuals specifically, the activation of CD39$^+$ NK cells, which was comparable to that in HC, was positively correlated with viral load but inversely correlated with CD4$^+$ T-cell count, indicating the failure of CD39$^+$ NK cells to suppress HIV-1 replication in the acute phase of infection. This observation suggests that CD39$^+$ NK cells are functionally ineffective at suppressing HIV-1 replication during the acute phase of infection and may even contribute to CD4$^+$ T-cell depletion, a hallmark of HIV-1 pathogenesis.

IL-10 is a multifunctional cytokine that orchestrates the balance between protective and pathogenic immune responses. While it plays a critical role in limiting excessive inflammation, IL-10 can also hinder pathogen clearance and promote chronic disease by suppressing the activity of effector immune cells (Rojas et al., 2017). In our study, CD39$^+$ NK cells exhibited a greater capacity to secrete IL-10 than CD39$^-$ NK cells across all study groups. However, a lower proportion of CD39$^+$ IL-10$^+$ NK cells compared to CD39$^-$ IL-10$^+$ NK cells was observed in HC and HIV-1-infected individuals. In both AHI and CHI individuals, the frequency of CD39$^+$ NK cells positively correlated with the overall IL-10-secreting capacity of NK cells, and this IL-10 production further correlated with HIV-1 viral load, with a positive association observed in CHI individuals. These findings suggest that CD39$^+$ NK cells impair HIV-1 suppression by promoting the secretion of IL-10, thereby potentially inhibiting the antiviral activity of multiple immune cells, such as Th1 and Th2 cells, NK cells, macrophages and dendritic cells (Rojas et al., 2017; Zhong et al., 2021).

Given the well-established inhibitory role of TIGIT in regulating NK cell function, we further explored whether TIGIT expression modulates IL-10 secretion in CD39$^+$ NK cells. Across HCs, AHI, and CHI individuals, TIGIT expression on NK cells was associated with elevated IL-10-secreting capacity, a phenomenon further amplified by HIV-1 infection. This is contrary to our prior observation that TIGIT$^+$ NK cells exhibit impaired secretion of the proinflammatory cytokines IFN-γ and TNF-α (Zhang et al., 2021). Notably, CD39$^+$ NK cells exhibited lower TIGIT expression than CD39$^-$ NK cells across all groups; however, coexpression of CD39 and TIGIT (in CD39$^+$TIGIT$^+$ NK cells) correlates with elevated IL-10 secretion. These observations align with prior

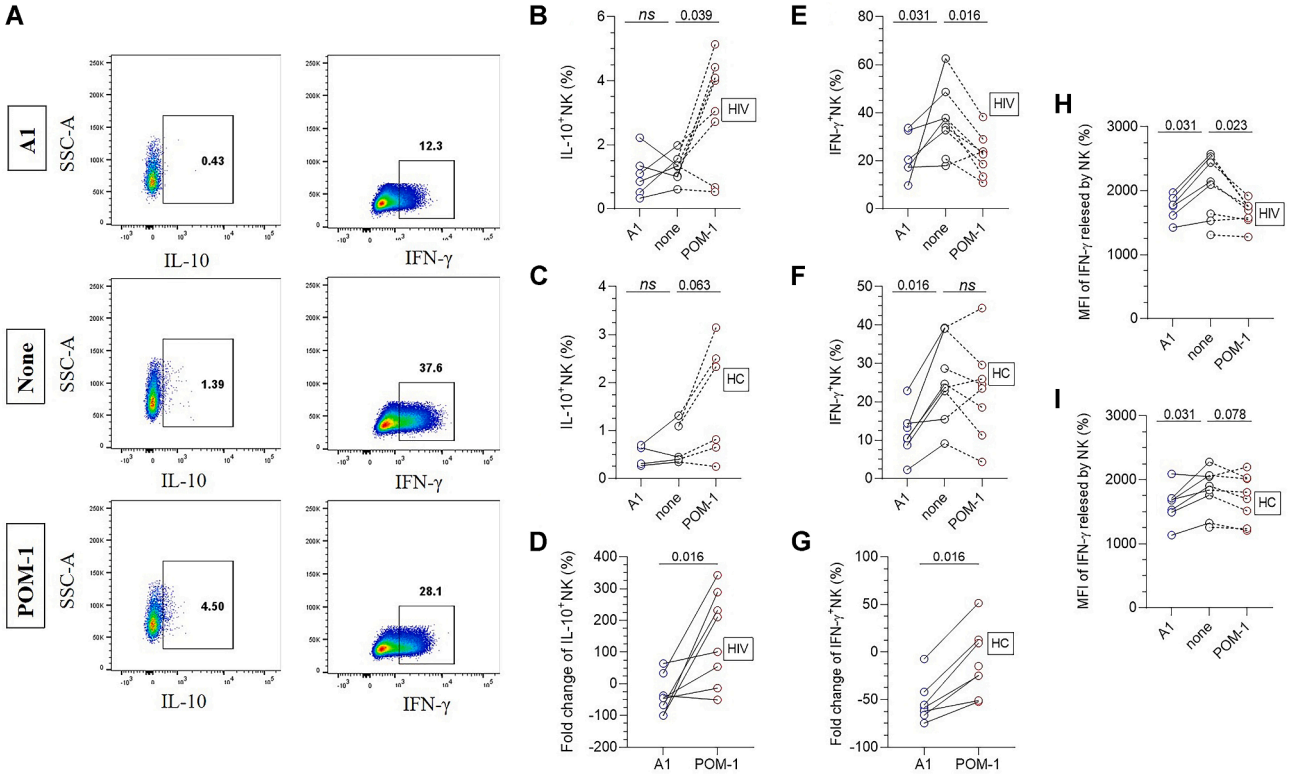


**Fig. 5.** Differential effects of CD39-targeting agents of POM-1 and A1 on NK cell secretion of IL-10 and IFN-γ. **A** Representative flow cytometry plots showing IL-10 and IFN-γ secretion by NK cells under three conditions: CD39 blocking (A1), ectonucleotidase inhibition (POM-1), and no blocking agent (none). **B, C** Comparison of IL-10$^+$ NK cell frequencies in HIV-1 individuals (**B**) and healthy controls (**C**) across three treatment conditions; HC: healthy controls with negative HIV-1 antibodies. HIV: HIV-1-infected individuals. HIV: A1 (n = 6), POM-1 (n = 8), none (n = 8); HC: A1 (n = 4), POM-1 (n = 6), none (n = 6). **D** Comparison of IL-10$^+$ NK cell frequency fold changes in HIV-1 individuals between A1 and POM-1 conditions. **E, F** Comparison of IFN-γ$^+$ NK cell frequencies in HIV-1 individuals (**E**) and healthy controls (**F**) across three treatment conditions; HIV: A1 (n = 6), POM-1 (n = 8), none (n = 8); HC: A1 (n = 7), POM-1 (n = 8), none (n = 8). **G** Comparison of IFN-γ$^+$ NK cell frequency fold changes in healthy controls between A1 and POM-1 conditions. **H** IFN-γ MFI of NK cell secretion in HIV-1-infected individuals: comparison among A1 (n = 6), POM-1 (n = 8), and none (n = 8). **I** IFN-γ MFI of NK cell secretion in HC: comparison among A1 (n = 7), POM-1 (n = 8), and none (n = 8). The fold change of IL-10$^+$ NK or IFN-γ$^+$ NK cell frequency was calculated as: (frequency under the indicated condition − frequency without blocking agent)/frequency without blocking agent. For paired variables, statistical analysis was performed with the Wilcoxon matched-pairs signed rank test (e.g., A1 vs. none; POM-1 vs. none; A1 vs. POM-1). *ns*: not significant.

studies showing that single or combined blockade of TIGIT and CD39 enhances the cytotoxicity of NK-92 cells against acute myeloid leukemia cells *in vitro* (Brauneck F. et al., 2021), suggesting that coexpression of TIGIT and CD39 impairs NK cell effector function.

However, the distinct modulatory effects of POM-1 and A1 on NK cell secretion of IL-10 and IFN-γ, as presented in Fig. 5, shed critical light on the functional complexity of CD39 in regulating NK cell immune responses during HIV-1 infection and in healthy status. For IL-10 secretion, POM-1 consistently enhanced the frequency of IL-10-producing NK cells in both treatment-naïve HIV-1-infected individuals and most healthy controls, whereas A1 had no such effect. This discrepancy suggests that CD39's ectonucleotidase activity, rather than its mere presence on the NK cell surface, is a key regulator of IL-10 production. By inhibiting CD39's ability to catalyze eATP into AMP, POM-1 may disrupt the adenosine-mediated signaling cascade that normally restrains IL-10 secretion. In contrast, the two agents exerted overlapping yet distinct effects on IFN-γ secretion. A1 significantly reduced IFN-γ-producing NK cells in both HIV-1-infected individuals and healthy controls, while POM-1 only attenuated IFN-γ secretion in HIV-1 individuals. This divergence implies that CD39 modulates IFN-γ production through dual mechanisms: one dependent on its surface expression (targeted by A1) and another linked to its ectonucleotidase activity (targeted by POM-1), with the latter mechanism becoming relevant only in the context of HIV-1 infection. The HIV-1 infection-specific effect of POM-1 on IFN-γ may reflect altered eATP/adenosine metabolism in HIV-1-related immune microenvironments. Additionally, the reduced MFI of IFN-γ in both A1-and POM-1-treated NK cells from HIV-1-infected individuals further confirms that CD39 targeting impairs not only the frequency of IFN-γ-secreting NK cells but also the magnitude of IFN-γ production. These findings highlight that CD39-targeting strategies must be tailored to specific therapeutic goals. Nonetheless, future studies are needed to determine whether dual blockade of CD39 and TIGIT can reduce IL-10 and restore IFN-γ secretion by NK cells and enhance HIV-1 control in HIV-1-infected individuals.

## CONCLUSIONS

In conclusion, NK cell CD39 upregulation in untreated HIV-1 individuals correlates with high viral loads; CD39$^+$ NK cells show impaired activation but enhanced IL-10 secretion, with TIGIT coexpression linking to elevated IL-10 levels. Our findings reveal a novel CD39$^+$ NK cell-mediated mechanism of poor HIV-1 control, supporting CD39 (alone or with TIGIT) as a potential target to restore antiviral NK cell function in treatment-naive patients.

## MATERIALS AND METHODS

### Subjects

The subjects in this study were recruited from the Beijing Primary Infection Monitoring & Observation (PRIMO) clinical cohort as reported in our previous study (Zhang et al., 2021). The enrolled subjects were

monitored every two months for HIV antibodies, HIV RNA levels, and clinical signs of acute/early infection, as previously described. Peripheral whole-blood collection was performed at weeks 1, 2, 4, 8 and 12 and then every three months after seroconversion identification, and peripheral blood mononuclear cells (PBMCs) were isolated and cryopreserved in liquid nitrogen. The subjects for whom HIV antibodies were either unidentified or indeterminate but for whom a nucleic acid amplification test was positive were defined as acutely infected with HIV (Huang et al., 2012). Alternatively, acute HIV infection was estimated to have been established at the midpoint between the last seronegative test and the first seropositive test. The date of acquisition of HIV infection was defined as follows. (1) HIV infection was determined to be 17 days before a sample was first found to be positive for HIV RNA but negative for HIV antibody. (2) When western blotting was indeterminate, HIV infection was estimated to have occurred 30 days prior to the index measurement or subject enrolment. (3) The date of infection was estimated the date of the positive test date minus 15 days for subjects who were negative for the anti-HIV antibody and negative for HIV-1 RNA followed by seropositivity and RNA positivity with a time between tests <2 months (Fiebig et al., 2003).

Thirty-four individuals from the Beijing PRIMO cohort who did not receive antiretroviral treatment during the first to third months of HIV-1 infection were enrolled in this study. In addition, we also enrolled thirty-eight individuals with an infection duration of more than nine months but less than three years, who were classified as having chronic infection and were not received antiretroviral treatment. Twenty-four healthy controls who were HIV-1-negative were enrolled as controls in this study.

## CD4 T-cell count and viral load measurement

Peripheral whole-blood samples were obtained from each patient, and routine blood $CD4^+$ T-cell counts (cells/μL) were determined *by* four-colour flow cytometry using human $CD45^+$, $CD3^+$, $CD4^+$ and $CD8^+$ T-cell markers Sample processing was performed using FACS-lysing solution according to the manufacturer's instructions. The plasma HIV-1 viral load (copies/mL) was quantified by real-time PCR, with a lower limit of viral RNA detection of 40 copies/mL in plasma.

## Profiles of natural killer cells were detected by staining and flow cytometry

PBMCs were thawed in RPMI 1640 medium supplemented with 10% foetal bovine serum. Then, the PBMCs were stained at room temperature for 20 min with the following fluorescent dye-conjugated human monoclonal antibodies (mAbs): CD3-Brilliant Violet 605™ (clone HIT3a, BioLegend, San Diego, USA), CD16-FITC (Clone 3G8, BioLegend, San Diego, USA), CD56-APC/Cyanine7 (clone HCD56, BioLegend, San Diego, USA), CD39-Brilliant Violet 785™ (clone A1, BioLegend, San Diego, USA), CD69-Brilliant Violet 711™ (clone FN50, BD Biosciences, San Diego, California, USA), and TIGIT-PE/Cyanine7 (clone A15153G, BioLegend, San Diego, USA). Dead cells were excluded by staining with the Zombie Violet™ Fixable Viability Kit (BioLegend, San Diego, USA). Then, the PBMCs were washed with phosphate buffer solution (PBS) and fixed with a fixative of PBS containing 4% formaldehyde. The cytometer was set up, and tracking of calibration particles (CST) was performed to ensure consistent fluorescence intensity measurements across all the experiments. Flow cytometric analysis was performed on a BD FACSLyric flow cytometer, and the data were analyzed with FlowJo Software version 10.8 (TreeStar, Ashland, OR, USA).

## Cytokine IL-10 released by NK cells was detected by flow cytometry

PBMCs were thawed and incubated for 2 h in RPMI 1640 medium supplemented with 10% foetal bovine serum. Then, IL-10-secreting NK cells were detected following the manufacturer's protocol (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, $1 \times 10^6$ PBMCs were stimulated overnight with 20 ng/mL PMA (phorbol 12-myristate 13-acetate) and 1 μM ionomycin at 37 °C in 5% $CO_2$. After washing, cells were incubated with cytokine capture reagent on ice for 5 min. The cells were then diluted in pre-warmed RPMI 1640 medium supplemented with 5% human AB serum and incubated for 45 min at 37 °C, with gentle mixing every 5 min to resuspend the settled cells. Subsequently, PBMCs were washed and stained at 4 °C for 15 min with IL-10-PE detection antibody and the following antibodies: CD3-Brilliant Violet 605™ (clone HIT3a, BioLegend, San Diego, USA), CD56-APC/Cyanine7 (clone HCD56, BioLegend, San Diego, USA), CD39-Brilliant Violet 785™ (clone A1, BioLegend, San Diego, USA), and TIGIT-PE/Cyanine7 (clone 15153G, BioLegend, San Diego, USA). Dead cells were excluded by staining with the Zombie Violet™ Fixable Viability Kit (BioLegend, San Diego, USA). After a final wash, the cells were analyzed with a BD FACSLyric flow cytometer. The data were analyzed with FlowJo Software version 10.8 (TreeStar, Ashland, OR, USA).

## Cytokines of IL-10 and IFN-γ released by NK cells after CD39 was blocked with A1 and POM-1

PBMCs were thawed and incubated for 2 h in RPMI 1640 medium supplemented with 10% foetal bovine serum. A1 antibody (Biolegend, San Diego, USA) and POM-1 inhibitor (MedChemExpress LLC, Monmouth Junction, NJ, USA) were added at final concentration of 0.5 ng/mL and 12.5 μM, respectively, followed by incubation at 37 °C for 30 min. Control cells were treated with medium alone in the absence of any inhibitors.

For IL-10 detection, PBMCs were stimulated with 20 ng/mL PMA and 1 μM ionomycin at 37 °C in 5% $CO_2$ for 16 h, and subsequent staining procedures were performed as described above. PBMCs were washed and stained at 4 °C for 15 min with IL-10-PE detection antibody and the following antibodies: CD3-PE/Cyanine7 (clone HIT3a, BioLegend, San Diego, USA), CD56-Brilliant Violet 421™ (clone HCD56, BioLegend, San Diego, USA), CD39-Brilliant Violet 785™ (clone TU66, BD Biosciences, San Diego, California, USA), and TIGIT-Brilliant Violet 711™ (clone 15153G, BioLegend, San Diego, USA).

For IFN-γ detection, PBMCs were stimulated with 40 ng/mL PMA and 1 μM ionomycin at 37 °C for 4–6 h in 5% $CO_2$. At the same time, brefeldin A was added into the medium according to the introductions of manufacturer. PBMCs were washed and stained with the following antibodies for 15 min: CD3-PE/Cyanine7 (clone HIT3a, BioLegend, San Diego, USA), CD56-Brilliant Violet 421™ (clone HCD56, BioLegend, San Diego, USA), CD39-Brilliant Violet 785™ (clone TU66, BD Biosciences, San Diego, California, USA), and TIGIT-Brilliant Violet 711™ (clone 15153G, BioLegend, San Diego, USA). Then PBMCs were fixed and permeabilized at 4 °C for 20 min. After washing, cells were stained with IFN-γ-FITC antibody (clone 4S.B3, BioLegend, San Diego, USA) at 4 °C for 30 min. Dead cells were excluded using the Zombie Violet™ Fixable Viability Kit (BioLegend, San Diego, USA). After a final wash, PBMCs were analyzed with a BD FACSLyric flow cytometer.

## Statistical analysis

Data are presented as the median and interquartile range in figures. Statistical analyses were performed using GraphPad Prism software version 6.0. For comparisons among three variables (i.e., parameters of individuals in the HC, AHI, and CHI groups), either the parametric One-Way ANOVA or the nonparametric Kruskal-Wallis test was used. If a significant difference was identified, multiple pairwise comparisons were conducted: the Tukey test was used for post-hoc analysis following One-Way ANOVA, while Dunn's test was applied for comparisons after the Kruskal-Wallis test. Paired variables were analyzed using the

Wilcoxon matched-pairs signed-rank test. For comparisons of parameters between the AHI and CHI groups in Table 1, either the parametric Student's *t*-test or the nonparametric Mann-Whitney *U* test was employed. Spearman's rank correlation analysis was performed to assess the relationship between two variables. Differences were considered statistically significant when the *P* value was $<0.05$ in two-tailed tests.

## DATA AVAILABILITY

Data supporting the findings are available from the corresponding author upon reasonable request.

## ETHICS STATEMENT

This study and all the relevant experiments were approved by the Beijing Youan Hospital Research Ethics Committee, and written informed consent was obtained from each participant in accordance with the Declaration of Helsinki. All participants provided written informed consent for the collection of information and for clinical samples to be stored and used for research. The methods used conformed to approved guidelines and regulations.

## AUTHOR CONTRIBUTIONS

Xin Zhang: conceptualization, methodology, data curation, formal analysis, visualization, writing-review & editing; Qianqian Xu: methodology, data curation, visualization; Junyan Jin: methodology, data curation and visualization; Hongxia Yan: data curation, software; Xiaofan Lu: data curation, visualization; Zhen Li: software and visualization; Zhiying Liu: data curation, visualization; Rui Wang: collecting samples; Lin Yuan: collecting samples; Zhenglai Ma: investigation, collecting samples; Tong Zhang: supervision; Hao Wu: supervision, funding acquisition; Bin Su: conceptualization, methodology, supervision, writing-review & editing, funding acquisition.

## CONFLICT OF INTEREST

The authors declare no competing financial interests.

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## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2026.03.006.

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