EVALUATION OF THE EFFECTOR CAPACITY OF NK CELLS IN MEN WHO HAVE SEX WITH MEN AT HIGH-RISK OF HIV-1 INFECTION FROM MEDELLIN

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Dedicatoria

A mis padres que son mi mayor referente de lo que se trata la vida: “vivir bajo la premisa del querer ayudar, del querer hacer un poquito más fácil la vida a los demás”, porque como ellos dicen, “mijo, de por sí la vida ya suele ser dura, ¿por qué no ayudar y que lo sea un poquito menos?

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Abstract

Introduction: Repeated exposure to HIV-1 usually result in infection; however, some individuals, under constant exposure to the virus and against the statistical probability, do not show serological or clinical evidence of infection. These individuals are known as HESN (HIV-Exposed Seronegative) and have turned into a key population to search of the natural resistance phenomenon against HIV-1 infection. HESN studies include persons with high-risk sexual behaviors, such as Men Who Have Sex with Men (MSM), who experience biological and sociocultural issues that contribute to a higher risk of acquiring the HIV-1 infection.

MSM has been poorly studied compared to other cohorts, such as commercial sex workers (CSW) or intravenous drug users (IDU). Two decades ago, evidence regarding the role of NK cells in natural resistance to HIV-1 infection such as higher effector capacity and higher cytokine production was described in HESN. However, little is known about the effector and antiviral potential of NK cells in the elimination of HIV-infected $CD4^+$ T cells in high-risk MSM. For this, we propose to evaluate the role of NK cells in the natural resistance to HIV-1 infection in this high-risk population.

Methodology: MSM at high-risk (HR-MSM) and low risk (LR-MSM) of HIV-1 infection were included in the study. The effector and antiviral capacity of NK cells were evaluated in co-cultures with HIV-infected $CD4^+$ T cells. The p24 viral protein was quantified by intracellular cytometry in HIV-1-infected $CD4^+$ T cells and the supernatants of the co-culture, by ELISA. Activation markers, cytokine production, and functional capacity of NK cells were evaluated by flow cytometry, and the quantification of cytokines in the co-culture was measured by Cytometric Bead Array (CBA).

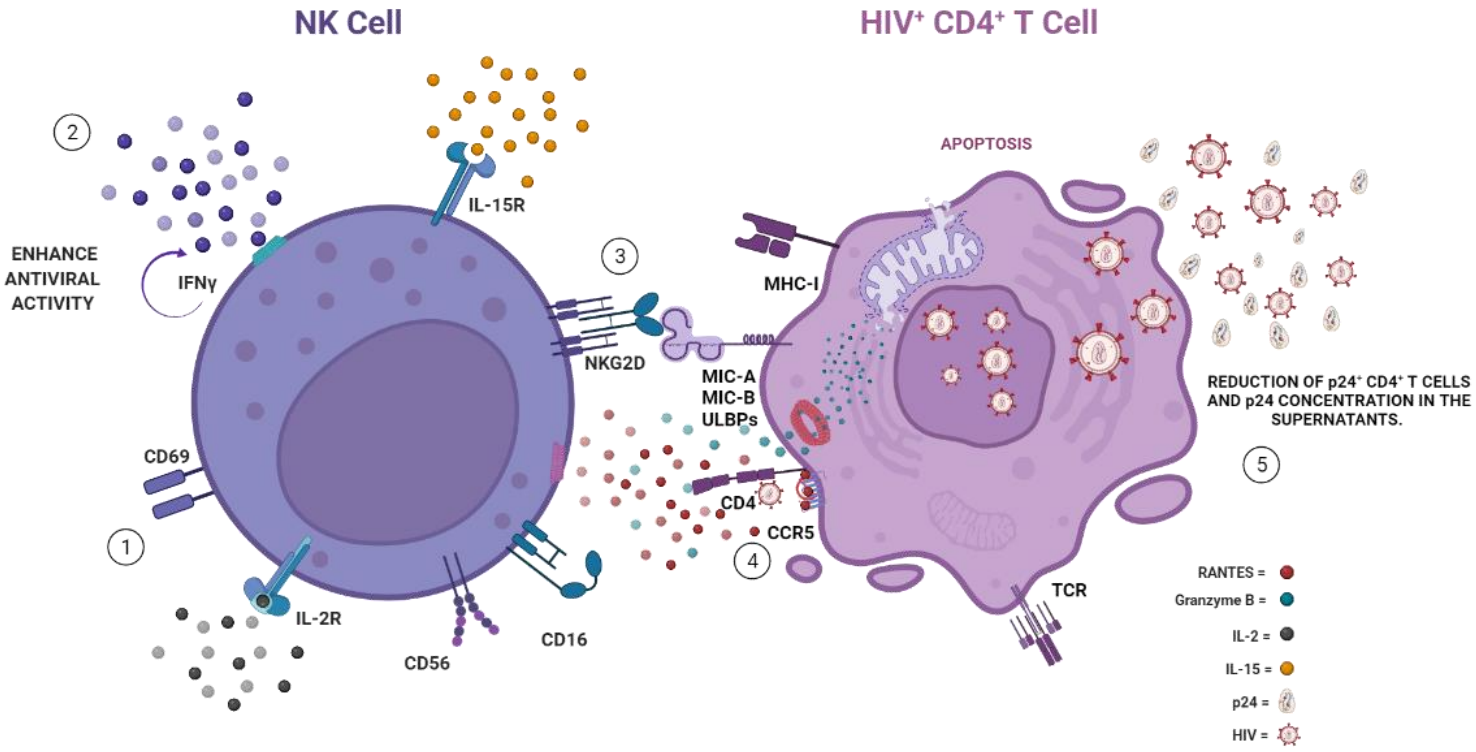
Results: MSM at high-risk of HIV-1 infection exhibit a higher capacity to eliminate HIV^+ $CD4^+$ T cells, a reduced percentage of $CD69^+$, and a higher percentage of $NKG2D^+$ NK cells. Despite the lower levels of $CD69^+$ NK cells, within this population, higher percentages of $CD69^+$ IFN-γ^+ and $CD69^+$ $NKG2D^+$ NK cells were found in the HR-MSM group. In addition, higher levels of RANTES and Granzyme B were found in the supernatants of the co-cultures of HR-MSM; these molecules are linked to the

antiviral activity, which could explain the lower percentages of $p24^+$ cells and the reduced concentration found for this protein in the evaluated supernatants. Finally, correlations among a reduction in the percentage of $p24^+$ cells, RANTES concentration, and percentage of positive NKG2D NK cells were found with the number of sexual partners in the last three months.

Conclusion: NK cells from HR-MSM individuals had a higher antiviral capacity, resulting in a lower concentration of p24 protein in the co-cultures and the percentage of $p24^+$ $CD4^+$ T cells. Changes on the antiviral capacity could be driven by differences found in the activation markers and the chemokines and cytokines production compared to LR-MSM. The production of some antiviral molecules was also correlated with the number of sexual partners in the last three months.

Altogether, this information suggests that higher exposure to HIV-1 in high-risk individuals could be included in a “training” state of NK cells to face the virus, influencing the natural resistance to HIV observed in this population.

Graphical Abstract



NK cells of HR-MSM had higher antiviral activity against autologous HIV⁺ CD4⁺ T cells

HR-MSM individuals shown a lower percentage of CD69⁺ NK cells than LR-MSM; the possible transition into an adaptive phenotype could explain this result (**1**). Despite this, higher percentages of CD69⁺ IFN-γ⁺ NK cells were found in HR-MSM individuals, which could enhance their antiviral activity against infected cells (**2**). Additionally, a higher percentage of cells expressing NKG2D was found within CD69⁺ NK cells of HR-MSM, reflecting a higher capacity to recognize stress proteins expressed on HIV-1-infected CD4⁺ T cells such as MIC-A, MIC-B and ULBP proteins (**3**). Finally, higher concentrations of RANTES and Granzyme B were found in the supernatants of HR-MSM co-cultures. RANTES binds to the CCR5 coreceptor, blocking viral entry, and Granzyme B works as a potent inductor of apoptosis on infected CD4⁺ T cells (**4**). Altogether, these mechanisms reduce the numbers of p24⁺ cells and p24 levels in the supernatants of the co-cultures of HR-MSM (**5**).

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1. Presentation

The development of this thesis begins with the introduction. This paragraph approaches key aspects around HIV-1 infection and natural resistance mechanisms against HIV-1. This background includes fundamental aspects of HIV epidemiology and pathogenesis. The definition of HESN cohorts and the features proposed for their study along with a summary of the natural resistance mechanisms described to date in the HESN and MSM population, underlying the potential of NK cell effector and its antiviral capacity against HIV-1 infection.

Moreover, the problem statement inquires why, despite the little information around HESN, compared to other cohorts, MSM represents a significant population for the study of natural resistance mechanisms. This thesis presents the results with their respective figures and tables grouped by the most relevant findings. The discussion section summarizes our main findings in the context of the relevant literature. In addition, we proposed how our findings could contribute to the generation of knowledge around NK cells and its implications for HIV-1 natural resistance.

2. Introduction

Opportunistic infections such as pneumonia by *pneumocystis jirovecii* rarely occurred in absence of immunosuppressive therapy; however, everything changed since Gottlieb and colleagues observed four MSM (Men who have Sex with Men) with pneumonia, mucosal candidiasis, and a marked lymphopenia [1]. These observations were one of the first sights of acquired immunodeficiency syndrome (AIDS) and Human Immunodeficiency Virus (HIV) infection. Today, HIV infection is considered a pandemic with significant prevalence and incidence numbers. AIDS is the outcome of HIV infection; this disease is diagnosed when an HIV-infected patient experience an AIDS-defining condition or the number of $CD4^+$ T cells drops below 200 cells/mm^3 (regardless of whether the person has an AIDS-defining condition).

Since the beginning of the HIV epidemic, around 79 million people have become infected, and 36 million people have died of AIDS-related illnesses worldwide. In 2020, 37.7 million people were living with HIV globally [2], and this data is equivalent to the entire population of a country like Canada. Despite considerable efforts to implement the Highly Active Antiretroviral Therapy (HAART) to decrease HIV mortality, around 700.000 deaths related to HIV occurred worldwide in 2020 [2].

In Colombia, the government statistics reported 124.000 people living with HIV in 2020. However, UNAIDS estimated 180.000, representing an increase of 45% for the same data. Nevertheless, Colombia has been characterized as a country with a HIV epidemic focused on high-risk populations, which includes men who have sex with men (MSM), transgender women, commercial sex workers (CSW), and Injection Drug Users (IDUs). The prevalence of HIV-1 for these populations corresponds to 17, 21.4, 1.2 and 2.8%, respectively; these numbers are significantly higher than the general population prevalence in Colombia, which is 0.4% [2].

In the first stage of the infection, HIV-1 infects resident dendritic cells and $CD4^+$ T cells of the Gut-Associated Lymphoid Tissue (GALT), the largest T cell compartment in the human body. After infection, due to the high viral replication, an abrupt decrease in $CD4^+$ T cells counts in GALT and peripheral blood occurs; however, as the instauration of the adaptive immune response arises, the viral replication is

partially controlled, and it is possible to establish a viral set point. At this stage, the chronic phase of the infection begins and a clear recovery in the number of $CD4^+$ T cell counts in peripheral blood appears because of the viral control. At these early stages of HIV-1 infection, the immunological system of most individuals experiences a general activation that persists overtime. Initially, this is caused by viral products, produced during the peak of viral replication, but then induced by the translocation of microbial products from the intestinal lumen to systemic circulation as a consequence of GALT damage, resulting in a vicious activation circle [3]. In addition, HIV-1 RNA interaction with the TLR-7 and TLR-9 at the entry site, induces the production of IFN-α by plasmacytoid dendritic cells and other proinflammatory cytokines, sustaining the activation and inflammation at the infection site [4]. Likewise, the products of abortive HIV replication such as free HIV DNA in the cytoplasm of $CD4^+$ T cells may induce the production of IL-1β contributing to this state [5], suggesting that the HIV-1 infection *per se* could act as an additional contributor to immune activation.

These events lead to a chronic activation state that marks the beginning of immunological exhaustion followed by AIDS, where HIV-infected individuals exhibit impaired immunological responses, making them susceptible to opportunistic infections and malignancies, eventually leading to death [6]. However, evidence from different cohorts of HIV-1 infected individuals has hown that HIV-1 progression is a heterogeneous process. In fact, HIV-infected individuals have been classified based on the time of progression to AIDS. In line with this, the typical progressors develop AIDS in approximately 5-10 years in absence of HAART, some individuals progress to AIDS in less than 5 years and are known as rapid progressors; moreover, subjects infected for 10 years or more with stable CD4+ T cell counts and clinical parameters, are known as Long Term Non-Progressors (LTNP) [7]. The study of LTNP to determine mechanisms of resistance to AIDS progression implies a significant challenge due to their low frequency and the extended follow-up time required to identify them. Therefore, a new classification criterion was defined, and HIV-infected individuals known as elite controllers (ECs) and viremic controllers (VCs) have emerged. ECs drives the viral replication to levels below the detection limit of

standard clinical assays, with HIV RNA levels <50 RNA copies/mL in the absence of antiretroviral therapy for at least one year and without evidence of chronic immune activation [8]. The VCs are individuals with a minor degree of virologic control, < 2000 RNA copies/mL, but the infection courses with stable CD4+ T cell counts [9]. The existence of these individuals glimpses a new vision regarding mechanisms mediating a prolonged control of HIV replication and eventually delaying progression to AIDS in absence of antiviral therapy.

The existence of these populations has encouraged the search for different cohorts in which, immunological mechanisms of natural resistance to HIV-1 infection could be leading to protection. In this context, another group of individuals has become of particular interest, this group is known as HIV-exposed seronegative individuals or HESN. These individuals maintain multiple and repeated exposures to HIV-1 but remain without serological or clinical evidence of the infection. The study of HESN individuals has settled the start of investigating a possible link between immunological responses and natural resistance to HIV-1 infection.

HESN Individuals in the study of natural resistance mechanisms to HIV-1 infection

Repeated contact with HIV-1 likely results in infection. However, some individuals remain uninfected despite multiple high-risk exposures or repeated high-risk behavior; this population is known as HESN. They are essential for the study of potential factors mediating natural resistance to HIV-1 infection [10]. HESN can be classified under three major groups: i) serodiscordant couples; ii) individuals with high-risk sexual behaviors, including commercial sex workers (CSW) and Men who have Sex with Men (MSM); iii) Individuals exposed non-sexually, including IDU, infants born to HIV-infected mothers, hemophiliacs, and others exposed to contaminated blood products [11].

Seronegative individuals from discordant couples were the first ones whose HIV-1-specific T cell responses were characterized [12]. Today, constitute one of the largest HESN cohorts of study, contributing to considerable advances in natural resistance comprehension. However, serodiscordant couples in long-term

relationships, lack some key characteristics compared to other sexually exposed HESN cohorts such as the possibility of encountering different HIV-1 quasispecies [11]. This supposes an obstacle to understanding the resistance focused on developing therapeutic alternatives or vaccines, in which a cross-reactive response is desirable.

In contrast, CSW and MSM with high-risk sexual behavior constitute a promising group for studying natural resistance to HIV-1 infection considering their higher probability of cross-reactive responses against different HIV-1 quasispecies. Multiple studies have focused on the study of CSW; such as the Pumwani cohort from Nairobi, Kenia, which was established in 1985 [13]. The participants are monitored twice a year generating a significant impact on the knowledge of mechanisms implicated in the transmission and immune response at the systemic level and the site of infection in women exposed through heterosexual intercourse.

In the distribution among critical populations, by 2019, MSM individuals reported 23% of new HIV-1 infection cases worldwide, representing the higher incidence among these groups [2]. Differences in sexual behaviors, such as the high number of sexual partners and the nature of sexual activities, represent a high-risk practice. For instance, in the case of sexual exposure to HIV-1, the epithelium is the first barrier that HIV-1 virions need to cross to reach the submucosa. However, the epithelium differs in its conformation depending on the tissues, which may have implications for viral transmission. In this context, the anorectal epithelium exhibits the highest probability of HIV transmission (0.3–5%) compared to female (0.05–0.5%) and male genital epithelium (0.04–0.14%), followed by the oral mucosa (0.01%) being the least susceptible [14, 15]. The risk of infection increases in MSM individuals since they suffer from a social stigma which may impair their access to healthcare, together, this could act as an endless loop, where the serological status remains unknown in high percentages of this population, impacting the risk of infection [2].

In Colombia, 53.05% of the new HIV-1 cases and 39.74% of the prevalent cases were reported in the MSM population; this scenario is also true for several countries

around the world in which MSM play a central role in HIV-1 transmission, highlighting the need to inquire about the natural resistance phenomena in this population [2].

Mechanisms of natural resistance to HIV infection

HESN cohorts are useful for the study of immunological and genetic features related to natural resistance to HIV infection. These features include CCR5 Δ32 mutation, immunological quiescence, HIV-1 specific IgA, HLA-KIR allele expression, HIV-1 specific cytotoxic lymphocytes (CTL), and production of soluble factors, among others [16]. Overall, natural resistance to HIV seems to be a complex multifactorial phenomenon with several mechanisms acting in concert, which varies among different cohorts.

The homozygosity for the CCR5Δ32 mutation is the most important genetic mechanism of natural resistance to HIV-1 infection. Homozygous individuals express a truncated protein that remains in the cytoplasm, rendering cells resistant to HIV, particularly to the R5 HIV strain that uses CCR5 as a co-receptor for viral entry [17]. However, this mutation only explains a minority of resistant cases, and is uncommon in the worldwide population, being particularly rare in South American countries [18]. In fact, the prevalence of homozygous Δ32 individuals, among HESN within serodiscordant couples, was estimated to be 2.6% in Colombia [19].

The fact that HIV-specific CD8+ T cells play a critical role in viral control during acute and chronic HIV infection allowed us to glimpse a possible mechanism associated with natural resistance reported in HESN individuals [20, 21]. For instance, a recent study in a HESN cohort showed a higher proportion of effector memory CD8+ T cells and higher numbers of CD8+ T cells expressing IFN-γ, TNFα, and IL-2 in response to Env and Gag peptides compared to HIV-1 progressors and HIV-1-unexposed seronegative controls [22]. Interestingly, CSW from the Pumwani cohort evidenced IFN-gamma production by mononuclear cells obtained from the cervix and peripheral blood in response to class I HLA-restricted CTL epitope, with the cervix-derived cells being the largest producers [23].

In addition, mechanisms relying on humoral responses have also been identified in HESN individuals; antibodies against HIV have been widely detected in the mucosa of this population [24]. In accordance, in 2003, Caputo *et al* reported mucosal HIV-1-specific IgA in males of serodiscordant heterosexual couples after urethral swabs analysis [25]. Additionally, a previous report in HESN women showed a similar trend, with a higher frequency of HIV-specific IgA at the genital tract than infected and uninfected controls [23]. The above information emphasizes the importance of the acquired immune response in natural resistance to HIV-1. Although these mechanisms have been reported in different cohorts, HIV-specific adaptive immune responses are absent in many HESN individuals. Moreover, this response requires time to develop fully; therefore, innate immunological mechanisms must be involved in the viral control responsible for avoiding infection.

In this line, different types of cells can secrete soluble factors, essential in mucosal protection. Some of these factors are polypeptides that exhibit antiviral activity under prevailing conditions at the tissues of origin. The two prominent families of these peptides are defensins and cathelicidins, in which defensins have been associated with antiviral activity against HIV1 replication for more than a decade [26, 27].

Defensins are small cationic peptides (α, β, and θ), in which the human β-defensins (HBD) are the most broadly studied in the HIV context, particularly HBD-1, HBD-2, and HBD-3. These 3 β-defensins are constitutively expressed in the oral mucosa [28]. Moreover, Zapata et al. reported a higher HBD molecules expression in oral mucosa of HESN compared to healthy controls and an overrepresentation of the single nucleotide polymorphism A692G in the *DEFB1* gene, which was related to a higher expression of HBD-1 [29]. The function of α-defensins in the context of HIV-1 infection has been studied. For instance, the α-defensins HNP1–3 prevent HIV-1 entry into the CD4+ T cells by inhibiting the envelope-mediated cell-fusion of viral gp120 [30]. In addition, higher concentrations of HNP1–3 in the breast milk of HIV-1 positive women were linked to a lower probability to transmit the virus to the child [31].

Apolipoprotein B mRNA-editing catalytic polypeptide-like 3G (APOBEC3G or A3G) is a cytidine deaminase, which drives hypermutations in the proviral DNA during reverse transcription. A3G and APOBEC3F have been implicated in the restriction of HIV-1 replication *in vitro* [32]. However, this viral restriction is countered by the virion infectivity factor of HIV (Vif) by recruiting an E3 ubiquitin ligase complex mediating ubiquitination and proteasomal degradation of both apolipoproteins [33]. Despite this, the haplotype 1 (Hap1) of APOBEC3H, which results in a less stable protein compared with other haplotypes of the same protein, seems to be resistant to Vif mediated degradation [34]. Interestingly, Hap1 was overrepresented in HESN compared to HIV-1 positive individuals [35].

Another soluble factor involved in HIV-1 antiviral activity in HESN is the Secretory Leukocyte Protease Inhibitor (SLPI). It is a soluble nonglycosylated cationic protein mainly secreted by epithelial cells covering mucosal surfaces [36]. Its antiviral activity against HIV-1 was firstly observed in oral mucosa, where a high concentration of this protein was linked with the low transmission rates of HIV in this epithelium [37]. In addition, evidence in perinatal studies in Africa showed that higher transmission rates were associated with low levels of SLPI in the vagina of infected mothers [38]. In accordance, Farquhar and colleagues demonstrated that higher salivary levels of SLPI were associated with a reduced risk of HIV-1 infection among newborns exposed to the virus via breastfeeding [39]. Our group reported higher levels of SLPI mRNA in the oral mucosa of HESN compared to chronically infected individuals and healthy controls [29, 40].

Serpins and Elafins are serine protease inhibitors and highly conserved proteins involved in different biological roles such as inflammatory control. For instance, Serpins regulate the activity of serine proteases produced by cytotoxic cells, which, if not properly controlled, can lead to chronic inflammation favoring a rapid progression [41, 42]. In addition, elafin works as an epithelial protector factor against excessive production of neutrophil elastase, and its downregulation was observed in multiple inflammatory diseases [43]. In the HIV-1 infection context, these molecules have been associated with antiviral activity by inhibiting infection, attachment, and

transcytosis through genital epithelial cells [44]. Likewise, the HESN individuals within a serodiscordant couple showed higher mRNA levels of these molecules in peripheral blood, GALT, and genital mucosa compared to healthy controls [45].

Recent studies, reviewed by Ganser-Pornillos, showed that the cytoplasmic protein TRIM5-α is an important factor in type 1 interferon-mediated suppression of HIV-1 replication, triggered via immunoproteasome stimulation. This information is in accordance with the hypothesis that TRIM5-α has a positive impact on controlling HIV during acute and chronic phases of the infection [46]. In 2015, our group reported that Colombian HESN had a higher expression of TRIM5-α mRNA in genital mucosa compared to healthy controls [45].

In the last two decades, considerable interest in describing the immunological state of HESN has emerged. Some authors suggest that several host factors needed for HIV-1 replication are mainly expressed in activated cells; this activation level is a hallmark during the infection, inducing exhaustion and disease progression [47, 48]. Hence, a lower activation of target cells could be related to reduced susceptibility to HIV-1 infection due to a lesser capacity to sustain viral replication [49]. This phenomenon has been named immunological quiescence.

Immunological quiescence or quiescent profile have been described in different HESN cohorts. In peripheral blood of the Pumwani CSW cohort, Card and colleagues reported a reduced percentage of $CD4^+$ $CD69^+$ and $CD8^+$ $CD69^+$ T cells with an elevated number of Treg cells [50]. Likewise, in the cervicovaginal epithelium of this cohort, it was found lower levels of $CD4^+$ $CD69^+$ and $CD8^+$ CD69+ T cells, with no difference in the ectocervical epithelial thickness when compared to women with low risk of infection [51]. In HESN MSM, a dampened innate immune response in rectal mucosa was evidenced by a low IL-6, and IL-1β production after stimuli with agonists for TLR-4, TLR-7, TLR-9 and NOD-2, but similar production of no inflammatory cytokines IL-10, IL-4, and IL-5 were found compared to control individuals (Seronegative women with lower HIV-1 exposure) [52].

In contrast, other studies showed protection against HIV infection under an immune activation state. Studies carried out in a HESN-IDUs cohort have shown a marked immunological activation profile, with lower percentages of naive $CD8^+$ T cells and higher percentages of $CD8^+CD25^+$ and $CD8^+CD38^+HLA\text{-}DR^+$ T cells in comparison to unexposed individuals [53]. Similarly, Biasin et al. described a higher mRNA expression of pro-inflammatory cytokines such as IL-6, TNF-α, IFN-γ and TNF-β in cervical biopsies of HESN serodiscordant women when compared to healthy controls [54]. Likewise, higher expression of the activation markers CD69 and CD83 was reported in myeloid dendritic cells of HESN-IDU subjects than non-sharing-needles IDU subjects with low risk of infection [55].

The role of the immune activation profile is not broadly accepted as a resistance mechanism in HIV-1 infection. A chronic activation state could lead to exhaustion, which is an undesirable outcome during an immune response. However, particularly in the case of an established infection by HIV-1, quiescence seems to have some problems as well. As it is known, HIV can establish a latent infection in some quiescent T cells, remaining as a transcriptionally inactive provirus. Thus, contributing to the rebound of the viral load in the case of antiviral treatment failure.

Some HLA alleles have been involved in slow progression to AIDS and HIV-1 natural resistance. The HLA-B allele is divided into two groups, HLA-Bw4 and HLA-Bw6, implicated in viral peptide recognition. Homozygous individuals for HLA-Bw4 alleles have been linked to slow progression to AIDS in the absence of CCR5-Δ32 mutation, showing a prolonged capacity to suppress viremia and preserved $CD4^+$ T cell count [56]. Besides, HLA-Bw4 can act as a ligand of NK cells inhibitory receptors (KIR), and some combined genotypes of these KIR alleles with Bw-4 have been linked to HIV-1 natural resistance. For instance, in a cohort of serodiscordant couples in Argentina, Habegger *et al.* reported that *KIR3DS1/L1* in combination with Bw4 could be associated with natural resistance [57].

Moreover, some specific variants of HLA-B, such as HLA-B*27 and B*57 have been related to enhanced capacity to control viremia. HIV-specific CTLs HLA-B*27– and HLA-B*57, showed lower activation than other haplotypes, and they were resistant

to Treg cell–mediated suppression. These cells could kill Treg cells in a granzyme B–dependent manner which could be associated with lower exhaustion and increased capacity to control HIV-1 viral load [58]. In addition, other HLA alleles like HLA-B*18 has been linked to HIV-1 protection during vertical transmission, and B*35 and B*58:02 alleles have been linked with impairment of HIV replication control [59].

The role of NK cells in natural resistance to HIV infection

NK cells have been also implicated in natural resistance to HIV-1 infection. The diverse functions of these cells are an important link between innate and adaptive immune responses through the production of cytokines and chemokines. In addition, NK cells can eliminate infected cells by the secretion of lytic granules containing perforin and granzymes. Upon activation, these cells are important producers of chemokines such as CCL3, CCL4, and CCL5, which are ligands for the HIV-1-co-receptor CCR5, inhibiting viral entry by blocking viral co-receptors [60]. Relevant implications around the impact of these cells in HIV-1 resistance have been evaluated in the HESN population.

Classically, human peripheral blood NK cells have been divided into different subsets according to the expression of the surface markers CD56 and CD16. $CD56^{bright}$ NK cells correspond to approximately 10% of the total NK cells in peripheral blood, express none or low levels of the FC receptor CD16, and have a high capacity to produce cytokines. The counterpart $CD56^{dim}$, represents around 90% of NK cells in peripheral blood, express high levels of CD16 and their potential to produce cytokines is limited; however, the cytotoxic capacity and the antibody-dependent cell-mediated cytotoxicity (ADCC) potential is higher than $CD56^{bright}$ NK cells [61, 62]. Interestingly, high HIV-1 viremia does not affect the numbers of peripheral blood NK cells but induces a redistribution of their subsets, contributing to dysfunctional phenotypes of these cells [63, 64].

Phenotypical characteristics around NK cells have been described in other viral infections such as human cytomegalovirus (HCMV). HCMV infection can induce an increased frequency of $CD57^{+}$ NK cell population with the expression of the activation receptor complex CD94/NKG2C [65]. This peculiar subset is associated

with an “adaptive” phenotype, a higher lifespan, and an enhanced effector function [66]. HIV-1 studies showed that a higher phenotypical frequency of NKG2C^{+} NK cells was related to a lower viral set point [67]. Our research group recently reported that MSM at high-risk of acquiring HIV-1 infection exhibited a higher frequency of CD57+/NKG2Chigh NK cells than MSM at low risk. In addition, these individuals showed a higher cytotoxic capacity against K562 cells and a positive correlation between higher mRNA levels of IFN-γ and CD57^{+}/NKG2C^{high} NK cells [68].

Likewise, almost two decades ago, Scott-Algara *et al.* reported similar results in a HESN-IDU cohort; in that report, a higher cytotoxic activity of NK cells was found against K562 cells when compared to healthy controls and other IDU seroconverters. In addition, a higher percentage of positive CCL3, CCL4 and CCL5 and IFN-γ^{+} NK cells were found [69]. This work was the first evidence of the role of NK cells in HIV-1 resistance mechanisms and since then, similar results have been published in several HESN cohorts [70-72].

Another weapon of NK cells against viral agents includes ADCC; this mechanism allows NK cells to lyse target cells after binding with specific antibodies attached to membrane-surface antigens. In the last two decades, ADCC mediated by NK cells has been widely studied and linked to natural resistance to HIV-1 infection. In fact, *Chung et al.* demonstrated specific degranulation by NK cells in response to HIV-1 antibodies and a correlation between NK cells effector activities such as IFN-γ, and CCL4 production and these non-neutralizing HIV-specific antibodies after HIV vaccination [73, 74]; these results suggested that ADCC could have a protective potential against HIV infection. Altogether, the previous information gives us insights on the role of NK cells during HIV-1 exposure. Differences of these cells on HESN individuals may help to clarify their potential role in natural resistance to HIV-1 infection.

3. Problem statement

Different cohorts of HESN individuals around the world, represents a big opportunity to clarify mechanisms of natural resistance to HIV-1. Among these cohorts, serodiscordant couples are the most common source of information in the study of HESN individuals. However, this type of cohort is generally recruited under clinical follow-up of the positive individuals while their partners receive medical psychotherapy. As described by Horton *et al.,* this situation reduces behavior risks and the possibility to be exposed to different HIV-1 quasispecies, despite the beneficial effect for these couples [11].

In the case of CSW cohorts, they constitute an important source of information on HIV-1 resistance mechanisms, nevertheless most of CSW are women, and most of the information provided by these cohorts cannot be extrapolated to men. Generation of knowledge around HIV-1 resistance in men is very important due to the magnitude of cases of HIV-1 worldwide, including Colombia, in which men represent 83.3% of people living with HIV [2]. This scenario highlights the importance of studying MSM as a natural resistant cohort. The sociocultural difficulties experienced by these individuals in our country, in addition to their biological characteristics place them as a very high-risk sexual population. In fact, according to the World Health Organization, globally, the risk of acquiring HIV is 26 times higher among MSM compared to the general population [75].

MSM represents the 23% of new adult HIV-1 infections around the world [2]. Similarly, In Latin America, MSM accounted for the 23% of the new infections in 2019, which is higher than the percentage of HIV-1 incidence of other key populations [2]. According to UNAIDS, the prevalence of HIV-1 cases for MSM population in our country corresponds to 21.4%, which are 53.5 times higher than in the general population (0.4%). This scenario is similar in other countries around the world in which the prevalence of HIV-1 cases in MSM population is at least 15 times higher than in general population [2]. Little information stratified by cities is available in Colombia; however, recent published data showed that in Medellin, the general

HIV prevalence is 0.27%, and in MSM is around 5.36%, around 20 times higher when compared to the general population [76].

Moreover, the receptive anal sex has a risk of infection around ten times higher than receptive vaginal sex [14, 15]. The highly increased risk of infection in the anorectal epithelium can be explained by the large number of $CD4^+$ T cells that are present in the gastrointestinal mucosa; in fact, the majority of these cells are located in this anatomical compartment representing the largest reservoir and replication site for HIV. In addition, the epithelium layer of the rectal mucosa is a single layer of columnar epithelium, more susceptible to lacerations than the stratified epithelium coating the vagina, which facilitates viral entry [77]. In addition to $CD4^+$ T cells, dendritic cells and macrophages expressing CCR5 and CXCR4 are present in this compartment, which can enhance or augment the probability to infection [78].

The concomitance of biological and some sociocultural factors contributes to a higher risk of acquiring the infection on MSM individuals when compared to the general population [2]. In fact, the use of condom is marked as an effective practice to diminish the sexual exposition to HIV-1; however, one of the last reports of UNAIDS in a study conducted between 2010-2018, showed a decline in the use of condom in MSM individuals in 5 of 9 African countries [2]. Moreover, in 33 of the 87 countries studied, more than 40% of MSM reported not to use the condom in their last anal sex encounter [2]. Furthermore, the awareness of serological status is also a key point in the high/steady numbers of HIV-1 incidence for this population. In accordance, a study conducted in 412 MSM in Brazil, showed that the 32.3% of these individuals were unaware of their serological status, impacting directly on the incidence of the infection [79]. These low levels of HIV testing are related to sociocultural factors such as homophobic behaviors, violence and discrimination; these behaviors continue marking a tendency along our country [80].

Nowadays, some of the mentioned mechanisms of HIV-1 resistance in serodiscordant couples and CSW have also been associated with MSM [52]. Recently, another mechanism, such as the effector capacity of NK cells described in HESN-IDU by Scott- Algara *et al.,* was associated in our research group with an

adaptive/memory NK phenotype in MSM [68]. However, these cells are still of particular interest in the study of resistance mechanisms; their antiviral and effector capacity against HIV-1 and other resistance mechanisms found in HESN cohorts should be explored and maybe extrapolated in MSM individuals.

MSM from Medellin city may reflect the high-risk state of MSM in our country and around the world; exploring the NK cell activity in this population can be an initial step in defining the role of these cells in natural resistance against HIV.

4. Objectives

General objective

- To evaluate the role of NK cells in the natural resistance to HIV-1 infection in MSM from Medellin, Colombia.

Specific objectives

- To describe the sociodemographic and sexual behavior of MSM from Medellin, Colombia.
- To evaluate the antiviral capacity of NK cells obtained from peripheral blood of MSM in co-cultures with autologous infected $CD4^+$ T cells.
- To characterize, by flow cytometry, the expression of CD69, NKG2D, IFN-γ Granzyme B, Perforin, and CD107a as markers of NK cell activation, obtained from MSM, in response to autologous infected $CD4^+$ T cells.
- To quantify by CBA the production of RANTES, MIP-1β, MIP-1α, IFN-γ, Perforin, Granzyme B, associated with the antiviral activity of NK cells, from the supernatants of the co-cultures of NK cells with autologous infected $CD4^+$ T cells.

5. Hypothesis and research question

Based on the evidence from previous studies conducted in different HESN cohorts, we hypothesize that, MSM individuals at high-risk of HIV-1 infection have a higher cytotoxic/antiviral activity of NK cells against autologous CD4+ T cells infected *in vitro* with HIV-1 when compared to low-risk MSM individuals. In addition, NK cells from high-risk MSM exhibit a higher expression of activation markers and produce higher levels of soluble antiviral factors compared to low-risk MSM.

Our study aims to provide data on the role of NK cells in the natural resistance to HIV-1 infection observed in high-risk MSM from the city of Medellín, Colombia.

6. Materials and methods

Study population

For this study, a total of 22 MSM from Medellin city were recruited. MSM were divided into two groups: i) MSM at high-risk of infection: MSM with more than 15 different sexual partners in the last 3 months with reported unprotected sexual intercourse (HR-MSM), and ii) MSM at low risk of infection: MSM with less than 4 different sexual partners in the last 3 months with reported unprotected sexual intercourse (LR-MSM). MSM younger than 18 years of age, positive for HIV 1/2 rapid test (SD BIOLINE), positive proviral PCR or Homozygous for Δ32 mutation were excluded.

After a detailed explanation of the study, all individuals provided a written informed consent and a survey for risk behavior was applied to everyone. Later, 50mL of peripheral blood were taken with a disposable syringe with EDTA. The ethics committee from the Universidad de Antioquia approved the present study.

NK cells anti-HIV activity

Peripheral blood mononuclear cells (PBMCs) were isolated through density gradient using Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO, USA) by centrifugation at 400g for 30 minutes. PBMCs were washed 3 times with PBS 1X to eliminate platelets. After, cells were counted and frozen until they were used.

PBMCs were thawed and let in culture in RPMI with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY) for 24h before each experiment. NK and CD4+ T cells were isolated in parallel from the PBMCs of each donor by negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany). The NK cell isolating antibody cocktail included monoclonal antibodies against T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes, and erythroid cells. The isolating antibody cocktail for CD4+ T included CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, and CD235a (Glycophorin A). After isolation, CD4+ T cells were stimulated for 48h with 8 µg/mL of Phytohemagglutinin- PHA (Sigma-Aldrich, St. Louis, MO) and 50 UI/mL IL-2 (Peprotech, Rocky Hill, CT). CD4+ T cells were infected by spinoculation with HIV-1BaL for 90min at 700 x g with 1ng of p24/million cells. After

spinoculation, cells were washed twice with PBS 1x to remove free virions. $8x10^4$ HIV-1-infected CD4+ T cells and $2x10^4$ NK cells were co-cultured and plated in duplicate in a 96-V bottom well plate at an Effector-Target ratio of 1:4 (one NK cell per four infected CD4+ T cells) and left in culture for seven days in RPMI plus 10% FBS supplemented with 15 UI/mL of IL-2. Viral production was assessed in the supernatant by HIV-1 p24 ELISA (Xpressbio, Ballenger Creek, Maryland, USA).

In addition, antiviral activity was also assessed by measuring intracellular p24 in cells harvested. Briefly, co-cultured cells were stained with antibodies against CD56 (CMSS; Thermo Scientific, Wilmington, DE, USA) and CD3 (UCHT1; Thermo Scientific, Wilmington, DE, USA) for 20 minutes in the dark. Later, cells were treated with Foxp3 / Transcription Factor Staining Buffer Set (Thermo Scientific, Wilmington, DE, USA) according to the manufacturer´s guidelines to permeabilize then, and then were stained with anti CD4 (RPA-T4; BD Biosciences, San Jose, CA, USA) and anti p24 (KC57; Beckman Coulter, Pasadena, CA, USA). After infection, downregulation of $CD4^+$ marker was observed on co-cultured lymphocytes, for that reason, to avoid underestimation, the percentage of $p24^+$ $CD3^+$ cells were measured instead. Data were analyzed using FlowJo version 10.5.3 (FlowJo, LLC, Oregon, USA), and normalized with infected $CD4^+$ T cells in absence of NK cells.

Evaluation of NK cell activation

NK cell activation was assessed by flow cytometry 12h after establishing the co-culture. Prior staining, 6ug/mL of Brefeldin A, 2mM of Monensin (both from Thermo Scientific, Wilmington, DE, USA) and 1uL of anti-CD107a (BD Biosciences, San Jose, CA, USA) were added to the culture and incubated at 37°C, 5% CO_2. Then, cells were stained with antibodies against CD56 (CMSS); NKG2D (1D11); and CD69 (FN50); (all of them from Thermo Scientific, Wilmington, DE, USA), and incubated for 20 minutes in the dark. In addition, cells were also treated with Foxp3 / Transcription Factor Staining Buffer Set and then stained with CD3 (UCHT1; Thermo Scientific, Wilmington, DE, USA), IFN-γ (4S.B3, Biolegend), Granzyme B (BD Biosciences, San Jose, CA, USA) and Perforin (δG9; BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo version 10.5.3 (FlowJo, LLC, Oregon,

USA). Data were normalized with NK cells co-cultured with uninfected $CD4^+$ T cells and SPICE platform were used for NK poly-functionality tests.

Quantification of antiviral molecules by Cytometric Bead Assay (CBA)

Supernatants of NK cell activation assays were collected and stored at -80°C until they were used. Then, supernatants were thawed at 4°C right before running the CBA assay. The panel for the CBA flex set included: TNF-α, Granzyme, IFN-γ, MIP-1α and RANTES (BD Biosciences, San Jose, CA, USA); CBA were done according to manufacturer´s instructions. The beads complex was acquired using LS Fortessa (BD Biosciences, San Jose, CA, USA). Obtained data were normalized with NK cells co-cultured with uninfected $CD4^+$ T cells and analyzed using FlowJo version 10.5.3 (FlowJo, LLC, Oregon, USA).

Statistical analysis

HR-MSM and LR-MSM data were compared with Mann–Whitney *U, Wilcoxon,* or Student's *t*-test, depending on the bivariate normality assumption according to the Shapiro–Wilk normality test. A p-value < 0.05 was considered statistically significant. Statistical tests were performed using GraphPad Prism Software version 7.02 and SPICE platform were used for NK poly-functionality tests. Only data with a representation higher than 0.1% were included for this analysis.

7. Results

MSM socio-demographic data

Twenty-two MSM fulfilled the inclusion criteria, with 11 individuals categorized for each group. Socio-demographic data are summarized in the ***Table 1***. Statistical differences were found in the age of the individuals when compared LR-MSM and HR-MSM. The median of sexual partners in the last three months were 2 and 25 and the median of sexual partners in a lifetime were 27 and 1708 for LR-MSM and HR-MSM, respectively. In addition, the percentage of HIV-1 positive partners was higher in the HR-MSM group, denoting a higher sexual exposure to HIV-1 infection not only in the previous months of being included in the study but throughout their sexual life. In addition, no differences were found when comparing the percentage of unprotected sex in the last three months between both groups.

Finally, one individual belonging to HR-MSM group, had a heterozygous genotype for *Δ 32* mutation.

Table 1. Socio-demographic data of MSM

	HR-MSM	LR-MSM	p value
n	11	11	
Age, median	34	26	0.0199[a]
Sexual partners in last 3 months, median	25	2	<0.0001[a]
% of unprotected sex in the last three months, median	50	39	ns
Sexual partners in a lifetime, median	1708	27	0.0019[a]
Frequency of STI*	86.5%	55.3%	<0.0001[b]
Frequency of HIV-1 positive partners	64%	45 %	0.0109[b]
Frequency of Δ 32 heterozygous	9% (1/11)	0% (0/11)	0.0032[b]
a: Mann-Whitney test **b: Fisher's exact test** ***Sexually Transmitted Infections**			

Among HR-MSM individuals, gonorrhea was the most common STI (***Figure 1***). In the LR-MSM population, a similar proportion of gonorrhea, syphilis and condyloma were found, 18.18% in each STI and, 45.46% of these individuals have not reported STIs ***(Figure 1 A)***. In contrast, 86.5% of HR-MSM reported STIs, including chlamydia and herpes, which were only found in this group (***Figure 1B***).

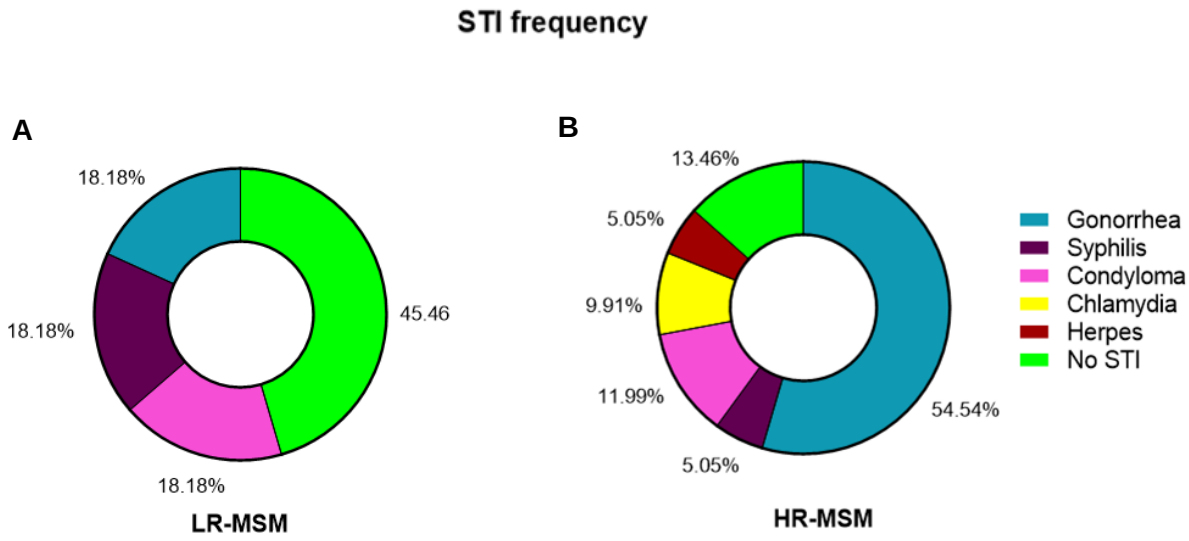


Figure 1. HR-MSM reported higher STI frequency compared to LR-MSM

Distribution of STI in LR-MSM (**A**) and HR-MSM (**B**). The history of STI was self-reported by individuals in the study survey.

NK cells of HR-MSM showed higher antiviral activity compared to LR-MSM

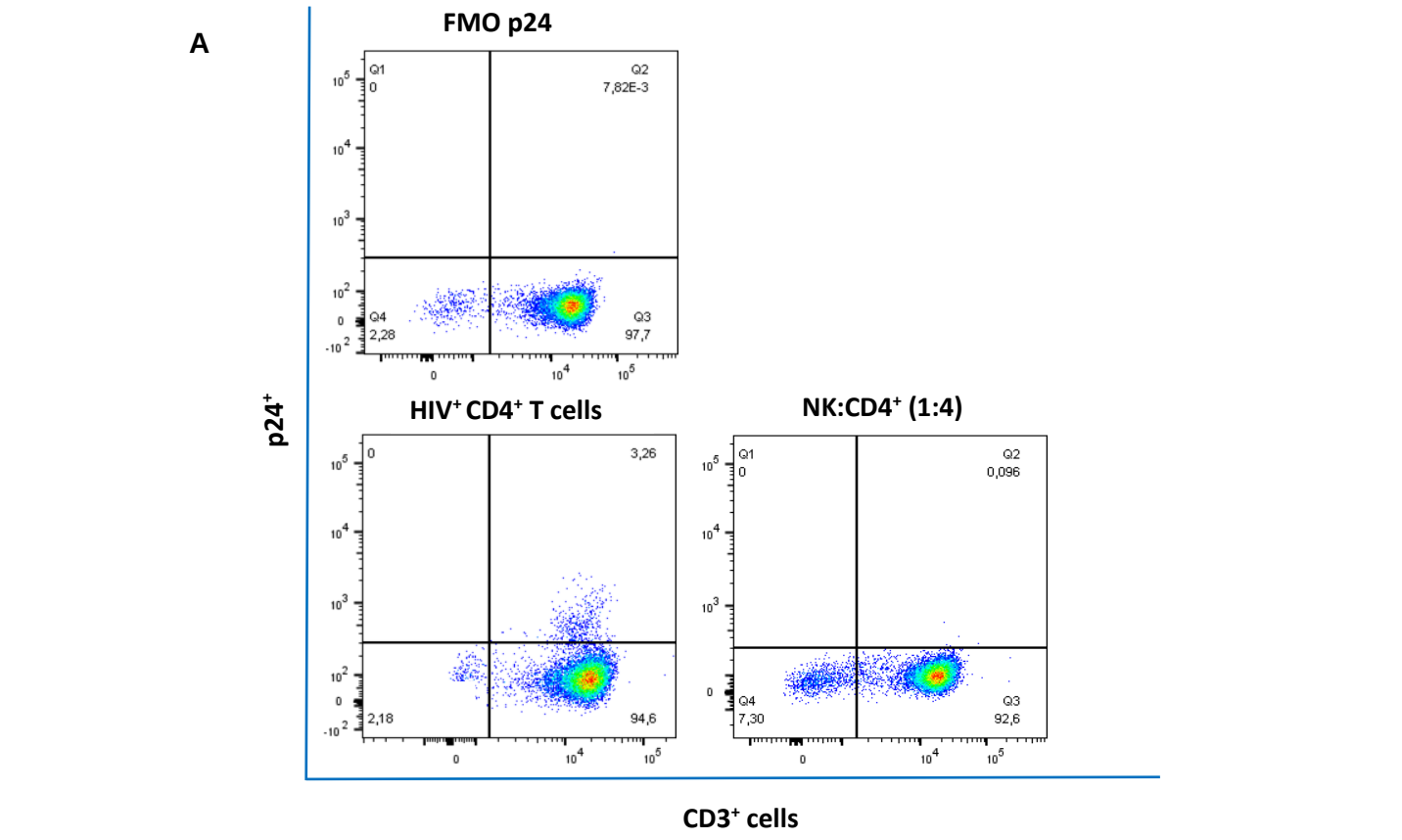
After activation and HIV-1 infection of purified autologous $CD4^+$ T cells, these cells were co-cultured with autologous NK cells in an effector-target ratio of 1:4. The antiviral activity of NK cells were measured after seven days of co-culture through the evaluation of the percentage of $p24^+$ cells by flow cytometry and, the detection of p24 protein in the supernatants by ELISA (***Figure 2***). The representative gating strategy of the flow cytometry analysis is shown in **Figure 2A.**

These results showed a marked reduction in the percentage of $p24^+$ cells in the co-culture with NK cells when both MSM groups were analyzed in combination against

their respective infected control without NK cells (p=0.0009) (***Figure 2B***). Moreover, when LR-MSM and HR-MSM groups were compared, statistical differences were found in the percentage of $p24^+$ cells, with a lower percentage of these cells in the HR-MSM group (49% on HR-MSM vs 68% on LR-MSM) (p=0.0217) (***Figure 2C***). In addition, the reduction of p24 expression measured by Mean Fluorescent Intensity (MFI) denoted a higher antiviral activity on HR-MSM (20.02) group compared to LR-MSM (7.523) (p=0.0433) (***Figure 2D***).

Likewise, when the p24 protein was measured in the supernatants of the co-cultures by ELISA and compared against their respective infected control ($8x10^4$ LT $CD4^+$ HIV^+ without NK cells), a higher percentage of reduction of p24 protein was found in HR-MSM (33.6%) when compared to LR-MSM group (8.3%) (p=0.0232) (***Figure 2E***);

Finally, 4 LR-MSM and 4 HR-MSM were paired by age, and the percentage of $p24^+$ cells was measured and compared for both groups. A tendency to a lower percentage of $p24^+$ cells was observed on HR-MSM (37.9) when compared to LR-MSM individuals (58.2) (**Figure 2F**); however, no statistical differences were found (p=0.1250).


A
FMO p24
Q1
0
Q2
7,82E-3
Q4
2,28
Q3
97,7
p24+
HIV+ CD4+ T cells
3,26
2,18
94,6
NK:CD4+ (1:4)
Q1
0
Q2
0,096
Q4
7,30
Q3
92,6
CD3+ cells

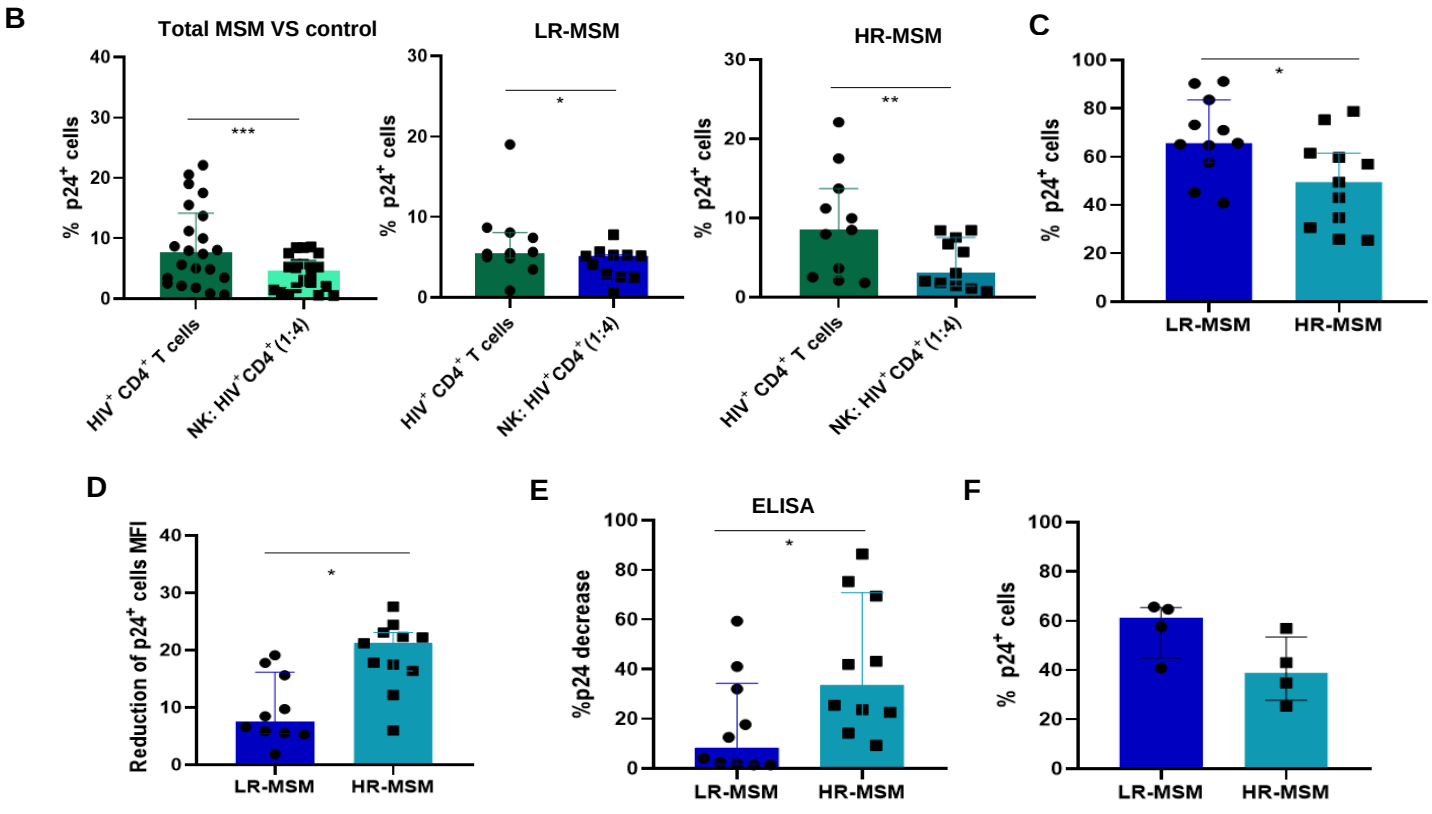

B
Total MSM VS control
LR-MSM
HR-MSM
% p24+ cells
HIV+ CD4+ T cells
NK: HIV+ CD4+ (1:4)
C
LR-MSM
HR-MSM
D
Reduction of p24+ cells MFI
E
ELISA
%p24 decrease
F

Figure 2. NK cells of HR-MSM showed a higher antiviral activity than LR-MSM.

A. Representative gating strategy of $p24^+$ cells evaluated in the co-cultures after seven days (FMO= Fluorescence Minus One control) (upper left panel), 80.000 HIV^+ $CD4^+$ T cells alone (lower left panel), and co-cultured NK with HIV^+ $CD4^+$ T cells in an effector:target ratio of 1:4 (lower right panel). **B.** Percentage of $p24^+$ cells in the co-cultures of all MSM individuals (LR-MSM and HR-MSM groups combined. Right bar) compared to infected control HIV^+ $CD4^+$ T cells (Left bar) and, LR-MSM and HR-MSM co-cultures compared to its respective infected control. **C**. Percentage of $p24^+$ CD4+ T cells in the co-cultures of LR-MSM (66%) and HR-MSM (46%). **D.** Reduction of $p24^+$ protein in the co-cultures expressed by MFI. **E.** Percentage of the p24 reduction measured by ELISA in the supernatants of the co-cultures after seven days n:10. **F.** Percentage of $p24^+$ CD4+ T cells in the co-cultures of LR-MSM (58.2%) and HR-MSM (37.9%) age paired. **B-D and F** were measured by flow cytometry and normalized with infected control data. MFI (Mean Fluorescence Intensity). The results are shown as mean± SD, n:11,11. Statistical evaluations were made with the Unpaired t-test and Mann-Whiney test. *p<0.05 and ***p<0.001.

HR-MSM showed a higher frequency of $NKG2D^+$ but a lower percentage of $CD69^+$ in total NK cells than LR-MSM after co-culture

The percentage of activation markers was evaluated by flow cytometry on the NK cells co-cultivated with HIV-1-infected autologous $CD4^+$ T cells after 12h of the co-culture. A lower percentage of $CD69^+$ NK cells was found in HR-MSM (7.5%) compared to LR-MSM (13.5%) (p=0.0050) (***Figure 3A***). However, this difference was not reflected in the production of perforin (0.33 vs. 0.60%), CD107a (2.26 vs. 2.50%), granzyme (1.8 vs. 1.5%) and IFN-γ (0.89 vs. 0.79%) (***Figure 3C-F***) production. Interestingly, a higher percentage of $NKG2D^+$ NK cells were observed in HR-MSM (1.88%) when compared to LR-MSM (0.84%) (p= 0.0161) (***Figure 3B***).

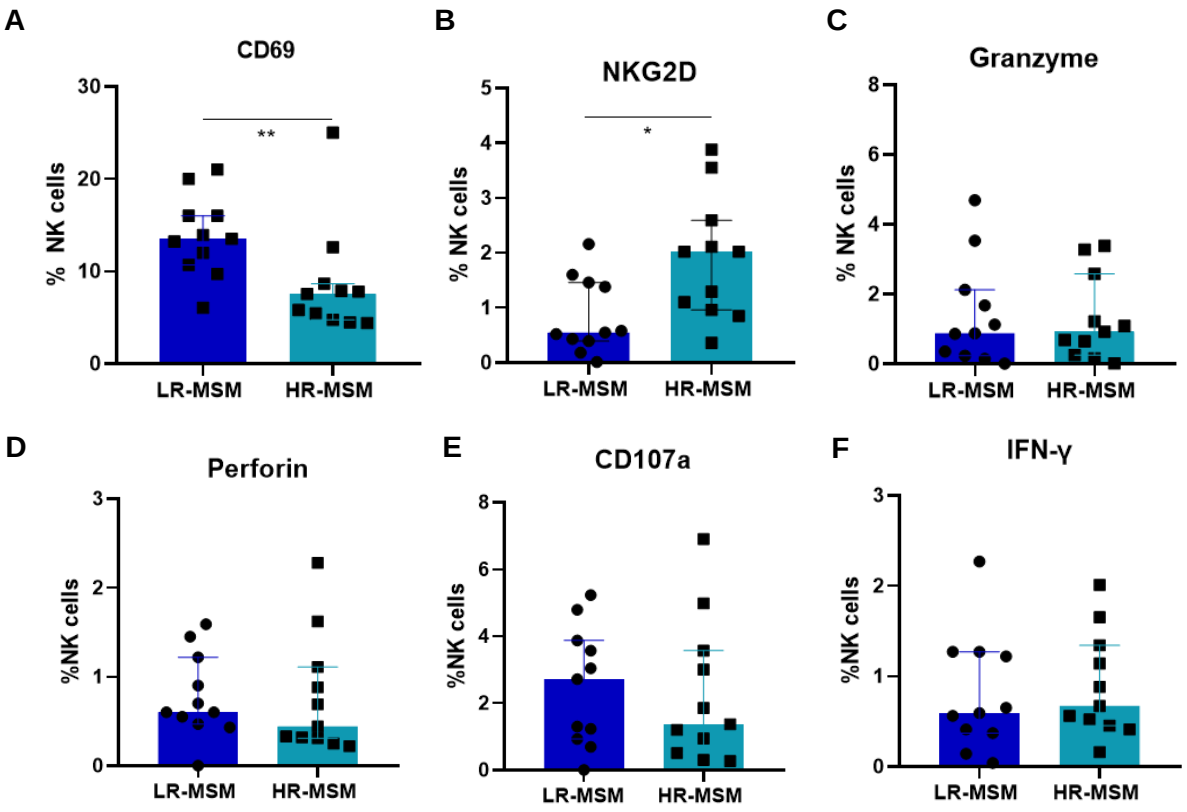


Figure 3. LR-MSM showed a higher percentage of CD69⁺ NK cells but lower percentages of NKG2D⁺ NK cells compared to HR-MSM.

Percentage of CD69+ NK cells evaluated in the co-cultures of LR-MSM and HR-MSM (p= 0.0050) (A). Percentage of NK cells expressing NKG2D (B) (*p= 0.0161), granzyme (C), perforin (D), CD107a (E) and IFN-γ (F) in the co-cultures of LR-MSM and HR-MSM. Bars represent the median with interquartile range. Statistical evaluations were made with Mann-Whitney U. *p<0.05, **p<0.01.

Co-culture of HR-MSM showed a higher percentage of CD69⁺ IFN-γ⁺ NK cells and high levels of IFN-γ production than LR-MSM.

HR-MSM showed a lower percentage of CD69⁺ NK cells than LR-MSM. However, the median of IFN-γ expression by CD69⁺ NK cells in HR-MSM (7.4%) were higher compared to the LR-MSM (3.9%) group (p= 0.0026) (***Figure 4A***). Suggesting that once activated, the NK cells from HR-MSM increase IFN-γ expression to exert its effector capacity.

Similarly, IFN-γ production was assessed by CBA in the supernatants of the co-cultures. Specifically, in comparison with the respective uninfected control, a higher significance was found in the HR-MSM group (p=0.0010) compared to the LR-MSM group (p=0.0117); (***Figure 4B-C***); however, no statistical differences were observed when LR-MSM and HR-MSM groups were compared (p=0.8383) (***Figure 3F***).

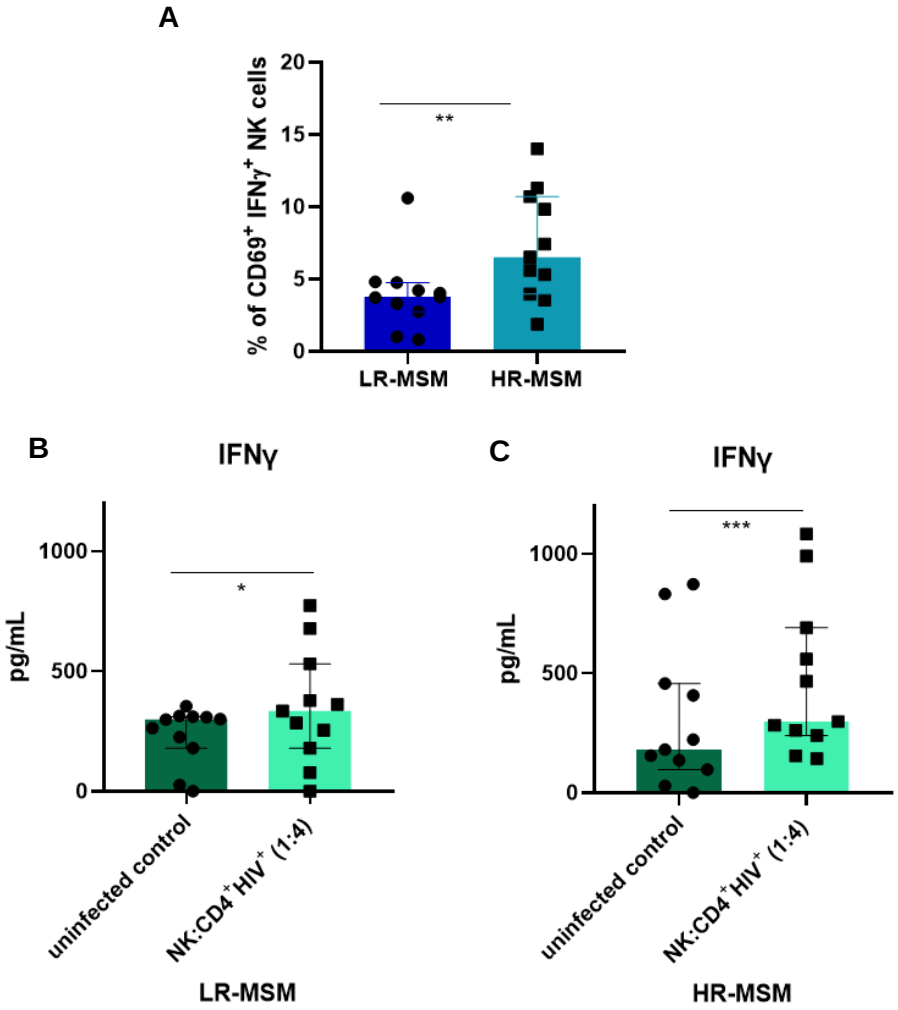


Figure 4. HR-MSM showed higher percentage of IFNγ⁺ activated NK cells and a tendency to higher production of IFN-γ compared to the LR-MSM.

A. Percentage of CD69⁺ IFN-γ⁺ NK cells measured after 12h of co-culture with CD4⁺ T cells infected with HIV (1:4). **B.** Concentration of IFN-γ (pg/mL) in the supernatants of the co-cultures measured by CBA in LR-MSM comparing uninfected co-cultures with HIV-infected CD4⁺ T cells. **C.** Concentration of IFN-γ (pg/mL) in the supernatants of the HR-MSM co-

cultures comparing uninfected control vs. HIV-infected $CD4^+$ T cells. The results are shown as mean± SD, n:11,11. Statistical evaluations were made with the Mann-Whiney test and Wilcoxon. *p<0.05, **p<0.01 and ***p≤0.001.

HR-MSM exhibited a distinct functional profile of $CD69^+$ NK cells than LR-MSM

After the evaluation of activation profile, and starting from $CD69^+$ subpopulation, the polyfunctional profile of NK cells was evaluated after 12h of co-culture by including the expression of NKG2D, IFN-γ and CD107a molecules (***Figure 5***).

The results showed differences between both groups. Accordingly, $CD69^+$/IFN-γ^+ NK cells and $CD69^+$/$NKG2D^+$ NK cells were more frequent in HR-MSM than LR-MSM (p=0.0114 for each analysis) (***Figure 5***). Additionally, a tendency was observed in $CD69^+$/IFN-γ^+ /$NKG2D^+$ and $CD69^+$/IFN-γ^+ /$CD107a^+$ profiles with a higher frequency in the HR-MSM group; however, no statistical differences were observed.

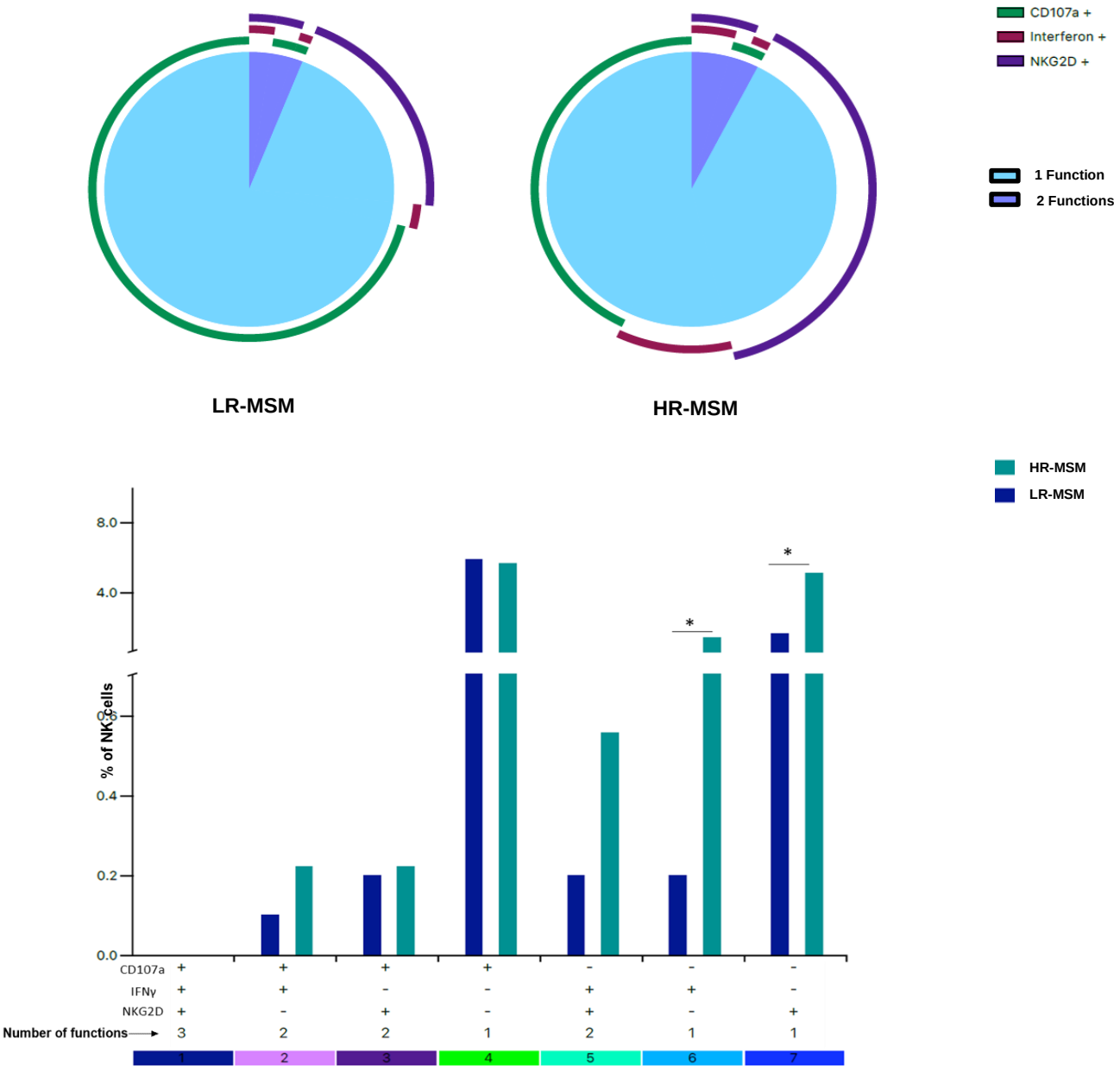


Figure 5. HR-MSM showed a higher frequency of NK cells with CD69$^+$/IFN-γ$^+$ and CD69$^+$/NKG2D$^+$ profiles. Polyfunctional profile analysis of NK cells of HR-MSM and LR-MSM after co-culture with CD4$^+$ T cell HIV$^+$ (1:4). The results are presented as mean, n:11,11 Statistical evaluations were made with Wilcoxon test *p< 0.05.

Supernatants of HR-MSM co-cultures showed higher Granzyme and RANTES levels than LR-MSM co-cultures.

After exploring NK cell activation, the ability of NK cells to produce effector molecules was evaluated by CBA in the supernatants of the co-culture after 12h. Statistical differences were observed in the concentration of granzyme for LR-MSM (72.81 pg/mL) compared to HR-MSM (237.70 pg/mL) (p=0.040); this difference was also observed in the MFI, with a median of fluorescence of 100.8 in the case of LR-MSM and 770.6 in HR-MSM group (p=0.0159) (***Figure 6A***). Similarly, a lower concentration of RANTES was observed in the LR-MSM group (26.27 pg/mL) compared to the HR-MSM (55.73 pg/mL) (p=0.0336). Again, this difference was also observed in the MFI, with a median of 267.2 for LR-MSM and 1.352 for HR-MSM (p=0.0192) (***Figure 6B***).

No statistical differences were found when IFN-γ, MIP1-α and TNF-α production were compared for both groups (***Figure 6C-E***).

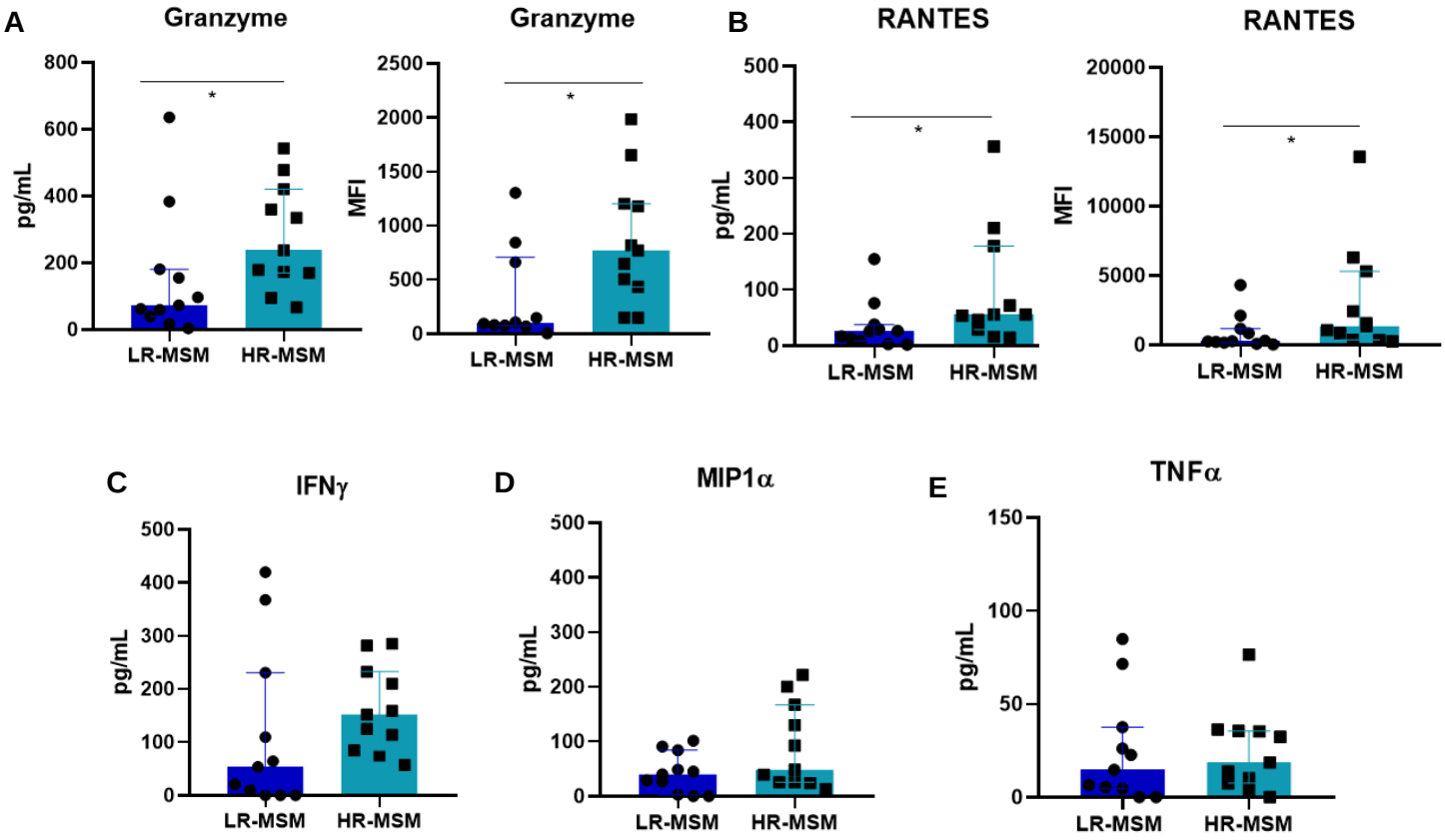


Figure 6. HR-MSM produces higher amounts of Granzyme and RANTES than LR-MSM. Effector molecules quantified in the supernatant after 12h the co-culture by CBA. The concentration of granzyme (A) and RANTES (B) in LR-MSM and HR-MSM expressed in pg/mL and MFI. **C-E.** IFN-γ, MIP1-α and TNF-α concentration in the supernatants of the co-cultures of LR-MSM and HR-MSM. Data were measured by CBA and are expressed as pg/mL and MFI (Mean Fluorescence Intensity). The results are presented as median. n:11,11. Statistical evaluations were made with the Mann-Whiney test. *p<0.05.

The number of sexual partners is correlated with reduction of $p24^+$ cells, RANTES production and the percentage of $NKG2D^+$ NK cells.

The number of sexual partners in the last three months was a key criterion for categorizing LR-MSM and HR-MSM groups. For that reason, correlations between this parameter and the antiviral capacity of NK cells, in terms of their ability to produce granzyme and RANTES, and the percentage of CD69+, IFN-γ+ and NKG2D+ NK cells were done. Remarkably, significant correlation was found with the percentage of $p24^+$ cells reduction (r=0.2734, p=0.0150) (***Figure 7A***), the percentage of $NKG2D^+$ NK cells (r=0.2094, p=0.048) (***Figure 7B***), and RANTES production (r=0.1859, p=0.0451) (***Figure 7C***).

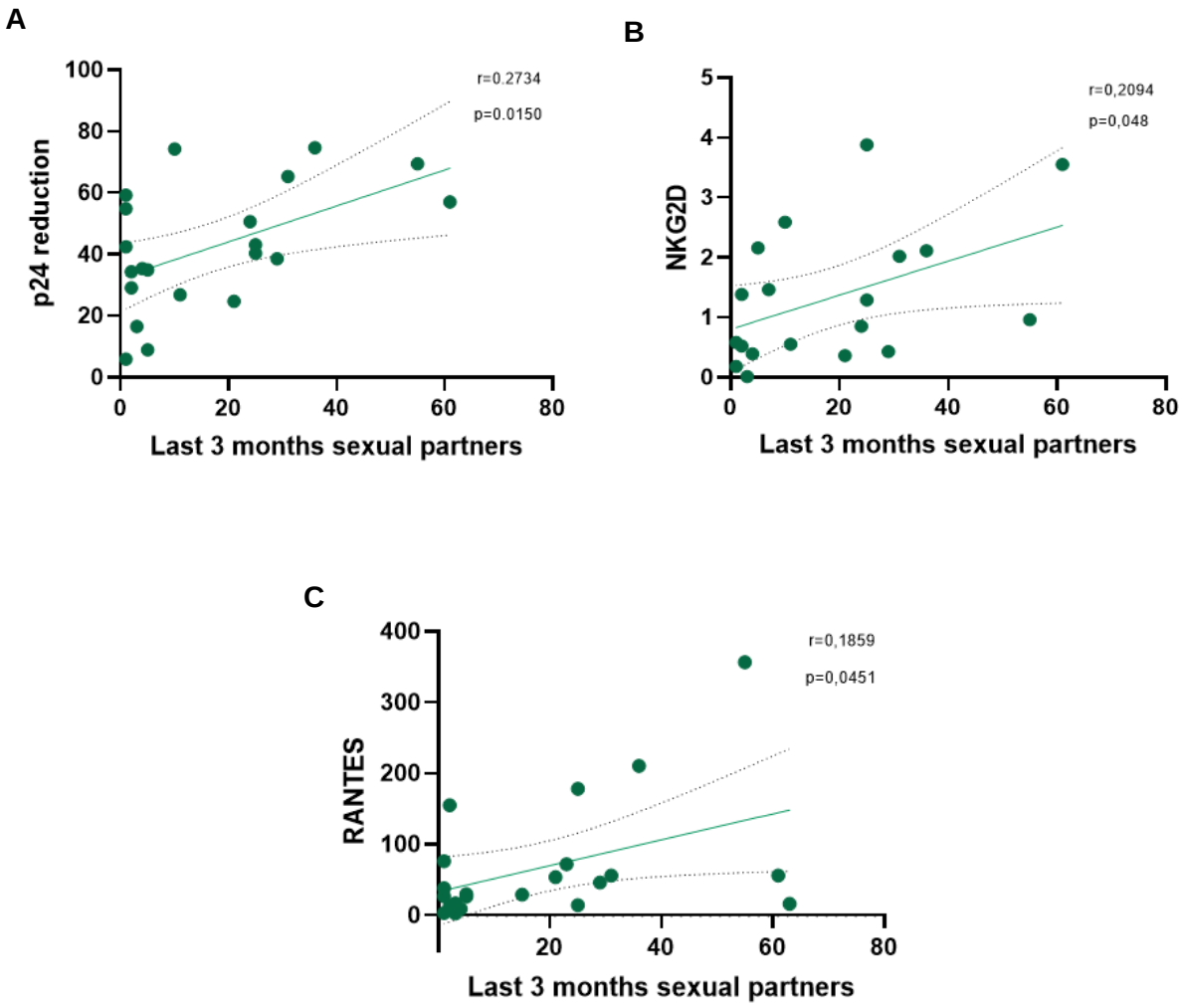


Figure 7. The number of sexual exposure in the last three months is correlated with reduction of p24⁺ cells, RANTES production and the percentage of NKG2D⁺ NK cells. Correlation of the number of sexual partners in the last three months in MSM individuals with: **A.** The percentage of reduction of p24⁺ cells. **B**. The percentage of NKG2D+ NK cells. **C.** Production of RANTES in the supernatants. Statistical evaluations were made with Spearman´s correlation test. *p<0.05.

8. Discussion

In recent years, the role of NK cells during HIV-1 infection has taken increasing importance. The antiviral activity of these cells is reflected in their ability to kill HIV-1 infected cells and the development of effector mechanisms that block viral entry, in addition to the enhancement of the immune response in some individuals. The experiments summarized in this work provide additional evidence on the protective function of NK cells in MSM individuals with a high-risk of acquiring HIV-1 infection. NK cells of HR-MSM exhibited a lower activation profile with higher antiviral capacity and, in accordance with previous reports in the same cohort [68], increased production of effector molecules and a higher cytotoxic capacity was also observed.

Other studies, including MSM individuals, have reported and categorized an average of 3.2 to 6 sexual partners within the last three months as a high-risk behavior [81, 82]. In fact, the median of the sexual partners in the last three months in the HR-MSM group was 25, and for the LR-MSM group, the median was two, reflecting a higher sexual exposure in our HR-MSM group. In addition, our LR-MSM group is outside of the mentioned range, indicating lower exposure and risk practices. Of note, in the present study, HR-MSM and LR-MSM shared biological factors and sociocultural background that directly influence the risk of acquiring HIV-1 infection, which are not present in other individuals, such as heterosexual men, supporting LR-MSM individuals as suitable controls to compare the evidence found in the HR-MSM group.

Moreover, in HR-MSM, a higher percentage of STIs (86,5%) were reported compared to LR-MSM (55,3%); the number of sexual partners in a lifetime could explain this difference, which is statistically different in both groups. There is a positive association between the number of STIs and the acquisition of HIV-1 infection. Indeed, several reports have linked herpes and syphilis to augmenting the probability of HIV-1 infection due to an increase of $CCR5^{+}$ T cells in the infected tissue [83]. Of notice, only one individual among both groups was heterozygous for the CCR5Δ32 mutation, and 21 individuals left were categorized as wild type, indicating that the most important genetic mechanism of natural resistance against

HIV-1 was absent in both groups. This information, along with the fact that 64% of the HR-MSM group reported at least one HIV-1 positive sexual partner, evidence the high-risk of acquiring the infection for this population.

The HR-MSM group had a median of 34 and for LR-MSM group was 26 years old. Some reports have concluded that NK cell maturation process is highly age-dependent, in which young people showed higher numbers of $CD56^{bright}$ cells compared to older people, which showed a higher frequency of $CD56^{dim}$ expressing maturation markers as CD57 [84]. Nevertheless, the differences in this phenotype can also be explained by accumulative exposure to different infections throughout the lifetime. For instance, in a study conducted in a Gambian population with high frequency of HCMV infection, it was reported that the percentage of terminally differentiated $CD56^{dim}$ $CD57^{+}NKG2C^{+}$ NK cells in young children population, were near to 70% at the age of six, which is higher than the median of the frequency of this fully mature phenotype reported in European population which is around 50% [85]. In addition, there is also evidence that the increase of $CD56^{dim}$ population in the circulation of elderly individuals does not correlate with an increase in overall cytotoxicity of NK cells [86], suggesting that despite age is involved in the stimulation and "training" of NK cells, there could be other mechanisms impacting the NK cells functionality.

We reported lower levels of $p24^{+}$ CD4+ T cells in the HR-MSM co-cultures compared to LR-MSM, and this result was validated with the levels of viral p24 protein in co-culture supernatants. The potential of NK cells for viral infected cells clearance has been of particular interest in the HIV-1 context. *Richard et al.* described that HIV-1 infection could induce the up-regulation of specific ligands for NKG2D receptor of NK cells, such as ULBP proteins induced by HIV-1 accessory protein Vpr, enhancing NK cell-specific lysis of infected cells [87]. Other mechanisms related to HIV-1 antiviral role of NK cells include the production of β-chemokines, ADCC, IFN-γ production, which influence the adaptive immunological response [88]. Notably, this antiviral activity against HIV of NK cells can be enhanced through stimuli with 25ng/mL of IL-15, promoting the elimination of latently infected $CD4^{+}$ T cells *in vitro,*

once they were induced to emerge from latency [89]. In the present work, this cytokine was used overnight, in a lower dose (20ng/mL), to maintain the viability of NK cells obtained from individuals of both groups before the co-culture. However, the differences between HR-MSM and LR-MSM groups were notorious, indicating that, despite being primed under the same conditions, NK cells of HR-MSM individuals had a higher antiviral capacity. Remarkably, the percentage of NK cells used in the co-cultures of the present study corresponds to 25% and 75% were infected $CD4^+$ T cells. This Effector-Target ratio (1:4) matches with the mean frequency of NK cells (15.8%) and $CD4^+$ T cells (43%) reported in peripheral blood of the Latin American cohort [90]. In addition, a tendency to a lower percentage of $p24^+$ cells was observed in HR-MSM compared to LR-MSM even after being paired by age, indicating that this parameter is not a direct correlate of the differences found in the antiviral capacity of NK cells of the MSM population on this study. Altogether, this evidence indicates that the higher antiviral capacity of HR-MSM NK cells could have a biological impact on the HIV-1 resistance observed in these individuals.

Some markers associated to NK cell activation were analyzed. Our results showed a lower percentage of $CD69^+$ NK cells in HR-MSM than LR-MSM group; however, no statistical differences were found when perforin, granzyme, IFN-γ and CD107a were compared in the total percentages of NK cells. This evidence was described in other HESN individuals in which, low percentages of $CD69^+$ NK cells were found in peripheral blood of CSW-HESN women [91]. Moreover, a reduced expression of this marker in $CD4^+$ T cells has been historically associated with quiescence phenotype, which is characterized by lower HIV target cell availability and susceptibility [92]. Remarkably, other markers associated with NK cell activation did not show statistical differences, indicating that a lower frequency of $CD69^+$ NK cells is not a synonym of activation impairment. In contrast, other reports have suggested that an increased expression of $CD56^{dim}$ $CD69^+$ NK cells prevail in HESN individuals [93]. However, recent evidence suggests that the differentiation into an adaptive phenotype such as $NKG2C^+$ NK cells seem to be linked to the expression of HLA-DR, unlike the expression of other activation markers such as CD69 and CD25 [94]. In agreement, more than two decades ago, it was demonstrated that the terminally differentiated

$CD56^{dim}$ NK cells population in older people, exhibits higher levels of HLA-DR and CD95, along with a decrease of the CD69 marker [95], also found in lower percentages in our study. We hypothesize that, NK cells from HR-MSM exhibit a lower activation profile and a higher expression of the NKG2C marker. In fact, previous data from our research group showed that HR-MSM had a higher percentage of $CD56^{dim}$ NK cells and higher frequency of adaptive $CD57^{+}$/$NKG2C^{high}$ cells NK were found compared to LR-MSM [68]. Likewise, this mature phenotype has been related to a better cytotoxic activity which can be linked the enhanced capacity to eliminate infected $CD4^{+}$ T cells found in this study.

Furthermore, higher expression of NKG2D was found in NK cells of HR-MSM, which could be linked to an enhanced capacity to recognize and eliminate HIV-1 infected cells. Classically, ligands to NKG2D such as MIC-A and MIC-B are expressed in infected CD4+ T cells during HIV-1 infection induced by Vpr protein, triggering NK cells-mediated lysis [96]. Moreover, NKG2D seems to act as a coreceptor during the ADCC process. *Parsons et al*. demonstrated a potential role of this receptor to enhance the ADCC mechanism against HIV-infected cells. In that work, peripheral blood NK cells were treated with an anti-NKG2D antibody, reducing the ADCC by 7.2% compared to the NK cells treated with an isotype antibody control [97]. Also, NKG2D seems to have a key role in the elimination of HIV-1 viral reservoirs during the shock and kill therapy; for instance, once the virus is reactivated, an upregulation of NKG2D ligands takes place on latently HIV-1 infected $CD4^{+}$ T cells promoting NK cytotoxic activity on infected cells [98, 99]. Unfortunately, under the approach of our work was not possible to measure any correlation between the higher expression of NKG2D and the ADCC mechanism. Further studies are required to clarify these issues.

In the HIV-1 infection context, IFN-γ seems to act on both sides of the coin; for instance, this cytokine can enhance $CD8^{+}$ T cell activity in HIV^{+} individuals, which is associated with early control of the infection and reduced viremia [100, 101]. In the case of HESN individuals, a higher percentage of IFN-γ^{+} NK cells were found compared to uninfected and HIV^{+} controls after the stimulation with PMA/Ionomycin

[72]. Another study in uninfected infants born to HIV-1 infected mothers showed that HIV-*gag* specific IFN-γ cellular response detected in breast milk was associated with decreased infant HIV-1 infection in HESN infants [102]. In fact, the effects of this cytokine in pro-inflammatory responses, immune activation and antiviral activity have converted IFN-γ into an interesting marker to evaluate immunological capability against HIV. In contrast, evidence also suggests that the role of this cytokine depends on its adequate regulation, which, if not properly controlled, is linked to chronic immune activation, leading to immunological exhaustion and rapid progression to AIDS [103]. As mentioned before, a lower frequency of $CD69^+$ NK cells were found in HR-MSM than LR-MSM. Despite this, a higher percentage of $CD69^+$ IFN-γ^+ NK cells were found in HR-MSM compared to LR-MSM, and this coincided with the tendency observed in the supernatants of the co-culture when IFN-γ was quantified by CBA. Therefore, it can be related to the augmented antiviral capacity of the NK cells in the HR-MSM evaluated. Although, no statistical differences were found in the production of IFN-γ among LR-MSM and HR-MSM; when IFN-γ levels were compared against its respective uninfected control, a higher statistical significance were found in HR-MSM group; this suggests wide biological differences in the basal production or regulation of IFN-γ among all individuals enrolled in this study.

The functional analysis of $CD69^+$ NK cells showed two different NK cell populations, $CD69^+$/IFNγ^+ and $CD69^+$/$NKG2D^+$ NK cells with higher frequency in HR-MSM. In the MSM-HESN population, little is known about the co-expression of these markers; however, all of them have been linked to HIV-1 antiviral activity in other HESN cohorts previously discussed [22]. Interestingly, in an HIV-1 infected cohort, *Nabatanzi et al.* reported an atypical activation pattern in NK cells among ART-treated individuals, with a higher $CD69^+$ expression and a lower expression of IFN-γ, NKG2D, and granzyme B, this functional impairment was evidenced despite of coursing with undetectable viral loads and steady CD4+ T cell counts for more than seven years [104]. In contrast, we reported a lower frequency of $CD69^+$ NK cells in HR-MSM individuals with an augmented functional capacity, reflected in the $CD69^+$/IFN-γ^+ and $CD69^+$/$NKG2D^+$ co-expression along with a tendency of higher

$CD69^+$/$IFN\gamma^+$ /$NKG2D^+$ NK population. Of note, the functional recovery of NK cells is a topic of special interest in positive individuals [64, 105], as well in the resistance mechanisms against HIV-1, in which IFN-γ^+ and $NKG2D^+$ expression seems to be involved [69, 106].

Furthermore, studies performed in a Colombian HESN cohort have shown a higher expression of granzyme by PBMCs, in a basal state and even after seven days of *in vitro* infection with HIV-1, compared to HIV^+ positive individuals and uninfected controls [107]. Remarkably, the serine protease granzyme B acts in a wide range of biological activities, including a widely studied antiviral potential [108]. The pro-apoptotic action of this protease is considered the most potent and rapid among all the granzymes [109]. Indeed, studies performed on elite controllers have linked higher levels of granzyme B with the capacity to control viral replication [110]. In HESN cohorts, recent reports have found high levels of granzyme B, TNF-α, and IFN-γ in cervicovaginal lavages compared to unexposed-healthy women [22]. Our findings also showed a higher concentration of granzyme B in the supernatants of the co-cultures of HR-MSM, with a tendency to higher levels of TNF-α and IFN-γ, which could be linked to differences in the percentages of $p24^+$ CD4+ T cells observed. Together, these results suggest that granzyme B could act as an important mechanism of resistance to HIV-1 infection.

The evaluation of molecules with antiviral activity in supernatants of the co-cultures by CBA showed that RANTES (CCL5) was produced in a higher concentration for HR-MSM compared to LR-MSM. Interestingly, this β-chemokine is associated with inhibiting HIV-1 entry through binding to the CCR5 coreceptor, interrupting the interaction with the HIV envelope glycoprotein gp120 [111]. Similarly, RANTES was reported to be in higher percentages on NK cells of peripheral blood of HESN-IDU. Indeed, this difference remains after co-culture with K562 cells compared to seropositive controls before and after infection. This evidence indicates that a higher frequency of $CCL5^+$ NK cells could be involved in natural resistance [69]. Likewise, a higher production of RANTES has been correlated with protection against HIV infection in different biological compartments such as peripheral blood, saliva, or

genital mucosa among different cohorts of HESN around the world [112-114]. These findings suggest that higher production of RANTES by NK cells could reduce the HIV-1 infected $CD4^+$ T cells, which is reflected in the percentages of $p24^+$ CD4 T cells in the evaluated co-cultures of HR-MSM individuals.

Finally, p24 levels reduction, NKG2D and RANTES expression were correlated to the magnitude of sexual exposure in the last three months, suggesting that higher exposure to HIV-1 infection in a recent period in HR-MSM individuals could prolong the “trained” state of NK cells, allowing these cells to respond more robustly to different microbial infections [115]. However, it is important to clarify that MSM-HESN individuals are not “created” from higher sexual exposure to HIV-1, its resistance could be a multifactorial state based on intrinsic biological differences, such as the capacity of their immunological system to clear HIV-1 infected cells. For that reason, the evaluation of NK cells antiviral capacity could be promissory to elucidate the natural resistance to HIV.

9. Concluding remarks

As we hypothesize, NK cells of HR-MSM display higher antiviral activity against HIV^+ $CD4^+$ T cells. This evidence is reflected in reducing the percentage of $p24^+$ $CD4^+$ T cells and the lower concentration of p24 protein detected in the supernatants of HR-MSM co-cultures. These results could be explained by the higher percentages of $CD69^+$/IFN-γ^+ and $CD69^+$/$NKG2D^+$ NK cells, and by the production of antiviral molecules such as RANTES and granzyme, key weapons against HIV-1 infected $CD4^+$ T cells. However, other mechanisms could be involved in the natural resistance to HIV-1.

Together, the differences observed in peripheral blood of MSM individuals highlight the role of NK cells as a possible target for strategies that enhance antiviral response against HIV-1 in seronegative and seropositive individuals.

10. Limitations and future perspectives

As was mentioned before, the Colombian MSM population lives under a challenging scenario of discrimination and social stigma, making difficult the approach to this population and the subsequent enrolment to different studies. A higher number of enrolled patients would have been desirable.

Moreover, in the ideal scenario, the cytotoxic/antiviral effects of NK cells should be measured within the first 4 hours of being in contact with the target cells; however, under the methodology applied in this work, this effect was measured at the seven days after co-cultivation, showing a late outcome of the initial effect of NK cells on infected $CD4^+$ T cells. It would be interesting to apply a different methodological strategy focused on the first hours of co-cultivation.

Moreover, all the methodological procedures were made starting from frozen cells, making it difficult to analyze some phenotypical markers such as CD56. However, the availability of these samples was limited and due to restrictions around the COVID-19 pandemic, a new recollection process was non-viable. In addition, there is growing information of a possible link between higher percentages of $NKG2D^+$ and ADCC; further investigations on HESN population around this mechanism and particularly on HESN-MSM individuals will be of particular interest to have a wider panorama of NK functionality and its role in HIV-1 resistance.

11. Additional information

The results of this study were shown in the Colombian Congress of Allergy, Asthma, and Immunology (ACAAI-ACOI) 2021. This work was awarded with the **first prize** in the poster category.

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Reconoce a:

DANIEL RINCÓN TABARES

Como ganador (a) del primer puesto de trabajos de investigación en MODALIDAD PÓSTER del Congreso Colombiano de Alergia, Asma e Inmunología ACAAI • ACOI realizado los días 9, 10 y 11 de septiembre de 2021 en modalidad virtual.

Título del trabajo EVALUACIÓN DE LA CAPACIDAD EFECTORA DE LAS CÉLULAS NK DE HOMBRES QUE TIENEN SEXO CON HOMBRES CON ALTO RIESGO DE INFECCIÓN POR EL VIH-1

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ACOI

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