PHENOTYPIC AND FUNCTIONAL EVALUATION OF NK CELLS IN MEN WHO HAVE SEX WITH MEN AT HIGH RISK OF HIV-1 INFECTION FROM MEDELLIN

LIZDANY FLOREZ ALVAREZ

Thesis submitted in fulfillment of the requirements for the degree of Master in Basic Biomedical Science, emphasis in Virology

ADVISOR

Wildeman Zapata Builes, Bact., MSc., DSc.

CO-ADVISOR

Juan Carlos Hernandez Lopez, Microbiol., DSc.

ADVISORY COMMITTEE

Maria Teresa Rugeles Lopez, Bact., MSc., DSc.

Paula Andrea Velilla Hernandez, Bact., MSc., DSc.

Angela Patricia Cadavid Jaramillo, M.D., MSc., DSc.

Universidad de Antioquia

Corporación Académica Ciencias Básicas Biomédicas

Medellin, Colombia

2019

PRESENTATION

This thesis begins with the introduction, which includes the background and theoretical framework. The background includes basic aspects of HIV epidemiology and pathogenesis, a definition of HIV-exposed seronegative individuals (HESN) and the cohorts proposed for their study, as well as a summary of natural resistance mechanisms previously described for this population, with particular emphasis on increased NK cell effector capacity associated with natural resistance against HIV-1 during viral exposure.

The theoretical framework is a review of the NK cells role during HIV infection published in Frontiers in immunology in 2018. This review covers basic aspects of NK cells biology, NK cell features associated with HIV control, the role of memory NK cells during HIV infection and vaccination strategies based on NK cells. Then, the problem statement highlights why despite being poorly studied compared to other HESN cohorts, men who have sex with men (MSM) represent a key population for the study of natural resistance mechanisms against HIV.

After, the general and specific objects are presented together with the hypothesis of the study. Later are material and methods, results and discussion. Results are presentedtogether with respective figures and tables grouped by the most relevant findings. The discussion presents argumented conclusions supported by scientific literature. In this section, we proposed how the findings contribute to generating knowledge in the field of natural resistance against HIV.

After, concluding remarks are presented, followed by limitations and future perspectives of the study. Finally, at the end of the text, some supplementary data are presented, in order to bring more information on methodological approaches and results.

ABSTRACT

Introduction: HIV exposed seronegative (HESN), are individuals that remain seronegative despite repeated exposure to HIV. Since the first reports of HIV resistance in humans in 1989, HESN individuals turn in to a key population for the research of the phenomenon of human natural resistance to HIV-1. Studies conducted in this population allowed to describe different immunological and genetic mechanisms associated with viral resistance. A higher effector capacity of NK cells has been related to natural resistance in different HESN cohorts. Besides, recently a population of NK cells with memory features has been described; these cells are increased in HESN individuals and they are involved in better control of HIV replication in primarily HIV infected subjects.

Although men who have sex with men (MSM) are one of the main cohorts for the study of HESN, this population has been poorly studied compared to others like commercial sex workers (CSW) and intravenous drug users (UDI). However, some mechanisms of natural resistance have been described in them. The role of NK cells on HIV resistance has not been studied in this population so far. This study evaluates the role of NK cells in the natural resistance to HIV-1 infection in MSM.

Methodology: Phenotypic and functional features were evaluated in NK cells from two groups of MSM, at different risk of HIV infection defined based on the number of sexual partners. Production of IFN-γ and β-chemokines were included in the analysis as well as the cytotoxic capacity and memory markers. Genetic features such as HLA and KIR alleles were also explored.

Results: High-risk MSM showed an increased frequency of fully mature NK cells ($CD56^{dim}CD57^{+}$) as well as memory NK cells ($CD56^{dim}CD57^{+}NKG2C^{high}$). High-risk MSM also show higher cytotoxic capacity and IFN-γ production against K562 stimuli. A subpopulation of NK cells with $CD107a^{+}$/IFN-γ^{+}/MIP-$1\beta^{-}$ functional profile was found in higher frequency among high-risk MSM compared to low-risk MSM. This NK cells population displays higher MFI of IFN-γ in MSM at high risk compared to low risk, characteristic absent in NK cells with other functional profiles. Protective KIR/HLA phenotype KIR3DL1/S1-HLA-B*Bw4 in a homozygous state was found in

30.7% of High-risk MSM. Of note, some of these functional features were related to higher frequencies of mature and memory NK cells, which in turn, were associated with the higher number of sexual partners found in high-risk MSM.

Conclusion: The changes observed in NK cells compartment can be driven by the magnitude of sexual exposure and immunological challenges of high-risk individuals, that could influence their resistance/susceptibility to HIV infection.

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Background

1.1.1 Epidemiology and pathogenesis of HIV infection

Human immunodeficiency virus (HIV) infection, represents a major public health problem worldwide. Since the beginning of the epidemic in 1981, more than 76.1 million people have been infected with the virus, and around 35 million people have died of HIV related causes (1). For 2018, UNAIDS reported an estimated 37.9 million people living with HIV in the world and 1.7 million new infections. Despite global efforts to reduce HIV related deaths and the implementation of combined antiretroviral therapy (cART), there are near to 770.000 deaths related to AIDS every year (1).

In Colombia, government statistics report near to 83.000 people diagnosed with HIV (2); however, UNAIDS estimate around 160.000 people living with HIV in the country (3). Colombia has been classified as a country with a concentrated HIV epidemic, where the infection has spread primarily in some high-risk populations. In these high-risk populations, HIV prevalence is around 17%, while the prevalence in the general population is 0.5%. High-risk populations include men who have sex with men (MSM), commercial sex workers (CSW), transgender women, and people deprived of their liberty (4, 5).

HIV is transmitted by sexual contact across mucosal surfaces, by maternal-infant exposure or by percutaneous inoculation. Sexual transmission is responsible for more than 70% of infections worldwide. The remain is attributable to maternal-infant infection and injection drug use (6). In the natural history of HIV infection, upon transmission to a new host, generally occurring at mucosa tissue, the virus infects resident dendritic cells and $CD4^+$ T cells, mostly those expressing the CCR5 molecule, which preferentially are located at the gut-associated lymphoid tissue (GALT) where 65% of body T cells are present. These cells are also enriched in other lymphoid tissues, facilitating virus dissemination, at early stages of infection when the virus can reach levels of 10^7 copies per mL, inducing a massive loss of

$CD4^+$ T cells. Then, the instauration of the adaptive immune response allows the partial control of HIV replication, where the infected individual reaches a steady-state know as the viral set point. At the chronic phase, the viral load remains controlled, allowing partial recovery of $CD4^+$ T cell counts in peripheral blood but more limited in the GALT.

During HIV infection, individuals experience a generalized immune activation state, generated by a high replication rate and then, maintained by a permanent release of microbial products from the intestinal lumen to blood, secondary to intestinal mucosa damage (7). Chronic immune activation leads to immune exhaustion, characterized by a loss of viral control and a decrease in $CD4^+$ T cells, along with a disfunction of other cell populations like $CD8^+$ T and NK cells (8-10). This phenomenon marks the beginning of the acquired immunodeficiency syndrome (AIDS) stage, where the immune system of HIV infected individuals is compromised, rendering them susceptible to opportunistic infections and neoplasias (11).

The course of HIV infection is complex and variable between individuals. For this reason, HIV infected individuals have been classified based on the time of progression to AIDS. Most of HIV infected individuals, known as typical progressors, progress to AIDS in a period between 5 and 10 years in the absence of cART; while some individuals instead, progress to AIDS in less than 5 years (rapid progressors). There is another group of individuals named long term non-progressors (LTNP), who despite being infected with HIV for more than 10 years show no signs of immune deterioration (12). Because of the low frequency, and the time required to classify an individual as LTNP, new classification criteria were defined. For instance, elite controllers are individuals who maintain undetectable viral load (<50 copies/mL) for one year in absence of cART, and viremic controllers who exhibit viral loads under 2000 copies/mL in the absence of cART (12, 13). The existance of these individuals points to the presence of natural mechanisms that can mediate a sustained control of HIV replication, delaying or eventually avoiding the progression to AIDS in the absence of the therapy.

There is an interesting group of individuals who has not serological or clinical evidence of infection despite repeated exposure to HIV. This group of individuals is known as HESN (HIV exposed seronegative). The discovery of HESN individuals marked the beginning of the research in the phenomenon of natural resistance to HIV infection in humans.

1.1.2 HESN individuals and the study of natural resistance to HIV infection

HESN individuals are described as “individuals who remain uninfected with HIV despite repeated exposure and against statistical probability”. Under this description, numerous cohorts that include HESN subjects have been defined. These cohorts are classified into three major groups: i) serodiscordant couples: couples with unprotected sexual intercourse, where one individual is seropositive, with detectable viral load, and the other seronegative; ii) individuals with high-risk sexual behaviors: CSW and MSM and, iii) individuals, non-sexual exposed: intravenous drug users (IDU), infants born to HIV positive mothers, hemophiliacs, and others, exposed to contaminated blood products (health personnel) (14). HESN individuals are relatively infrequent; they represent approximately 10% of HIV exposed subjects (15).

Serodiscordant couples are one of the largest exposed seronegative groups; studies on this cohort has provided a great deal of information regarding the natural resistance phenomenon. One of the first reports of resistant individuals was made in a cohort of serodiscordant couples in 1989 when the negative partner of a seropositive individual showed HIV-1 specific T cell responses (16).

The seronegative partner, within a serodiscordant couple, is repeatedly exposed to the same HIV quasispecies, which could represent an easier task for the immune system in the sense that it is responding to related strains. This fact can be a barrier if the understanding of natural resistance is routed to develop therapeutic alternatives or vaccines, because in these cases a cross-reactive response is more desirable; that is the case of individuals with high-risk sexual behaviors (14).

CSW are part of the high-risk sexual behaviors group and represent one of the most important cohorts for the study of natural resistance to HIV infection. These individuals have been classified as resistant based on epidemiological models carried out in CSW cohorts from Africa and Asia. One of the largest and extensively followed is the Pumwani cohort from Nairobi, Kenia (17). These women have a high frequency of unprotected sex, about 15 clients per day, in a population where, at the peak of the epidemic, the prevalence was 14% (currently 8%) (18). To date, many female CSW cohorts have been identified, and they represent a key source of information on natural resistance mechanisms in women.

It is important to mention that the mechanisms of transmission and immune response at the site of infection differ between men and women, and not always, the findings in these female cohorts can be relevant to men. This issue makes the research of the natural resistance phenomenon in men very important, mainly because men still carry a disproportionate burden of HIV infection in many countries.

MSM represent 18% of people living with HIV worldwide, above UDI (9%) and CSW (3%) (19). The differences in sexual behaviors make this group a unique cohort for studies on natural resistance to HIV infection, based on the high number of sexual partners and specially the nature of MSM sexual intercourse, which represents a high-risk practice by itself. Receptive anal sex has a risk of infection ten times higher than receptive vaginal sex (20), and this fact added to some social and biologic factors that will be mentioned further ahead make MSM a population at a really high risk of HIV infection.

1.1.3 Mechanisms of natural resistance to HIV infection

The study of cohorts including HESN has led to a better understanding of factors involved in natural resistance to HIV infection. Genetic and immune factors found in these cohorts include *CCR5* Δ32 mutation, HLA-KIR allele expression, immune quiescence, HIV-1 specific cytotoxic lymphocytes (CTL), HIV-1 specific IgA, soluble factors, among others. Resistance to HIV cannot be attributed to any of these factors by itself, because, none of them has been found in all HESN individuals. Currently, the natural resistance to HIV is recognized as a complex and multifactorial

phenomenon where resistance can be derived from distinct mechanisms acting together, that might vary in frequency along the different cohorts.

The *CCR5 Δ32* mutation is one of the most important genetic factors described to date. This mutation is present in about 2 to 4% of Caucasian individuals and causes deletion of 32bp in the *CCR5* gene, leading to the production of a truncated protein that is not expressed at the cell membrane (21, 22). Homozygous individuals for this mutation (Δ32/Δ32) are resistant to HIV infection by R5 strains, which use CCR5 as a co-receptor for viral entry into the target cell. Heterozygosity instead, has been related to slow progression to AIDS and protection to HIV infection only in some HESN cohorts (23). In Colombian HESN, the prevalence of Δ32/Δ32 individuals is 2.6%, explaining only a small fraction of natural resistance cases (24).

HIV-specific CTL was one of the first mechanisms associated with natural resistance in HESN individuals. IFN-ɣ production was observed in CWS from the Pumwani cohort in response to class I HLA-restricted CTL epitope peptides in mononuclear cells from the cervix and peripheral blood. CTL responses in the cervix were higher than in blood, and the HIV-specific response was higher in CSW compared with low-risk individuals who did not show any response (25). Similar results have been found in HIV-1 negative babies born to positive mothers and health care workers, who have been accidentally exposed to HIV but remain seronegative (26, 27)

In addition, the humoral response also has been detected in HESN. In 1999, Plummer *et al.* reported a higher frequency of HIV-specific IgA at the genital tract of HESN women (76%), compared to (26%) infected women(26%) and low-risk controls (11%)(28). Neutralizing IgA has been also found in plasma and saliva of HESN women but no in low-risk controls (29). These data suggest a role of HIV-specific IgA in natural resistance to HIV-1 infection, which has been highlighted by vaccination and passive immunization studies, where specific IgA showed the capacity to neutralize the virus, preventing transcytosis (30).

Adaptive immunity responses require time to develop and most likely rely on innate mechanisms to control the incoming virus. Additionally, approximately half of HESN

individuals lack any detectable HIV-specific adaptive immune response, suggesting that innate immune mechanisms are involved in natural resistance to HIV infection.

Soluble factors are part of antiviral mechanisms of innate immunity; these are secreted by a variety of cells, being an important part of mucosal defenses. Defensins are small cysteine-rich cationic peptides that display potent antiviral features (31); there are 3 groups of defensins (α, β, and θ), α and β have are associated with resistance in several HESN studies. The group of human β-defensins (HBD) are the most widely studied, such as HBD-1, HBD-2 and HBD-3. HBD-1 is constitutively expressed in oral mucosa, while 2 and 3 are induced by several stimuli including virus.

In 2016, our group reported an increase in HBD-2 and 3 expression in a cohort of exposed individuals (32); these molecules are able to inhibit HIV-1 infection (of both X4 and R5 tropic viruses) by direct virus inactivation and downregulating CXCR4 expression (33). In addition, it has been reported that polymorphisms in genes encoding HBD, *DEFB1, 2* and *3* are over-represented in HESN. That is the case of A692G that is correlated with an increased expression of HBD-1 (34). α-Defensins have an important role in protection against vertical transmission. Studies in seropositive mothers showed an association between high concentrations of α-Defensins and low risk of birth and postnatal HIV transmission. Other studies have shown that a lack of HBD expression allows transmigration of virions within oral mucosa and increases the risk of mother-to-child HIV-1 transmission through breastfeeding (35, 36).

The apolipoprotein B mRNA editing enzyme catalytic polypeptide like 3 (APOBEC3) family includes seven members; these genes encode for cytidine deaminases capable of inducing G to A hyper-mutation in the viral genome, inhibiting the correct viral replication. HESN individuals express higher levels of APOBEC3G mRNA at basal, and after IFN-α stimulation compared to healthy controls and HIV positive individuals (37). APOBEC3G antiviral function is counteracted by the HIV protein, virion infectivity factor (Vif), which leads APOBEC3 proteins to proteasome degradation. There is one APOBEC3H haplotype that is resistant to Vif action; this

haplotype, named Hap1 is over-represented in HESN compared to HIV positive individuals (38). Increased expression of other members of this family like APOBEC3F has been also associated with a decreased risk of HIV-1 infection and slow progression to AIDS (39).

Other soluble factors found increased in HESN include SLPI, Serpin, TRIM5-α, ELAFIN, and RNases. SLPI is secreted primarily by epithelial cells lining mucosal surfaces and it has an important role in protection against vertical transmission. Higher levels of SLPI in saliva were associated with a lower risk of HIV infection in babies born to positive mothers exposed via breastfeeding (40). Likewise, SLPI levels in the vaginal fluid of positive women who transmit HIV to their babies were lower compared with non-transmitting women (41). Our group reported that Colombian HESN had higher expression of this protein in oral mucosa compared to healthy controls (34).

TRIM5-α, a member of the tripartite motif (TRIM) family has shown to be a highly species-specific restriction factor against retroviruses. Although the anti-viral mechanism of this molecule is not fully understood, it is well known that TRIM5-α displays proteasome-dependent and independent restriction capacity. Higher expression of this molecule is associated with low HIV-1 viral loads (42). Our group also report an increase of TRIM5-α mRNA in the genital mucosa of HESN compared to healthy controls (43).

Serpin and Elafin are protease inhibitors that affect virus attachment and transcytosis in epithelial cells. Serpin, in addition, reduces the production of pro-inflammatory cytokines (44). HESN exhibit higher mRNA levels of these molecules in PBMCs, GALT, and genital mucosa compared to healthy controls (43). RNases are antimicrobial peptides with reported antiviral activity; we have previously reported that recombinant RNase-1 inhibits primary infection of T cell blasts (45). We have also reported increased levels of RNase-1 in cervical samples of HESN compared to healthy controls and seropositive individuals (46).

Immune activation, at the site of infection, is one of the critical factors for the establishment of HIV infection, and later on, is a significant factor involved in the

destruction of the immune system and disease progression (47, 48). Therefore, a lower immune activation state of target cells could be related to a lesser ability to sustain viral replication, resulting in reduced susceptibility to HIV infection. Lower immune activation together with basal lower production of proinflamatory citokines are part of the quiescent phenotype (49). The immune-quiescent profile has been found in cells from peripheral blood and mucosa of different HESN cohorts, such as serodiscordant couples, CSW, and MSM (50-52).

Studies carried out in CSW from the Pumwani cohort, have shown that reduced susceptibility to HIV was related to a lower frequency of $CD4^+$ T cells expressing CD69 activation marker, and an elevated number of Treg cells (53). Mucosal explants from MSM exhibit a lower production of pro-inflammatory cytokines, after stimulation with inactivated whole pathogens, while anti-inflammatory cytokines and frequency of immune cells were similar compared to controls (50). A decreased expression of genes implicated in T cell receptor signaling, leukocyte homing as well as genes associated with HIV integration and replication has been described (54-56).

Conversely, other studies have shown that the immune activation state is related to protection against HIV infection. In these studies, a higher number of activated T lymphocytes, higher release of pro-inflammatory cytokines and decreased percentage of naïve T cells have been repeatedly found in HESN cohorts. In 2000, Biasin *et al.* reported an increase in pro-inflammatory cytokines secretion and chemokine receptors expression in cervical biopsies of HESN women; in this study, specific IFN-ɣ secreting cells was also reported (57). More recently, *Tomescu et al*. reported an increased expression of activation markers CD69 and CD83 in NK and myeloid dendritic cells in HESN, IDU subjects, compared to low-risk non-sharing needles IDU subjects (58).

Although there is no consensus on the role of immune activation in HIV protection, there is a line of work, that attributes these contrasting results to differences in the intensity of exposure among studied cohorts; where occasional contacts resulted in

immune activation, while massive and repeated exposure resulted in immune quiescence, as observed in Nairobi CSW cohort (59).

Alleles of MHC involved in protection against HIV infection and slow progression to AIDS have been also reported. HLA-B alleles can be divided into two groups, based on the expression of molecular epitopes, HLA-Bw4 and Bw6. Both, have a role in presenting viral peptides for immune recognition. HLA-Bw4 alleles have been associated with slow progression to AIDS; individuals homozygous for Bw4 alleles exhibit a profound suppression of HIV viremia and a normal $CD4^+$ T cell count (60, 61).

In addition to present viral peptides, Bw4 but no Bw6 act as a ligand of natural killer cell inhibitory receptors (KIR). The combined genotype of Bw4 with *KIR3DS1/L1* has been associated with natural resistance to HIV infection in several studies (62, 63). In a cohort of female CSW, in the context of this genotype, the absence of ligands to inhibitory KIRs reduced the activation threshold of NK cells, leading to an increased activation (64). These data support the important role of KIR/HLA interaction for HIV infection control.

Specific variants like HLA-B*57 and B*27 has been associated with better control of the infection; these molecules are restricted to gag epitopes, and they induce a more efficient response of CTL and delayed escape mutations (65-67)

Other alleles like HLA-B*18 has been related to protection against HIV-1 infection during mother to child transmission; in contrast, B*35 and B*58:02 have been associated with inefficient control of HIV replication and rapid progression to AIDS. HLA-C alleles. Although less frequent, it has been also associated with viral control (68)

1.1.4 NK cells in natural resistance to HIV infection

An increase in NK cells effector capacity has been described within the innate immune mechanisms associated with natural resistance to HIV-1 infection. These cells may contribute to the control of HIV infection in several ways. NK cells are essential in the induction of adaptive immune response. Besides, they can eliminate

infected cells trough cytotoxic mechanisms and they are major producers of β-chemokines, which avoid the infection of new cells by blocking viral co-receptors.

In 2003 Scott-Algara *et al.* reported, for the first time, an increase in the effector capacity of NK cells in IDUs who remained uninfected despite several years of high-risk practices. NK cells from HESN IDU shown a higher cytotoxic capacity, compared with NK cells from healthy controls and other IDUs who seroconverted during the study (69); similar findings were published by Tomescu *et al*. (58). Not only natural cytotoxicity, but soluble factors production and antibody-dependent cellular cytotoxicity (ADCC) has been related to natural resistance to HIV infection. High levels of IFN-ɣ production by NK cells have been associated with the maintenance of seronegative status in infants born to HIV positive mothers, IDU and serodiscordant couples, similar results have been published for β-chemokines (69-73). The role of these molecules in the context of HIV infection is mentioned in depth further in the theoretical framework.

ADCC mediated by NK cells has gained importance in the field of natural resistance to HIV infection in the last years. In 2015, *Chung et al*. reported a correlation between NK cells effector activities (degranulation, IFN-ɣ, and CCL4 production) and non-neutralizing HIV-specific antibodies, produced after HIV vaccination (74). These antibody-dependent functions have been associated with spontaneous control observed in elite controllers (75). These results suggest that HIV specific non-neutralizing antibodies, mediating ADCC, may play a protective role in HIV infection, offering a new field for HIV treatment and vaccine research.

In 2006, NK cells with memory characteristics were described in animal models (76). Later, in 2015, *Reeves et al.* reported the ability of these cells to eliminate dendritic cells pulsed with vaccine proteins from SIV vaccinated rhesus macaques (77). These memory NK cells have been found in higher frequencies in HESN individuals compared with healthy controls and HIV infected individuals, in this same study, the frequency of memory NK cells was positive correlated to HCMV titers (78). HCMV infection seems to be the main trigger for NK cells differentiation to memory NK cells.

In HIV infected individuals, the frequency of memory NK cells correlates with a lower viral set point establishment, suggesting that memory NK cells can contribute to the control of HIV replication, pointing towards a resistant phenotype (79). The role of NK cells during HIV infection, as well as recent findings related to the memory NK cells, are further explained in detail in the theoretical framework section.

1.2 Theoretical Framework

Review: NK Cells in HIV-1 Infection: From Basic Science to Vaccine Strategies.

Flórez-Álvarez L, Hernandez JC, Zapata W. NK Cells in HIV-1 Infection: From Basic Science to Vaccine Strategies. Front Immunol. 2018;9:2290. Published 2018 Oct 17. doi:10.3389/fimmu.2018.02290

frontiers in Immunology

REVIEW
published: 17 October 2018
doi: 10.3389/fimmu.2018.02290

NK Cells in HIV-1 Infection: From Basic Science to Vaccine Strategies

*Lizdany Flórez-Álvarez[1,2], Juan C. Hernandez[2] and Wildeman Zapata[1,2]**

[1] *Grupo Inmunovirología, Facultad de Medicina, Universidad de Antioquia UdeA, Medellín, Colombia,* [2] *Infettare, Facultad de Medicina, Universidad Cooperativa de Colombia, Medellín, Colombia*

NK cells play a key role in immune response against HIV infection. These cells can destroy infected cells and contribute to adequate and strong adaptive immune responses, by acting on dendritic, T, B, and even epithelial cells. Increased NK cell activity reflected by higher cytotoxic capacity, IFN-γ and chemokines (CCL3, CCL4, and CCL5) production, has been associated with resistance to HIV infection and delayed AIDS progression, demonstrating the importance of these cells in the antiviral response. Recently, a subpopulation of NK cells with adaptive characteristics has been described and associated with lower HIV viremia and control of infection. These evidences, together with some degree of protection shown in vaccine trials based on boosting NK cell activity, suggest that these cells can be a feasible option for new treatment and vaccination strategies to overcome limitations that, classical vaccination approaches, might have for this virus. This review is focus on the NK cells role during the immune response against HIV, including all the effector mechanisms associated to these cells; in addition, changes including phenotypic, functional and frequency modifications during HIV infection will be pointed, highlighting opportunities to vaccine development based in NK cells effector functions.

Keywords: natural killer cells, HIV-1, HIV resistance, HIV vaccine, memory NK cells

OPEN ACCESS

Edited by:
Aurelio Cafaro,
Istituto Superiore di Sanità (ISS), Italy

Reviewed by:
Guido Ferrari,
Duke University, United States
Domenico Mavilio,
Università degli Studi di Milano, Italy
Robin Parihar,
Baylor College of Medicine, United States
Kamalakannan Rajasekaran,
Genentech, Inc., United States

****Correspondence:***
Wildeman Zapata
Wildeman.zapatab@campusucc.edu.co

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *22 June 2018*
Accepted: *14 September 2018*
Published: *17 October 2018*

Citation:
Flórez-Álvarez L, Hernandez JC and Zapata W (2018) NK Cells in HIV-1 Infection: From Basic Science to Vaccine Strategies. Front. Immunol. 9:2290. doi: 10.3389/fimmu.2018.02290

INTRODUCTION

Natural killer (NK) cells play a key role in the host defense given their cytotoxic activity against tumors and virus-infected cells (1). In addition, they produce several cytokines including interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF) (2) and chemokines (macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4) and CCL5, also known as RANTES (regulated on activation, normal T cell expressed and secreted) (3).

These cells, were named as natural killer cells because of their capacity to destroy tumor cells without prior sensitization (4). The identification of several donors with NK cell deficiencies, who were susceptible to severe and recurrent viral infections, provided evidence on the role of NK cells in the antiviral response, especially on herpes virus, hepatitis virus and poxvirus (5, 6). NK cells constitute about 10% of the mononuclear cells in human peripheral blood and their function are regulated by a number of germline-encoded activating/inhibitory receptors that together orchestrate their activation (7, 8).

These receptors can be divided into three groups, (i) killer immunoglobulin-like receptors (KIRs), (ii) natural cytotoxicity receptors (NCR) and (iii) NKG2/CD94 heterodimer family (C-type Lectin-like receptors). The KIRs include activating and inhibitory receptors that

recognize major histocompatibility complex (MHC) class I associated with peptides, while NCRs a family of activating receptors, recognize viral derived products (9). NK cells are able to recognize host stress proteins up regulated during viral infections through C type Lectin-like receptor family (10). In addition, NK cell surveys the expression of MHC class I molecules through inhibitory receptors like NKG2A and KIRs to protect healthy cells from NK cell-mediated killing. NK cells also express Toll-like receptors (TLRs), and *in vitro* assays shows that TLR agonists can activate them, revealing their role in early defense against other pathogens than the virus (11).

In addition to the antiviral immune response, NK cells are implicated in tumor surveillance. Besides down regulation of HLA, NK cells can recognize several MHC-related ligands that are up-regulated on various tumors (12), including UL16-binding proteins (ULBP1-6) and MHC class I-chain-related proteins A and B (MICA and MICB) (13, 14). NK cells are also involved in regulatory functions, by improving $CD8^+$ T cell responses against viral infection (15), inhibiting the size/functionality of the T cell response and regulating crosstalk network with dendritic cells (DCs) and neutrophils to promote or hamper the immune response (16, 17).

The effector capacity of NK cells in the context of HIV-1 infection is not restricted to cytotoxic elimination of target cells. NK cells activation by the recognition of HIV-1-infected cells, may also lead to secretion of IFN-γ and MIP-1β, influencing the antiviral response and limiting viral spread (18). NK cells can also modulate adaptive response by a crosstalk with DCs (19), and shape the induction of antibodies through elimination of follicular T cells (Tfh) (20), demonstrating the multiple facets of NK cell in HIV-1 infection (**Figure 1**).

The antiviral response against HIV has been evaluated in different cohorts, that is the case of HIV controllers who maintain lower levels of HIV-1 replication in the absence of antiretroviral therapy, slow progressors and HIV-1-exposed seronegative individuals (HESN) who remain uninfected despite repeated exposure to the virus (21–23). Finding characteristics that explain their singularities, including an increased NK cell effector capacity, among other immune and genetic conditions, which opens a new field for HIV research with special attention in treatment and vaccination development, given the fall of classical approaches based on neutralizing antibodies.

This review will be focus on NK cells effector function during immune response against HIV infection, and the effect of this infection on NK cells number, phenotype and functionality highlighting the new field in HIV vaccine research based on NK cells.

EFFECTOR FUNCTIONS OF NK CELLS DURING HIV-1 INFECTION

Cytokine and Chemokine Production

Studies carried out in HESN cohorts, have shown that high levels of IFN-γ are associated with the seronegative status in uninfected infants born from HIV-1 infected mothers (HESN-infants) (24). Scott-Algara, et al. (25) reported an increased in IFN-γ and TNF-α production by NK cells from HIV-1-exposed seronegative intravenous drug users (HESN-IDU) compared with healthy controls (25). Similar results have been reported in different cohorts of serodiscordant couples (one partner is HIV negative and the other is HIV seropositive) (26).

A protective role of these cytokines could be explained by their ability to promote DCs maturation, up regulation of MHC molecules that favor antigen presentation and skew the adaptive response toward a Th1 profile, favoring the early control of HIV infection (27). However, these cytokines seem to have deleterious effects on chronically HIV-1 infected subjects. *in vitro* studies showed that TNF-α promotes HIV-1 gene expression via nuclear factor kappa B (NF-κB) pathway (28); in addition, there is a positive correlation between TNF-α levels and disease progression (29); this correlation may be due to HIV-1 capacity to induce TNF-α production through Tat, gp120 and Nef proteins, enhancing viral replication and inducing apoptosis of uninfected bystander cells, leading to immune escape by lacking recognition (30). Currently, the use of TNF-α inhibitors has been evaluated for the treatment of HIV-1 positive individuals, resulting in a reduction of the inflammatory state (31). IFN-γ is also involved in the chronic immune activation state that leads to immune exhaustion (32). These results show the differential role of these cytokines depending on the phase of infection.

NK cells produce large amounts of β-chemokines, CCL3, CCL4, and CCL5, natural ligands for CC-chemokine receptor 5 (CCR5), one of the coreceptors used by HIV to enter target cells. A study carried out on HIV-1-infected subjects showed that after stimulation with IL-2 and IL-15, the NK cells dramatically increased β-chemokines production, inhibiting viral entry to $CD4^+$ T cells, limiting its spread (33). β-chemokines are also important in preventing mother to child transmission of HIV-1; studies developed with decidual NK cells from healthy pregnant woman, reported a partially inhibition of macrophages infection through CCL3, CCL4, and CCL5 production *in vitro* (18); this may be an explanation to the low transmission rate of HIV-1 from mother to child during the first trimester of pregnancy despite the permissiveness of the placenta and the decidual macrophages to be infected by HIV-1. PBMCs of elite controllers, showed specific *in vitro* resistance to R5 HIV strains while remaining susceptible to X4 strains, given higher levels of CCL3 compared with healthy donors (34). In addition, CCL3, CCL4, and CCL5 have been associated with natural resistance to HIV-1 infection in HESN-IDUs, who showed higher percentage of chemokines-producing NK cells, compared with seroconverted IDUs and healthy donors (25). Recently, genetic variants in CCL3 and CCL5 and their receptors have been associated with natural resistance to HIV-1 in a Colombian HESN cohort (35, 36).

Cella et al. (37) described an NK subpopulation named NK-22 with poor cytotoxic capacity and IFN-γ production, but a great ability to produce IL-22, IL-26 and leukemia inhibitor factor (LIF); cytokines that stimulate epithelial cells proliferation, expression of anti-apoptotic molecules and IL-10 (37). Currently, it is well known that these are innate lymphoid cells (ILC) which belongs to ILC3 group, while conventional NK cells belong to ILC1 group. However, there is evidence that

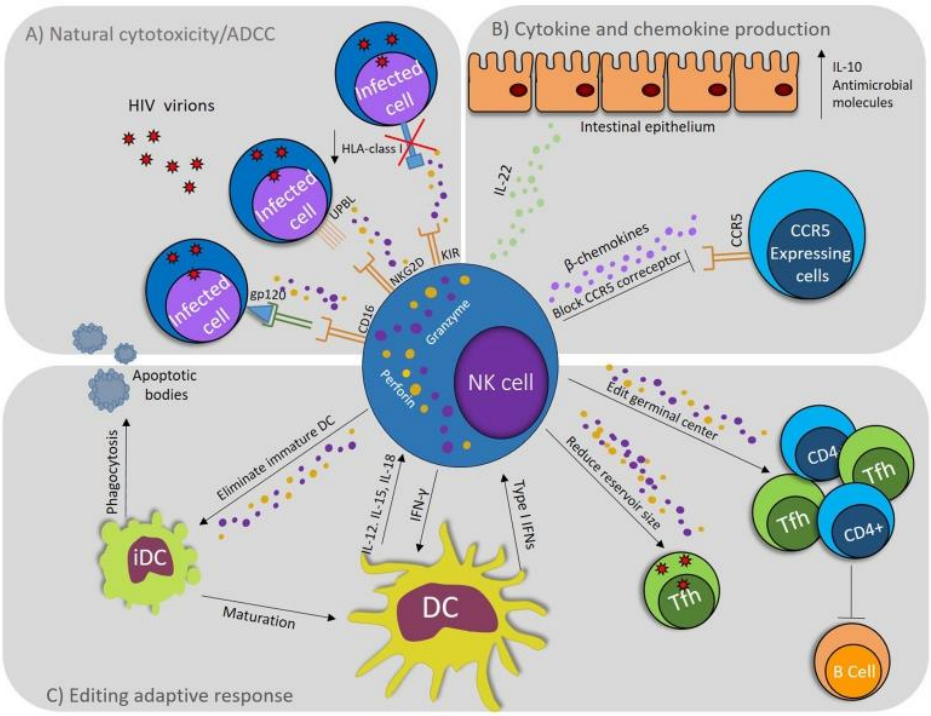


FIGURE 1 | NK cell role during HIV-1 infection. **(A)** NK cells degranulate in response to activating signals via CD16 (FcγRIII), which binds Abs recognizing HIV proteins; also, by activating signals via NKG2D that binds stress signals like UPBL1, 2 and 3, which are up regulated on infected cells. Down regulation of HLA class I molecules induces activation by absence of inhibitory signals through KIR. **(B)** NK cells produce IL-22, which induce the production of antimicrobial molecules and IL-10 by epithelial cells. NK cells produce β-chemokines, which exert anti–HIV-1 activity *in vitro* by displacing the viral envelope glycoprotein gp120 from binding to CCR5 and by promoting CCR5 endocytosis. **(C)** iDCs uptake apoptotic bodies produced by NK cells activity inducing their maturation. NK cells realize DC editing eliminating iDCs to select mature DCs. DCs induce the activation of NK cells by producing IL-12, IL-18, and type I IFNs and NK cells produce IFN-γ inducing maturation of DCs. NK cells can eliminate CD4+ T cells and follicular helper T cells (Tfh), editing germinal center and affecting Abs production, but at the same time, by eliminating the Tfh, they reduce the HIV reservoirs.

conventional NK cells can also produce IL-22 (38–40) in order to regulate inflammation and immunity at the gastrointestinal tract and mucosal-associated lymphoid tissues (41). IL-22 is a member of the IL-10 family, which protects the epithelial cell barrier in the gut and other mucosal tissues of pathogens. IL-22 receptor is expressed almost exclusively on epithelial cells, where it initiates the STAT3 signaling pathway, inducing the production of antimicrobial molecules such as calcium-binding proteins of the S100 family, RegIII β, RegIII γ, lipocalin-2, IL-10 (42, 43), and β-defensins that have shown anti-HIV activity (44, 45).

Animal models of Simian immunodeficiency virus (SIV)/Simian-Human immunodeficiency virus (SHIV) have shown the association of IL-22 secretion in mucosa with microbial translocation. The amount of IL-22 secreted in mucosa was lower in the chronic phase than the early acute phase of SIV infection and the IL-22 levels were increased before microbial translocation occurred, suggesting that a decrease in IL-22 production favors microbial translocation (46).

Interestingly, transcriptome analysis carried out in HESN individuals have shown that IL-22 participates in the phenomenon of natural resistance to HIV. In this study, the expression of Granzyme B, Peroxiredoxin II (PRDX) and IL-22 was up-regulated in HESN compared with their HIV positive partners and healthy donors (47). Whereas PRDX is an enhancing factor for NK cells cytotoxicity to induce strong anti-HIV-1 activity, the IL-22 induce the production of acute phase serum proteins such as the serum amyloid A (SAA), a molecule that inhibits HIV-1 infection *in vitro,* modulating CXCR4 and CCR5 expression (47). This evidence suggests that NK cells promote a strong and well-directed adaptive anti-HIV response, including the modulation of HIV coreceptors. Besides promote the survival of epithelial cells in the gut mucosa, preventing microbial translocation, a key contributor to immune activation/exhaustion during HIV infection.

Natural Cytotoxicity and Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

Cytotoxicity is one of the main functions of NK cells; the binding of activating receptors with their ligands and the absence of inhibitory signals, results in NK cell activation and formation

of lytic immunological synapse for polarized release of cytotoxic molecules stored in secretory lysosomes (48).

The effector ability of NK cells improves after immune licensing or education. In the immune licensing model, the acquisition of inhibitory KIRs specific for self HLA class I molecules, besides preventing auto-reactivity, is associated with the development of functional competence during maturation. Thus, exposure to target cells lacking HLA-I results in increased response rates of NK cells expressing inhibitory KIR for that missing HLA-I, whereas NK cells lacking self-inhibitory KIR remain hyporesponsive (49). On viral infections, down modulation of HLA molecules on infected cells can induce the activation of NK cells by absence of signals trough inhibitory KIR receptors. During HIV-1 infection the virus induces down modulation of HLA A/B expression to avoid recognition by cytotoxic lymphocytes (CTL) while leaving no classical HLA molecules unaltered, preventing NK cell activation (50).

Increased cytotoxicity in response to HLA down regulation has been associated with protection against HIV infection. Scott Algara et al. (25) reported that NK cell lytic activities against cell lines with reduced HLA expression (K562 and Daudi cells) were significantly increased in HESN-IDUs compared with either healthy donors or IDU who seroconverted during the study. In addition, HIV induces up regulation of specific ligands for NKG2D receptor, such as ULBP-1, 2, and 3 by Vpr accessory protein enhancing the susceptibility of HIV-1 infected cells to NK cell–mediated killing (51).

NK cells can also be activated by antibody-dependent stimuli in two ways; (i) NK cell activation dependent of antibodies (ADNKA) and (ii) Antibody-dependent cellular cytotoxicity (ADCC). Although both require the presence of opsonized target cells, ADNKA refers to NK cell activation measured by CD107a, IFN γ, and MIP1β expression; and ADCC point out the lysis of target cells by NK cells in the presence of an antibody bridge between NK cell and target cell, both are important in the context of HIV infection (52).

In this regard, several studies have demonstrated multiple antibody-dependent responses apart from neutralization that can be involved in protection against HIV infection and other viruses, these extra neutralizing functions include antibody-dependent compplement deposition, ADNKA, ADCC and antibody-mediated respiratory burst (53–55). A clinical trial for HIV/AIDS vaccine designed to evaluate non-neutralizing functions of vaccine-induced antibodies confirmed the correlation between HIV-specific antibodies and secretion of IFN-γ, CCL4 and expression of the degranulation marker CD107a on NK cells, from vaccine recipients (56). These extra-neutralizing antibody functions have been described in HIV elite controllers. Ackerman et al. (56) reported that spontaneous control of HIV infection showed by these subjects is related with polyfunctional antibody profile defined as the capacity to coordinately mediate ADCC and other NK antibody-dependent functions like IFN-γ secretion and maintained levels of neutralizing antibodies (57). These results suggest that HIV specific non-neutralizing antibodies, which mediate ADCC and ADNKA, may play a protective role against HIV infection, enhancing cytokine/chemokine production and lysis of target cells by NK cells, these mechanisms could be the base of several treatment and vaccine strategies for HIV infection, which will be mentioned further in this review.

Finally, given the effective activity of ADCC to eliminate infected cells, the HIV has developed strategies to evade this mechanism by inducing its dysfunction and limiting the presence of Env protein in the infected-cells surface through the accessory protein Vpu, which decrease the expression of tetherin, a cellular host restriction factor that tethers HIV virions on the cell surface, sequestering viral particles (58). Thus, decreased expression of tetherin reduces the ADCC, protecting infected cells from the NK cell activity (59).

Editing the Adaptive Immune Response

NK cells and DCs have a central role in antiviral immunity by modulate the adaptive immune response. Crosstalk between NK cells and DCs result in maturation of DC and in turn, DC cells up regulates NK cell effector function. This "co-activation" is given by cytokine production and cell to cell contact. IL-12 and IL-18 produced by activated conventional DCs promote IFN-γ production by NK cells, favoring Th1 response (60, 61), while plasmacitoid DCs (pDCs) produce type I IFN, which promotes NK cell proliferation and cytotoxicity. On the other hand, lysis of infected cells by NK cells provides a source of apoptotic bodies that immature DCs uptake, leading to their maturation and promoting viral antigen presentation to T cells (**Figure 1**) (61).

Mature DCs are the main antigen presenting cell (APCs) that initiate adaptive immune response, whereas, immature DCs are involved in tolerance and induction of regulatory T cells. *In vitro* models shown that NK/DC interaction results in lysis of immature DCs and preservation of mature DCs, in a process called "DC editing." In this process, activated NK cells via NKp30 kill and secrete IFN-γ in response to immature DCs while mature DCs remain protected by up regulation of HLA molecules that induced inhibitory signals trough KIR receptors (62, 63). During HIV-1 infection, quantitative and qualitative alterations in DC and NK cells have been reported, affecting all these processes of reciprocal activation and preventing the development of adequate response (63).

In vitro studies have shown that HIV-1 accessory protein Nef has the capacity to affect NK/DC crosstalk. In presence of Nef-pulsed DCs, $CD56^{bright}$ cells exhibit high capacity to produce IFN-γ, while $CD56^{dim}$ showed a reduction in the cytotoxic capacity. Likewise, NK cells showed a significant reduction of IL-10, GM-CSF, MIP-1α, and RANTES secretion but increased TNF-α production, resulting in increased viral replication (19).

In addition, NK cells can directly modulate T and B cell responses. Immunoglobulin class switching and generation of memory by T cells can be enhanced by the cytotoxic activity and IFN-γ production of NK cells (64, 65). At the same time, studies carried out in mouse models have shown that in early moments of infection, NK cells can inhibit the generation of long-lived specific memory T and B cells, as well as specific antibodies by eliminating $CD4^+$ activated T cells and Tfh. In mouse models, the NK cells depletion result in higher numbers of $CD4^+$ and $CD8^+$ memory T cells with polyfunctional profile during acute infection (20). In addition, (20) reported that in absence of NK cells, the infected mouse shown higher proportion of Tfh

cells during acute infection and better formation of germinal centers (GC), which was reflected in higher numbers of antibody-secreting cells compared with NK cells sufficient mouse who shown reduced frequencies antibody secreting cells, even for months after infection (20).

The elimination of Tfh cells by NK cell affects the GC formation preventing the establishment of humoral response during acute phase of infection; however, it has been reported that elimination of Tfh cell during HIV infection could be beneficial, because these cells support HIV-1 replication in viremic individuals (66); therefore, NK cells may limit the size of the reservoir that is established after the acute phase of infection. This phenomenon has been described in animal models of SIV were natural hostess (animals that do not progress to disease despite presenting high viremias) like african green monkeys shown an enhanced capacity to control viral replication in lymph nodes compared with non-natural hostess like macaques that progress to disease. This capacity was associated with higher number of NK cells present in the lymph node (LN), specifically into the B-cell follicle of african green monkeys, this pattern of migration was associated with higher levels of CXCR5 expression on NK cells from african green monkeyscompared with macaques (67). The capacity of natural SIV hostess to control viral replication in LN has been related with a better control of inflammation, absence of Tfh infection and preservation of LN architecture and function that are some of the principal differences between pathogenic and non-pathogenic SIV/HIV infection (68).

This evidence suggests the important role of NK cells in different phases of HIV infection and offers a plausible target for immune modulation to avoid the elimination of cells forming part of GC in early moments of HIV infection, which could increase the antibodies production but promoting elimination of reservoirs in chronic infection. The beneficial effect of NK cells on adaptive immunity depends in great manner on the context, highlighting the need for addressing the activity of NK cells in a careful way during HIV infection. For instance, during curative intervention, targeting Tfh cells could lead to reduce the reservoir, but after vaccination is necessary to disable their suppressive effects to promote adequate humoral responses (69). Therefore, the impact of NK cells interventions, which could have a repercussion on adaptive immune response development, offers a new alternative in the design of methodologies for HIV prevention and cure.

NK CELL PHENOTYPE AND FREQUENCIES DURING HIV-1 INFECTION

NK cells have been classified in two major subsets based on the surface expression of CD56 (neural cell adhesion molecule, N-CAM). $CD56^{bright}$ NK cells represent around 10% of peripheral blood NK cells, these cells are $CD16^{neg}$ and express CD25 (High affinity IL-2R), whereas $CD56^{dim}$ NK cells (around 90%) are $CD16^{pos}$ and express CD122/132 (intermediate affinity IL-2R) (70, 71); differences in IL-2 receptor expression are reflected in their proliferative capacity.

Differences between these two subsets also include homing molecules and effector capacity. $CD56^{bright}$ express CCR7 and CD62L, allowing them to migrate to secondary lymphoid organs; in fact, they are the most common NK cells in this tissue (70). $CD56^{bright}$ are also characterized by low presence of lytic granules and the production of high amounts of IFN-γ, TNF-α, and GM-CSF (72). In contrast, $CD56^{dim}$ preferentially migrate to inflamed peripheral tissues by CXCR1, CX3CR1, and ChemR23 and they express higher amounts of lytic granules. $CD56^{dim}$ NK cells are considered the mature stage of NK cells; these cells come from a differentiation process involving loss of inhibitory receptors, like NKG2A and gain of CD16, KIR, and CD57 receptors (73). Despite the vast majority of NK cells can be included in these two groups, another subpopulation have been defined, $CD56^{-}$ NK cells that express high density of CD16 molecules; these cells are NK cells with low cytotoxic activity and cytokines production; this functional altered subpopulation increase during chronic viral infections like HIV-1 (74).

Several changes in NK cell receptor repertoire and functionality are observed during HIV infection, some of them have been related with progression but others are still under study. Recently, it has been observed that treatment-naïve HIV-infected individuals showed a significant loss of the $CCR7^{+}/CD56^{bright}$ population, this change is associated with higher viral loads (75). Studies carried out in HIV and hepatitis C virus (HCV) coinfected individuals, showed that $CD27^{+}/CD56^{bright}$ NK cells can help to clear of acute HCV infection by IFN-γ-mediated suppression, thus the alterations in $CD56^{bright}$ subset can be related, not only with the loss of HIV control, but also with decreased capacity to eliminate other pathogens (76).

The loss of $CD56^{dim}$ NK cell subset during HIV-1 infection has been also observed, especially in less differentiated $CD56^{dim}$ cells, such as $CD57^{-}$ and $CD57^{dim}$ subpopulations (77, 78). The loss of $CD56^{+}$ NK cells is associated with expansion of $CD56^{neg}/CD16^{+}$ NK cell population; this aberrant population expresses low levels of NKp30 and NKp46, reduced IFN-γ production, impaired cytotoxicity and poor ADCC response (79); in contrast, they produce significant amounts of MIP-1β (80).

Additional to the reduction in the CD56 expression, the expression of immunoregulatory molecules such as Siglec-7 and Tim-3 are also altered during HIV-1 infection. Siglec-7 is an adhesion inhibitory receptor, preferentially expressed by mature NK cells. Siglec-7^{+} NK cells displayed higher levels of activating receptors, increased CD107a expression and IFN-γ production than Siglec-7^{-} NK cells (81). Siglec-7 is considered one of the early surface markers down regulated in HIV-1 infection, associated with the expansion of dysfunctional NK cell subsets. Siglec-7 expression is suppressed throughout the course of infection, and its down regulation is associated with higher viremia (82). On the other hand, NK cells expressing high amounts of Tim-3 are fully responsive by cytokine production and cytotoxicity; this molecule is also an exhaustion marker (83). Tim-3 is also reduced in HIV-1 infected individuals; moreover, these individuals also showed high plasma levels of Gal-9, a Tim-3 ligand (84). Persistent Gal-9 stimuli via Tim-3, might result in loss of functionality and accumulation of NK $Tim\text{-}3^{low}$

population, this might by an explanation to NK cell dysfunction observed in chronic HIV-1 infection (84).

Animal models of SIV shown accumulation of $CD56^{-}CD16^{+}$ NK cells in lymph nodes of infected animals compared with naïve animals. NK cells from lymph nodes demonstrated an enhanced cytotoxic capacity in acute infection models. Furthermore, Tim-3 was up regulated on NK cells from lymph nodes in chronically infected animals (85), suggesting that NK cells present a cytotoxic phenotype during acute SIV infection but may become dysfunctional and exhausted in chronic phase of disease. In addition to the alteration of NK cells in peripheral blood and lymphoid organs, a depletion of NK-22 cells in mucosa of SIV-infected macaques also has been reported. The loss of this population was associated with increased intestinal mucosal damage and microbial translocation (46, 86, 87), both implicated in immune hyperactivation and immune exhaustion, which are characteristics of the final stage of HIV infection.

NK CELL REPERTOIRE IS ASSOCIATED WITH HIV PROGRESSION AND RESISTANCE

The study of long term non-progressors, controllers and HESN has revealed the existence of mechanisms for HIV resistance. NK cells are part of these mechanisms, exhibiting increased cytotoxicity and higher production of soluble mediators that have been associated with the expression of certain NK cells phenotypes.

The presence of the activator receptor KIR3DS1 on NK cells in combination with HLA-Bw4-I80, is one of the most reported phenotype associated with delayed AIDS progression and resistance to infection (32, 88–90). NK cells expressing KIR3DS1 are licensed by this receptor to activate in response to HLA-Bw4 expressed on target cells and they show an improved capacity of both cytotoxicity and IFN-γ production, apparently by HLA-Bw4 recognition in a peptide-dependent manner (91). However, the expression of KIR3DS1 in absence of HLA-Bw4-I80 allele has been associated with rapid progression to AIDS (92), probably because licensed NK cells through this KIR receptor are less responsive to target cells expressing another HLA allele, according with the immune licensing model.

The differential expression of the inhibitory lectin type C receptor, NKG2A, also has been related with an improved response against HIV-infected cells. $NKG2A^{+}$ NK cells exhibit higher response against HLA null cells, and infected CD4+ T cells compared to $NKG2A^{-}$ NK cells. In addition, these cells exhibit a poly-functional profile with co-expression of CD107a, IFN-γ, and CCL4, indicating that NKG2A receptors may have a role in the anti-HIV responses mediated by NK cells (93).

In 2006, a phenotype of NK cells with "memory" characteristics was described. O'Leary et al. showed that $rag^{-/-}$ mouse can be sensitized against hapten. In this model, NK cells mediated an antigen-specific long-lived immunological recall response called contact hypersensitivity reaction for at least 4 weeks (94).

Based on the capacity of a recall response, this subset of NK cells has been named "Memory like NK cells" and they represent a final stage of NK cells maturation. Classically, it has been thought that after $CD56^{bright}$ differentiation to $CD56^{dim}$, these cells retained the functional and phenotypic properties through the lifespan. However, some studies have shown that $CD56^{dim}$ continue to differentiate; in this process, NK cells lose NKG2A expression, acquire KIR and CD57 markers, changing the expression of homing molecules like CD62L, and they display a decline in proliferative capacity and also they show epigenetic modifications in some genes like IFN-γ, TNF-α, and IL-10 (95, 96). This phenotype of NK cells has been well characterized in human cytomegalovirus (CMV) infection, where a subset of NK cells with this phenotype $CD56^{dim}/CD57^{+}/NKG2A^{-}/NKG2C^{high}$ is selectively expanded during acute infection and exhibit characteristics like recall response and long lived capacity (97) (**Figure 2**). Memory like NK cells have shown the ability to mediate adaptive immune responses to other viruses, such as vesicular stomatitis virus and HIV, and even against other pathogens such as bacteria (98, 99).

Recently an association between memory NK cells and HIV infection has been described. Reeves et al. found that a subset of NK cells from SHIV and SIV infected macaques, specifically they lysed Gag and Env-pulsed DCs in a NKG2C dependent manner, in contrast to NK cells from uninfected macaques (96). Moreover, splenic and hepatic NK cells from vaccinated macaques efficiently lysed antigen-matched DCs but not "naïve" DCs, even 5 years after vaccination. These data demonstrate that robust, durable, and antigen-specific NK cell memory can be induced in primates after both, infection and vaccination (100). HESN, exhibited higher frequency of Memory like NK cells compared with HIV infected individuals and also a positive correlation between memory NK cells and IgG titles for HCMV, which appears to be the trigger by these cells (101). Finally, HIV infected individuals showed a negative correlation between frequency of memory NK cells and viral load (102), suggesting that Memory-like subset might constitute a ready-armed immune cells able to contribute to natural control of viral load.

This evidence suggests the existence of association between the Memory like NK cells (expanded in response to CMV infection) and HIV resistance, which opens a new field in the HIV vaccine research, based in the induction of Memory like NK cells with specific responses against HIV.

NK CELL BASED VACCINE "BOOSTING NK CELL ACTIVITY"

After more than 30 years of the discovery of HIV-1, the vaccine remains elusive. Difficulties in the HIV vaccine development include: (i) integration of viral genome into the host DNA, (ii) the ability to induce immune suppression and (iii) the development of viral variants that escape from the immune control. Many attempts to design prophylactic vaccines have been focused on the induction of neutralizing antibodies that

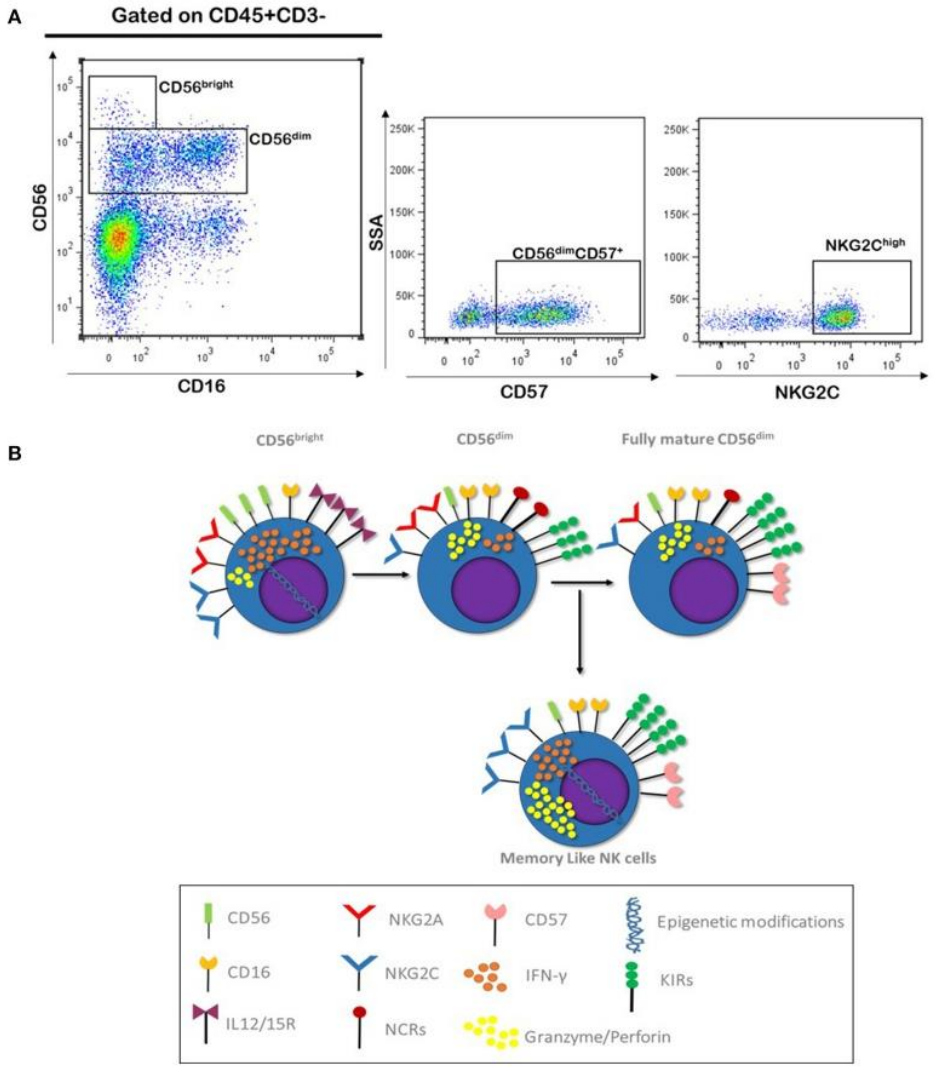


FIGURE 2 | NK maturation and Memory like phenotype. **(A)** Gating strategy used for the identification of NK cells subpopulation, which includes fully mature (CD56dim CD57+) and Memory like (NKG2Chigh) NK cells; blood sample correspond to healthy donor from our laboratory (Unpublished data). **(B)** $CD56^{bright}$ NK cells represent less mature stage of NK cells differentiation, these cells respond to IL12/15 stimulation. These also express NCRs and NKG2A/C and high affinity IL2R (CD25), which reflect their proliferative capacity. After immune licensing process, $CD56^{dim}$ gain the expression of KIRs and loss expression of cytokines receptor, making them less reactive to cytokine stimuli, they also gain CD16 expression together with an improved cytotoxic capacity. Fully differentiated $CD56^{dim}$ express CD57 and loss NKG2A and NCRs expression, they respond better trough KIR and NKG2C signaling. Memory like NK cells are thought to develop after CMV infection, they have a high cytotoxic capacity and present epigenetic modifications in *IFN-γ*, *TNF-α*, and *IL-10* genes allowing them to produce high amounts of these cytokines rapidly after stimuli through KIRs and NKG2C receptors.

block infection by free virions; nevertheless, in the case of HIV, virus-infected cells are more infectious than free virus in both *in vitro* and *in vivo* models (103). In addition, neutralizing antibodies do not block cell to cell transmission, thus, effective design of the vaccine against HIV needs a different approach.

Accumulated evidence of the protective role of NK cells during HIV-1 infection, leads to think that boosting NK cell activity during infection or even before can help to eliminate viral reservoirs or avoid infection. The idea of boosting NK cells to cure pathologic conditions is not exclusive of HIV; in fact, a growing number of studies have been developed to enhance NK cytotoxicity as immune therapy against cancer, based on the capacity to eliminate tumor cells (104).

Increased natural cytotoxicity and ADCC mediated by NK cells have been associated with protection to HIV infection and disease progression, because those are mechanisms capable to control cell-associated virus, blocking cell to cell transmission.

For this reason, the administration of molecules that can promote the activation and proliferation of NK cells have been evaluated in preclinical phase studies. For instance, it has been reported that IL-15 induce proliferation of NK and T cells in rhesus macaques (105). On 2015 Conlon et al. published the results of the first clinical trial with recombinant IL-15 in cancer patients; the administration of this cytokine resulted in increased NK cells proliferation with no toxic effects on patients and decreased of tumor size (106).

Currently, the administration of IL-15 has been considered as a part of the kick and kill strategy, which is based on the viral replication induction on latently infected cells and agents with the ability to boost NK cells activity to kill them in presence of antiretroviral therapy to avoid the infection of new cells, resulting in a diminished viral reservoir size and viral eradication in infected individuals. Garrido et al. (107) in a *ex vivo* model shown that IL-15 improves the NK cells function of healthy controls and HIV infected individuals with antiretroviral therapy, and more important these cells were able to clear latently infected cells after exposure to vorinostat (a latency reversal agent) (107).

Inducing Memory like NK cells will allow the development of new vaccine strategies based on cellular immunity, letting individuals to respond with strong cytotoxic activity and IFN-γ production in "specific" way during early moments of infection. Despite CMV infection seems to be the only trigger for Memory like NK cells development, cytokines cocktails induce the expansion of NK cells populations with some memory features (108) (**Figure 3**). Although cytokines can induce Memory like NK cells (CIML), they present a different phenotype with no expression of CD57 and KIR, and no epigenetic modifications in *IFN-γ* gene. These cells are being evaluated for cancer treatment in a clinical trial (NCT01898793) with promising results. Therefore, it is necessary to evaluate if this NK cell phenotype induced *in vitro* can be used to control HIV infection.

As mentioned before, the crosstalk between NK cells and DCs impact the establishment of both innate and adaptive

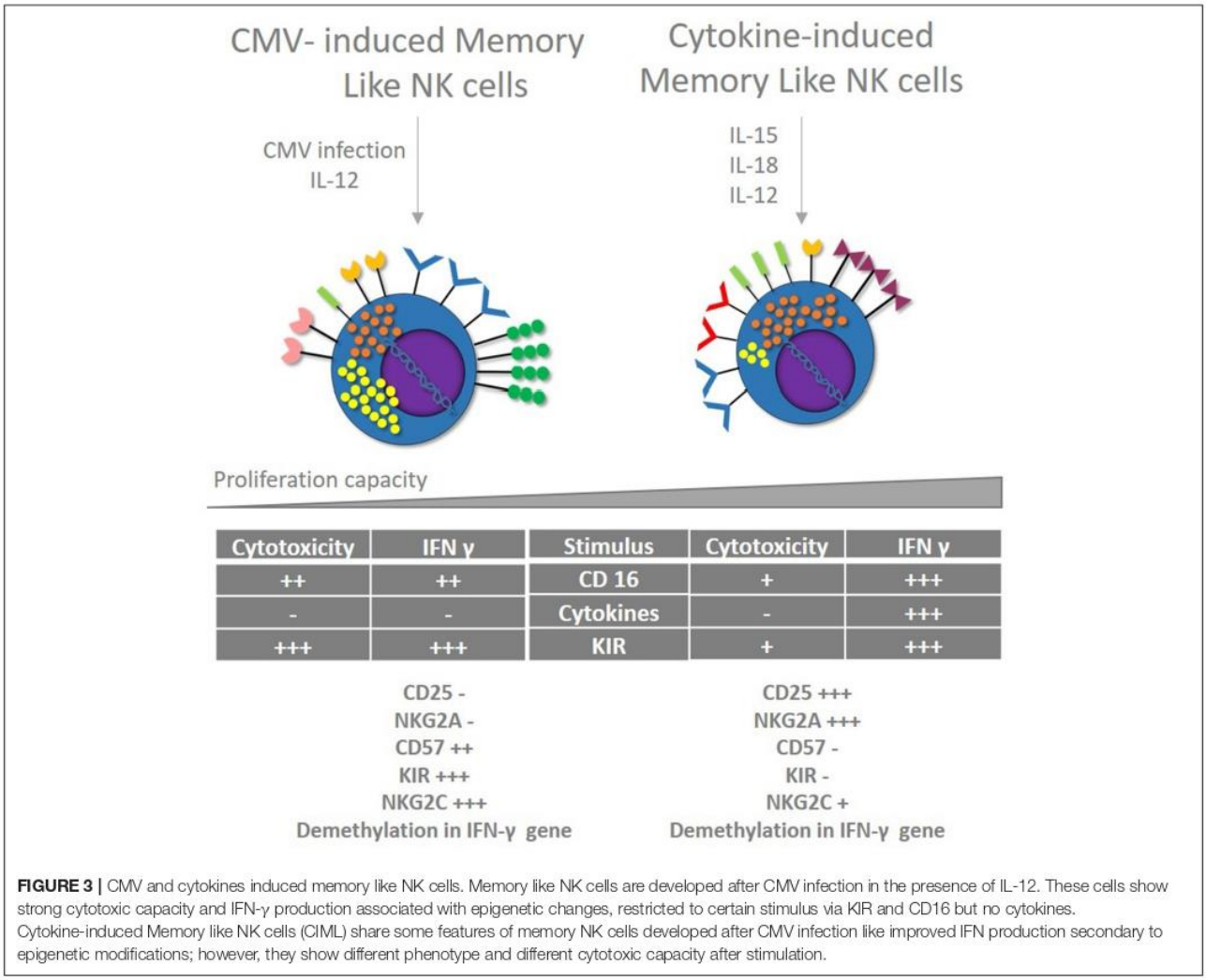


FIGURE 3 | CMV and cytokines induced memory like NK cells. Memory like NK cells are developed after CMV infection in the presence of IL-12. These cells show strong cytotoxic capacity and IFN-γ production associated with epigenetic changes, restricted to certain stimulus via KIR and CD16 but no cytokines. Cytokine-induced Memory like NK cells (CIML) share some features of memory NK cells developed after CMV infection like improved IFN production secondary to epigenetic modifications; however, they show different phenotype and different cytotoxic capacity after stimulation.

immune response; in this regard, vaccine candidates based on NK-DC interaction have been proposed. Cummings et al. reported that in cocultures of NK cells with autologous DCs infected with MVA-HIV (modified vaccinia virus containing Gag, Pol, and Nef proteins from HIV), NK cells showed enhanced proliferation and modulation of cell receptor repertoire; these changes were reflected in a higher ability to control HIV-1 infection. This activity appears to be specific to HIV, as MVA-HIV-primed NK cells did not have a better ability to control other viral infections or respond against tumoral cells (109). Despite the advances in this field, none of these NK cells boosting strategies have been tested in humans.

On the other hand, the induction of antibodies that mediate ADCC has been evaluated with promising results in both preclinical and clinical studies. In fact, the evidence shows that unlike neutralizing antibodies, which are rarely induced in HIV infected individuals, ADCC-mediating antibodies are found often and in high quantity in non-progressor individuals (110). In addition, Milligan et al. reported that the levels of passively acquired ADCC-inducing antibodies were associated with decreased mortality risk in infected infants born from HIV positive mothers (111), supporting the importance of ADCC-inducing antibodies in the control of HIV infection. Additionally, Lu et al. reported that the administration of broadly neutralizing antibody accelerate the elimination of HIV-1 infected cells acting on free virus clearance and contributing to elimination of infected cells by a mechanism involving Fcγ receptors (112). Antibodies used in this studio target envelope glycoprotein gp160 and led to rapid reduction of viral load by an average 1.48 $\log^{10}$ copies/ml, this study also shown that both neutralizing and non-neutralizing antibodies can support anti HIV ADCC that was associated with both control of replication and protection against infection (112). Gómez-Román et al. showed that priming with a recombinant vaccine consisting of replication competent adenovirus with mutant SIV, followed by boosting with SIVgp120 protein, elicited the production of antibodies with ADCC activity and these antibodies were related with reduction of acute SIV viremia after a mucosal challenge of SIV in rhesus macaques (113). Recently, another type of molecule called BiKE (bi specific killer engager) has been evaluated in order to direct the NK cell-mediated ADCC against HIV-infected targets. BiKE contain the Fab portion of broadly neutralizing antibodies linked to a nanobody that binds CD16 with high affinity and induce a strong activation signal; interestingly, the use of this construct improved the ADCC and IFN-γ production capacity of NK cells in co-cultures with HIV infected cell lines (114).

Besides the success in the preclinical studies, the induction of ADCC-mediating antibodies was demonstrated to be a useful strategy for HIV-1 vaccination in human trials. The first phase III clinical trial for HIV/AIDS vaccine (AIDSVAX), which aimed to induce neutralizing antibodies, show no efficacy for prevention of acquisition or modification in HIV infection (115, 116). After this fail, AIDSVAX was evaluated in combination with another failed vaccine in the ALVAC-HIV/AIDSVAX study that used a "primed-boost" strategy. This vaccines were developed for circulating HIV subtypes from Thailand and the ADCC activity showed a significant difference in the magnitude of the response and the frequency of responders in the group of vaccines recipients compared to placebo (117). From these results, the clinical trial RV144 was design to prove the vaccine combination efficacy in other populations. In this new study, the participants lowered the rate of HIV infection by 60% at 12 months and 31.2% at 42 months compared with placebo (118). This protection was not associated with the presence of neutralizing antibodies or cytotoxic T cell responses (119); rather, it was associated with the presence of anti-HIV Env specific IgG non-neutralizing antibodies able to mediate ADCC (120). In 2011, the comparison of different immune parameters between those who received the vaccine and contracted HIV, and those who did not become infected (121), reported that subjects who produced antibodies, which recognized the V2 loop in the HIV envelope protein were 43% less likely to become infected (120).

Recently, a new variation of ALVAC-HIV/AIDSVAX has been proof in animal models with promising results. This new vaccine trial includes the recombinant poxvirus from ALVAC and the pentavalent version of AIDSVAX to increase the diversity of gp120 motifs in the antibody response. Evaluation of the antibody responses identified: (i) plasma antibody binding to HIV-infected cells, (ii) peak of ADCC antibody titers, (iii) NK cell-mediated ADCC and (iv) antibody-mediated expression of MIP-1β in NK cells; four immunological parameters with important antiviral activity as mentioned before, resulting in 55% of protection from SIV challenge (122).

In addition to strategies to boost NK cells effector function, the adoptive transfer of NK cells has been evaluated for HIV treatment. CAR expressing NK cells are modified NK cells that express chimeric antigen receptors against tumor-associated or pathogen-associated antigens redirecting the effector function to specific cells (123). This strategy has been evaluated for tumor immunotherapy, initially with T cells showing remarkable success in hematological cancers and after with NK cells on hematological cancers and solid tumors (124). In HIV context, a study by Zhen et al. (125) reported that CAR modified hematopoietic stem cells differentiate to NK cells, these cells were resistant to HIV infection and suppress viral replication *in vivo* (125); currently, the safety and tolerability of NK cells transfer is evaluated in a phase II clinical trial NCT03346499 designed to study the effects of haploidentical NK cells infusion after the stimulation with IL-2 in HIV^+ individuals.Taken together, the evidences presented in this review highlight the role of NK cells in HIV infection; not only by the capacity to eliminate infected cells trough cytotoxic activity, but also producing soluble mediators that can block HIV entry to new cells, affecting the viral replication and spread. Beyond of these effector functions, NK cells are capable of shape the adaptive response and they are implicated in the maintenance of mucosal epithelia, a key activity to avoid the tissue damage and the subsequent microbial translocation during HIV infection. Finally, the possibility of boost the NK cells response offers a new strategy in the

design of vaccines, which represent a challenge for classical vaccination and should be a priority in research to find an HIV cure.

AUTHOR CONTRIBUTIONS

JH, WZ: Funding acquisition and Project administration. LF-Á, JH, and WZ: Writing-original draft. JH and WZ: Writing-review and editing. LF-Á, JH, and WZ: Approval of manuscript for publication.

FUNDING

This work was supported by Universidad de Antioquia, UdeA, Colciencias (code 111565740820) and Universidad Cooperativa de Colombia (code INV1896).

REFERENCES

1. Montaldo E, Del Zotto G, Della Chiesa M, Mingari MC, Moretta A, De Maria A, et al. Human NK cell receptors/markers: a tool to analyze NK cell development, subsets and function. *Cytom A* (2013) 83:702–13. doi: 10.1002/cyto.a.22302
2. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* (2010) 115:2167–76. doi: 10.1182/blood-2009-08-238469
3. Roda JM, Parihar R, Magro C, Nuovo GJ, Tridandapani S, Carson WE. Natural killer cells produce T cell–recruiting chemokines in response to antibody-coated tumor cells. *Cancer Res.* (2006) 66:517–26. doi: 10.1158/0008-5472.CAN-05-2429
4. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int J Cancer* (1975) 16:216–29.
5. Bukowski JF, Woda BA, Habu S, Okumura K, Welsh RM. Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis *in vivo*. *J Immunol.* (1983) 131:1531–8.
6. Lee SH, Miyagi T, Biron CA. Keeping NK cells in highly regulated antiviral warfare. *Trends Immunol.* (2007) 28:252–9. doi: 10.1016/j.it.2007.04.001
7. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol.* (2003) 3:781–90. doi: 10.1038/nri1199
8. Lanier LL. Evolutionary struggles between NK cells and viruses. *Nat Rev Immunol.* (2008) 8:259–68. doi: 10.1038/nri2276
9. Khakoo SI, Carrington M. KIR and disease: a model system or system of models? *Immunol Rev.* (2006) 214:186–201. doi: 10.1111/j.1600-065X.2006.00459.x
10. Jamieson AM, Diefenbach A, McMahon CW, Xiong N, Carlyle JR, Raulet DH. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* (2002) 17:19–29. doi: 10.1016/S1074-7613(02)00333-3
11. Sivori S, Falco M, Chiesa MD, Carlomagno S, Vitale M, Moretta L, et al. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: Induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proc Natl Acad Sci USA.* (2004) 101:10116–21. doi: 10.1073/pnas.0403744101
12. Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat Immunol.* (2000) 1:119–26. doi: 10.1038/77793
13. Salih HR, Antropius H, Gieseke F, Lutz SZ, Kanz L, Rammensee HG, et al. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. *Blood* (2003) 102:1389–96. doi: 10.1182/blood-2003-01-0019
14. Watson FS, Spendlove I, Madjd Z, McGilvray R, Green AR, Ellis IO, et al. Expression of the stress-related MHC class I chain-related protein MICA is an indicator of good prognosis in colorectal cancer patients. *Int J Cancer* (2006) 118:1445–52. doi: 10.1002/ijc.21510
15. Robbins SH, Bessou G, Cornillon A, Zucchini N, Rupp B, Ruzsics Z,et al. Natural killer cells promote early CD8 T cell responses against cytomegalovirus. *PLoS Pathog.* (2007) 3:e123. doi: 10.1371/journal.ppat.0030123
16. Crome SQ, Lang PA, Lang KS, Ohashi PS. Natural killer cells regulate diverse T cell responses *Trends Immunol.* (2013) 34:342–9. doi: 10.1016/j.it.2013.03.002
17. Campbell KS, Hasegawa J. Natural killer cell biology: an update and future directions. *J Allergy Clin Immunol.* (2013) 132:536–44. doi: 10.1016/j.jaci.2013.07.006
18. Quillay H, El Costa H, Durie M, Marlin R, Cannou C, Madec Y, et al. NK cells control HIV-1 infection of macrophages through soluble factors and cellular contacts in the human decidua. *Retrovirology* (2016) 13:39. doi: 10.1186/s12977-016-0271-z
19. Quaranta MG, Napolitano A, Sanchez M, Giordani L, Mattioli B, Viora M. HIV-1 Nef impairs the dynamic of DC/NK crosstalk: different outcome of CD56(dim) and CD56(bright) NK cell subsets. *FASEB J.* (2007) 21:2323–34. doi: 10.1096/fj.06-7883com
20. Rydyznski C, Daniels KA, Karmele EP, Brooks TR, Mahl SE, Moran MT, et al. Generation of cellular immune memory and B-cell immunity is impaired by natural killer cells. *Nat Commun.* (2015) 6:6375. doi: 10.1038/ncomms7375
21. Horton RE, McLaren PJ, Fowke K, Kimani J, Ball TB. Cohorts for the Study of HIV-1–exposed but uninfected individuals: benefits and limitations. *J Infect Dis.* (2010) 202:S377–81. doi: 10.1086/655971
22. Johansson SE, Rollman E, Chung AW, Center RJ, Hejdeman B, Stratov I, et al. NK cell function and antibodies mediating ADCC in HIV-1-infected viremic and controller patients. *Viral Immunol.* (2011) 24:359–68. doi: 10.1089/vim.2011.0025
23. Taborda NA, Hernández JC, Lajoie J, Juno JA, Kimani J, Rugeles MT, et al. Short communication: low expression of activation and inhibitory molecules on NK cells and CD4(+) T cells is associated with viral control. *AIDS Res Hum Retroviruses.* (2015) 31:636–40. doi: 10.1089/AID.2014.0325
24. Lohman-Payne B, Slyker JA, Moore S, Maleche-Obimbo E, Wamalwa DC, Richardson BA, et al. Breast milk cellular HIV-specific interferon γ responses are associated with protection from peripartum HIV transmission. *AIDS* (2012) 26:2007–16. doi: 10.1097/QAD.0b013e328359b7e0
25. Scott-Algara D, Truong LX, Versmisse P, David A, Luong TT, Nguyen VN,et al. Cutting edge: increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravascular drug users. *J Immunol.* (2003) 171:5663–7. doi: 10.4049/jimmunol.171.11.5663
26. Montoya CJ, Velilla PA, Chougnet C, Landay AL, Rugeles MT. Increased IFN-gamma production by NK and CD3+/CD56+ cells in sexually HIV-1-exposed but uninfected individuals. *Clin Immunol.* (2006) 120:138–46. doi: 10.1016/j.clim.2006.02.008
27. Alter G, Altfeld M. Mutiny or scrutiny: NK cell modulation of DC function in HIV-1 infection. *Trends Immunol.* (2011) 32:219–24. doi: 10.1016/j.it.2011.02.003
28. Osborn L, Kunkel S, Nabel GJ. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc Natl Acad Sci USA.* (1989) 86:2336–40.
29. Wada NI, Jacobson LP, Margolick JB, Breen EC, Macatangay B, Penugonda S,et al. The effect of HAART-induced HIV suppression on circulating markers of inflammation and immune activation. *AIDS* (2015) 29:463–71. doi: 10.1097/QAD.0000000000000545
30. Kumar A, Abbas W, Herbein G. TNF and TNF receptor superfamily members in HIV infection: new cellular targets for therapy? *Mediators Inflamm.* (2013) 2013:484378. doi: 10.1155/2013/484378

31. Gallitano SM, McDermott L, Brar K, Lowenstein E. Use of tumor necrosis factor (TNF) inhibitors in patients with HIV/AIDS. *J Am Acad Dermatol.* (2016) 74:974–80. doi: 10.1016/j.jaad.2015.11.043
32. Roff SR, Noon-Song EN, Yamamoto JK, Johnson HM. The significance of interferon-γ in HIV-1 pathogenesis, therapy, and prophylaxis. *Front Immunol.* (2014) 4:498. doi: 10.3389/fimmu.2013.00498
33. Oliva A, Kinter AL, Vaccarezza M, Rubbert A, Catanzaro A, Moir S, et al. Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication *in vitro*. *J Clin Invest.* (1998) 102:223–31. doi: 10.1172/JCI2323
34. Walker WE, Kurscheid S, Joshi S, Lopez CA, Goh G, Choi M, et al. Increased levels of macrophage inflammatory proteins result in resistance to R5-tropic HIV-1 in a subset of elite controllers. *J Virol.* (2015) 89:5502–14. doi: 10.1128/jvi.00118-15
35. Vega JA, Villegas-Ospina S, Aguilar-Jiménez W, Rugeles MT, Bedoya G, Zapata W, et al. Haplotypes in CCR5-CCR2, CCL3, and CCL5 are associated with natural resistance to HIV-1 infection in a Colombian cohort. *Biomédica* (2017) 37:267–73. doi: 10.7705/biomedica.v37i3.3237
36. Zapata W, Aguilar-Jiménez W, Pineda-Trujillo N, Rojas W, Estrada H, Rugeles MT. Influence of *CCR5* and *CCR2* genetic variants in the resistance/susceptibility to HIV in serodiscordant couples from Colombia. *AIDS Res Hum Retroviruses.* (2013) 29:1594–603. doi: 10.1089/aid.2012.0299
37. Cella M, Fuchs A, Vermi W, Facchetti F, Otero KJ, Lennerz KM, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* (2009) 457:722–5. doi: 10.1038/nature07537
38. Zenewicz LA, Flavell RA. Recent advances in IL-22 biology. *Int Immunol.* (2011) 23:159–63. doi: 10.1093/intimm/dxr001
39. Xu X, Weiss ID, Zhang HH, Singh SP, Wynn TA, Wilson MS, et al. Conventional NK cells can produce IL-22 and promote host defense in *Klebsiella pneumoniae* Pneumonia. *J Immunol.* (2014) 192:1778–86. doi: 10.4049/jimmunol.1300039
40. Colonna M. Interleukin-22-producing natural killer cells and lymphoid tissue inducer-like cells in mucosal immunity. *Immunity* (2009) 31:15–23. doi: 10.1016/j.immuni.2009.06.008
41. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. *Immunity* (2004) 21:241–54. doi: 10.1016/j.immuni.2004.07.007
42. Wolk K, Sabat R. Interleukin-22: a novel T- and NK-cell derived cytokine that regulates the biology of tissue cells. *Cytokine Growth Factor Rev.* (2006) 17:367–80. doi: 10.1016/j.cytogfr.2006.09.001
43. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med.* (2008) 14:282–9. doi: 10.1038/nm1720
44. Zapata W, Rodriguez B, Weber J, Estrada H, Quinones-Mateu M, Zimermman P, et al. Increased levels of human beta-defensins mRNA in sexually HIV-1 exposed but uninfected individuals. *Curr HIV Res.* (2008) 6:531–8. doi: 10.2174/157016208786501463
45. Zapata W, Aguilar-Jiménez W, Feng Z, Weinberg A, Russo A, Potenza N, et al. Identification of innate immune antiretroviral factors during *in vivo* and *in vitro* exposure to HIV-1. *Microbes Infect.* (2016) 18:211–9. doi: 10.1016/j.micinf.2015.10.009
46. Wang W, Wu F, Cong Z, Liu K, Qin C, Wei Q. The secretion of IL-22 from mucosal NKp44 $^{+}$ NK cells is associated with microbial translocation and virus infection in SIV/SHIV-infected Chinese macaques. *J Immunol Res.* (2014) 2014:1–13. doi: 10.1155/2014/387950
47. Missé D, Yssel H, Trabattoni D, Oblet C, Lo Caputo S, Mazzotta F, et al. IL-22 Participates in an innate anti-HIV-1 host-resistance network through acute-phase protein induction. *J Immunol.* (2006) 178:407–15. doi: 10.4049/jimmunol.178.1.407
48. Topham NJ, Hewitt EW. Natural killer cell cytotoxicity: how do they pull the trigger? *Immunology* (2009) 128:7–15. doi: 10.1111/j.1365-2567.2009.03123.x
49. Elliott JM, Yokoyama WM. Unifying concepts of MHC-dependent natural killer cell education. *Trends Immunol.* (2011) 32:364–72. doi: 10.1016/j.it.2011.06.001
50. Martin MP, Qi Y, Gao X, Yamada E, Martin JN, Pereyra F, et al. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat Genet.* (2007) 39:733–40. doi: 10.1038/ng2035
51. Richard J, Sindhu S, Pham NQ, Belzile JP, Cohen EA. HIV-1 Vpr up-regulates expression of ligands for the activating NKG2D receptor and promotes NK cell-mediated killing. *Blood* (2010) 115:1354–63. doi: 10.1182/blood-2009-08-237370
52. Bernard NF, Kiani Z, Tremblay-McLean A, Kant SA, Leeks CE, Dupuy FP. Natural Killer (NK) cell education differentially influences HIV antibody-dependent NK cell activation and antibody-dependent cellular cytotoxicity. *Front Immunol.* (2017) 8:1033. doi: 10.3389/fimmu.2017.01033
53. Barouch DH, Alter G, Broge T, Linde C, Ackerman ME, Brown EP, et al. Protective efficacy of adenovirus/protein vaccines against SIV challenges in rhesus monkeys. *Science* (2015) 349:320–4. doi: 10.1126/science.aab3886
54. Bournazos S, Klein F, Pietzsch J, Seaman MS, Nussenzweig MC, Ravetch JV. Broadly Neutralizing anti-HIV-1 antibodies require Fc effector functions for *in vivo* activity. *Cell* (2014) 158:1243–53. doi: 10.1016/j.cell.2014.08.023
55. DiLillo DJ, Tan GS, Palese P, V Ravetch J. Broadly neutralizing hemagglutinin stalk–specific antibodies require FcγR interactions for protection against influenza virus *in vivo*. *Nat Med.* (2014) 20:143–51. doi: 10.1038/nm.3443
56. Chung AW, Kumar MP, Arnold KB, Yu WH, Schoen MK, Dunphy LJ, et al. Dissecting polyclonal vaccine-induced humoral immunity against hiv using systems serology. *Cell* (2015) 163:988–98. doi: 10.1016/j.cell.2015.10.027
57. Ackerman ME, Mikhailova A, Brown EP, Dowell KG, Walker BD, Bailey-Kellogg C, et al. Polyfunctional HIV-specific antibody responses are associated with spontaneous HIV control. *PLOS Pathog.* (2016) 12:e1005315. doi: 10.1371/journal.ppat.1005315
58. Giese S, Marsh M. Tetherin can restrict cell-free and cell-cell transmission of HIV from primary macrophages to T Cells. *PLoS Pathog* (2014) 10:e1004189. doi: 10.1371/journal.ppat.1004189
59. Arias JF, Heyer LN, von Bredow B, Weisgrau KL, Moldt B, Burton DR, et al. Tetherin antagonism by Vpu protects HIV-infected cells from antibody-dependent cell-mediated cytotoxicity, *Proc Natl Acad Sci USA.* (2014) 111:6425–30. doi: 10.1073/pnas.1321507111
60. Borg C, Jalil A, Laderach D, Maruyama K, Wakasugi H, Charrier S, et al. NK cell activation by dendritic cells (DCs) requires the formation of a synapse leading to IL-12 polarization in DCs, *Blood* (2004) 104:3267–75. doi: 10.1182/blood-2004-01-0380
61. Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. Reciprocal activating interaction between natural killer cells and dendritic cells. *J Exp Med.* (2002) 195:327–33. doi: 10.1084/jem.20010938
62. Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, Münz C. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med.* (2002) 195:343–51. doi: 10.1084/JEM.20011149
63. Ferlazzo G, Moretta L. Dendritic cell editing by natural killer cells. *Crit Rev Oncog.* (2014) 19:67–75. doi: 10.1615/CritRevOncog.2014010827
64. Wilder JA, Koh CY, Yuan D. The role of NK cells during *in vivo* antigen-specific antibody responses. *J Immunol.* (1996) 156:146–52.
65. Krebs P, Barnes MJ, Lampe K, Whitley K, Bahjat KS, Beutler B, et al. NK cell-mediated killing of target cells triggers robust antigen-specific T cell-mediated and humoral responses. *Blood* (2009) 113:6593–602. doi: 10.1182/blood-2009-01-201467
66. Perreau M, Savoye AL, De Crignis E, Corpataux JM, Cubas R, Haddad EK, et al. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *J Exp Med.* (2013) 210:143–56. doi: 10.1084/jem.20121932
67. Huot N, Jacquelin B, Garcia-Tellez T, Rascle P, Ploquin MJ, Madec Y, et al. Natural killer cells migrate into and control simian immunodeficiency virus replication in lymph node follicles in African green monkeys. *Nat Med.* (2017) 23:1277–86. doi: 10.1038/nm.4421
68. Huot N, Bosinger SE, Paiardini M, Reeves RK, Müller-Trutwin M. Lymph node cellular and viral dynamics in natural hosts and impact for HIV cure strategies. *Front Immunol.* (2018) 9:780. doi: 10.3389/fimmu.2018.00780
69. Scully E, Alter G. NK Cells in HIV Disease. *Curr HIV/AIDS Rep.* (2016) 13:85–94. doi: 10.1007/s11904-016-0310-3

70. Michel T, Poli A, Cuapio A, Briquemont B, Iserentant G, Ollert M, et al. Human CD56bright NK cells: an update. *J Immunol.* (2016) 196:2923–31. doi: 10.4049/jimmunol.1502570
71. Taborda NA, Hernández JC, Montoya CJ, Rugeles MT. Las células natural killer y su papel en la respuesta inmunitaria durante la infección por el virus de la inmunodeficiencia humana tipo-1. *Inmunología* (2014) 33:11–20. doi: 10.1016/j.inmuno.2013.11.002
72. Moretta A, Marcenaro E, Parolini S, Ferlazzo G, Moretta L. NK cells at the interface between innate and adaptive immunity. *Cell Death Differ.* (2008) 15:226–33. doi: 10.1038/sj.cdd.4402170
73. Moretta L. Dissecting CD56dim human NK cells. *Blood* (2010) 116:3689–91. doi: 10.1182/blood-2010-09-303057
74. Alter G, Teigen N, Davis BT, Addo MM, Suscovich TJ, Waring MT, et al. Sequential deregulation of NK cell subset distribution and function starting in acute HIV-1 infection. *Blood* (2005) 106:3366–9. doi: 10.1182/blood-2005-03-1100
75. Hong HS, Ahmad F, Eberhard JM, Bhatnagar N, Bollmann BA, Keudel P, Ballmaier M, et al. Loss of CCR7 Expression on CD56bright NK Cells Is associated with a CD56dimCD16+ NK cell-like phenotype and correlates with HIV viral load. PLoS ONE (2012) 7:e44820. doi: 10.1371/journal.pone.0044820
76. Bhardwaj S, Ahmad F, Wedemeyer H, Cornberg M, Schulze zur Wiesch J, van Lunzen J, et al. Increased CD56bright NK cells in HIV-HCV co-infection and HCV mono-infection are associated with distinctive alterations of their phenotype. *Virol J.* (2016) 13:67. doi: 10.1186/s12985-016-0507-5
77. Hong HS, Eberhard JM, Keudel P, Bollmann BA, Ballmaier M, Bhatnagar N, et al. HIV infection is associated with a preferential decline in less-differentiated CD56dim CD16+ NK cells. *J. Virol.* (2010) 84:1183–8. doi: 10.1128/JVI.01675-09
78. Lopez-Vergès S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood* (2010) 116:3865–74. doi: 10.1182/blood-2010-04-282301
79. Milush JM, López-Vergès S, York VA, Deeks SG, Martin JN, Hecht FM, et al. CD56negCD16+ NK cells are activated mature NK cells with impaired effector function during HIV-1 infection *Retrovirology* (2013) 10:158. doi: 10.1186/1742-4690-10-158
80. Gonzalez VD, Falconer K, Bjorkstrom NK, Blom KG, Weiland O, Ljunggren G, et al. Expansion of functionally skewed CD56-negative NK cells in chronic hepatitis c virus infection: correlation with outcome of pegylated IFN- and ribavirin treatment. *J Immunol.* (2009) 183:6612–8. doi: 10.4049/jimmunol.0901437
81. Shao JY, Yin WW, Zhang QF, Liu Q, Peng ML, Hu HD,et al. Siglec-7 defines a highly functional natural killer cell subset and inhibits cell-mediated activities. *Scand J Immunol.* (2016) 84:182–90. doi: 10.1111/sji.12455
82. Brunetta E, Fogli M, Varchetta S, Bozzo L, Hudspeth KL, Marcenaro E, Moretta A, et al. The decreased expression of Siglec-7 represents an early marker of dysfunctional natural killer-cell subsets associated with high levels of HIV-1 viremia. *Blood* (2009) 114:3822–30. doi: 10.1182/blood-2009-06-226332
83. Ndhlovu LC, Lopez-Verges S, Barbour JD, Jones RB, Jha AR, Long BR, et al. Tim-3 marks human natural killer cell maturation and suppresses cell-mediated cytotoxicity. *Blood* (2012) 119:3734–43. doi: 10.1182/blood-2011-11-392951
84. Jost S, Moreno-Nieves U, Garcia-Beltran W, Rands K. Dysregulated Tim-3 expression on natural killer cells is associated with increased Galectin-9 levels in HIV-1 infection. *Retrovirology* (2013) 10:74. doi: 10.1186/1742-4690-10-74
85. Schafer JL, Li H, Evans TI, Estes JD, Reeves RK. Accumulation of cytotoxic CD16+ NK cells in simian immunodeficiency virus-infected lymph nodes associated with in situ differentiation and functional anergy. *J Virol.* (2015) 89:6887–94. doi: 10.1128/JVI.00660-15
86. Reeves RK, Rajakumar PA, Evans TI, Connole M, Gillis J, Wong FE, et al. Gut inflammation and indoleamine deoxygenase inhibit IL-17 production and promote cytotoxic potential in NKp44+ mucosal NK cells during SIV infection. *Blood* (2011)118:3321–30. doi: 10.1182/blood-2011-04-347260
87. Xu H, Wang X, Liu DX, Moroney-Rasmussen T, Lackner AA, Veazey RS. IL-17-producing innate lymphoid cells are restricted to mucosal tissues and are depleted in SIV-infected macaques. *Mucosal Immunol.* (2012) 5:658–69. doi: 10.1038/mi.2012.39
88. Körner C, Altfeld M. Role of KIR3DS1 in human diseases. *Front Immunol.* (2012) 3:326. doi: 10.3389/fimmu.2012.00326
89. Habegger de Sorrentino A, Sinchi JL, Marinic K, López R, Iliovich E. KIR-HLA-A and B alleles of the Bw4 epitope against HIV infection in discordant heterosexual couples in Chaco Argentina. *Immunology* (2013) 140:273–9. doi: 10.1111/imm.12137
90. Jackson E, Zhang CX, Kiani Z, Lisovsky I, Tallon B, Del Corpo A, et al. HIV exposed seronegative (HESN) compared to HIV infected individuals have higher frequencies of telomeric Killer Immunoglobulin-like Receptor (KIR) B motifs; Contribution of KIR B motif encoded genes to NK cell responsiveness. *PLoS ONE* (2017) 12:e0185160. doi: 10.1371/journal.pone.0185160
91. Carr WH, Rosen DB, Arase H, Nixon DF, Michaelsson J, Lanier LL. Cutting edge: KIR3DS1, a gene implicated in resistance to progression to AIDS, encodes a DAP12-associated receptor expressed on NK cells that triggers NK cell activation 1. *J Immunol.* (2007) 178:647–51. doi: 10.4049/jimmunol.178.2.647
92. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet.* (2002) 31:429–34. doi: 10.1038/ng934
93. Lisovsky I, Isitman G, Song R, DaFonseca S, Tremblay-McLean A, Lebouché B, et al. A higher frequency of NKG2A $^{+}$ than of NKG2A $^{-}$ NK cells responds to autologous HIV-infected CD4 cells irrespective of whether or not they coexpress KIR3DL1. *J Virol.* (2015) 89:9909–19. doi: 10.1128/JVI.01546-15
94. O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. T cell– and B cell–independent adaptive immunity mediated by natural killer cells. *Nat Immunol.* (2006) 7:507–16. doi: 10.1038/ni1332
95. Björkström NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood* (2010) 116:3853–64. doi: 10.1182/blood-2010-04-281675
96. Holder K, Comeau E, Grant M. Origins of natural killer cell memory: special creation or adaptive evolution. *Immunology* (2018) 154:38–49. doi: 10.1111/imm.12898
97. Lopez-Vergès S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, et al. Expansion of a unique CD57^{+}NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc Natl Acad Sci USA.* (2011) 108:14725–32. doi: 10.1073/pnas.1110900108
98. Paust S, Gill HS, Wang Z, Flynn MP, Moseman EA, Senman B, et al. Critical role for the chemokine receptor CXCR6 in NK cell–mediated antigen-specific memory of haptens and viruses. *Nat Immunol.* (2010) 11:1127–35. doi: 10.1038/ni.1953
99. Venkatasubramanian S, Cheekatla S, Paidipally P, Tripathi D, Welch E, Tvinnereim AR, et al. IL-21-dependent expansion of memory-like NK cells enhances protective immune responses against Mycobacterium tuberculosis. *Mucosal Immunol.* (2017) 10:1031–42. doi: 10.1038/mi.2016.105
100. Reeves RK, Li H, Jost S, Blass E, Li H, Schafer JL, et al. Antigen-specific NK cell memory in rhesus macaques. *Nat Immunol.* (2015) 16:927–32. doi: 10.1038/ni.3227
101. Lima JF, Oliveira MS, Pereira NZ, Mitsunari GE, Duarte JS, Sato MN. Distinct natural killer cells in HIV-exposed seronegative subjects with effector cytotoxic CD56dim and CD56bright cells and memory-like CD57+NKG2C+CD56dim Cells. *J Acquir Immune Defic Syndr.* (2014) 67:463–71. doi: 10.1097/QAI.0000000000000350
102. Gondois-Rey F, Chéret A, Granjeaud S, Mallet F, Bidaut G, Lécuroux C, et al. NKG2C+memory-like NK cells contribute to the control of HIV viremia during primary infection: Optiprim-ANRS 147. *Clin Transl Immunol.* (2017) 6:e150. doi: 10.1038/cti.2017.22
103. Anderson DJ, Politch JA, Nadolski AM, Blaskewicz CD, Pudney J, Mayer KH. Targeting trojan horse leukocytes for HIV prevention. *AIDS* (2010) 24:163–87. doi: 10.1097/QAD.0b013e32833424c8
104. Childs RW, Carlsten M. Therapeutic approaches to enhance natural killer cell cytotoxicity against cancer: the force awakens. *Nat Rev Drug Discov.* (2015) 14:487–98. doi: 10.1038/nrd4506

105. Bergamaschi C, Kulkarni V, Rosati M, Alicea C, Jalah R, Chen S, et al. Intramuscular delivery of heterodimeric IL-15 DNA in macaques produces systemic levels of bioactive cytokine inducing proliferation of NK and T cells. *Gene Ther.* (2015) 2284:76–86. doi: 10.1038/gt.2014.84
106. Conlon KC, Lugli E, Welles HC, Rosenberg SA, Fojo AT, Morris JC, et al. Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J Clin Oncol.* (2015) 33:74–82. doi: 10.1200/JCO.2014.57.3329
107. Garrido C, Abad-Fernandez M, Tuyishime M, Pollara JJ, Ferrari G, Soriano-Sarabia N, Margolis DM. Interleukin-15-stimulated natural killer cells clear HIV-1-infected cells following latency reversal *ex vivo*. *J Virol.* (2018) 92:JVI.00235-18. doi: 10.1128/JVI.00235-18
108. Romee R, Rosario M, Berrien-Elliott MM, Wagner JA, Jewell BA, Schappe T, et al. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Sci Transl Med.* (2016) 8:357ra123. doi: 10.1126/scitranslmed.aaf2341
109. Cummings JS, Moreno-Nieves UY, Arnold V, Gilbert A, Yarbrough K, Didier C, et al. ANRS HIV Vaccine Network (AHVN), Natural killer cell responses to dendritic cells infected by the ANRS HIV-1 vaccine candidate, MVA HIV. *Vaccine* (2014) 32:5577–84. doi: 10.1016/j.vaccine.2014.07.094
110. Ahmad R, Sindhu ST, Toma E, Morisset R, Vincelette J, Menezes J, Ahmad A. Evidence for a correlation between antibody-dependent cellular cytotoxicity-mediating anti-HIV-1 antibodies and prognostic predictors of HIV infection. *J Clin Immunol.* (2001) 21:227–33. doi: 10.1023/A:1011087132180
111. Milligan CB, Richardson AA, John-Stewart G, Nduati R, Overbaugh J, John-Stewart G. Passively Acquired Antibody-Dependent Cellular Cytotoxicity (ADCC) activity in HIV-infected infants is associated with reduced mortality. *Cell Host Microbe* (2015) 17:500–6. doi: 10.1016/j.chom.2015.03.002
112. Lu L, Murakowski DK, Bournazos S, Schoofs T, Sarkar D, Halper-Stromberg A, et al. Enhanced clearance of HIV-1-infected cells by broadly neutralizing antibodies against HIV-1 *in vivo*. *Science* (2016) 352:1001–4. doi: 10.1126/science.aaf1279
113. Gómez-Román VR, Patterson LJ, Venzon D, Liewehr D, Aldrich K, Florese R, et al. Vaccine-elicited antibodies mediate antibody-dependent cellular cytotoxicity correlated with significantly reduced acute viremia in rhesus macaques challenged with SIVmac251. *J Immunol.* (2005) 174:2185–9. doi: 10.4049/jimmunol.174.4.2185
114. Davis ZB, Lenvik T, Hansen L, Felices M, Cooley S, Vallera D, et al. A Novel HIV envelope Bi-specific killer engager enhances natural killer cell mediated ADCC responses against HIV-infected cells. *Blood* (2016) 128: 2517.
115. Flynn NM, Forthal DN, Harro CD, Judson FN, Mayer KH, Para MF, et al. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J Infect Dis.* (2005) 191:654–65. doi: 10.1086/428404
116. Pitisuttithum P, Gilbert P, Gurwith M, Heyward W, Martin M, van Griensven F, et al. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. *J Infect Dis.* (2006) 194:1661–71. doi: 10.1086/508748
117. Karnasuta C, Paris RM, Cox JH, Nitayaphan S, Pitisuttithum P, Thongcharoen P, et al. Thai AIDS Vaccine Evaluation Group, Thailand, Antibody-dependent cell-mediated cytotoxic responses in participants enrolled in a phase I/II ALVAC-HIV/AIDSVAX® B/E prime-boost HIV-1 vaccine trial in Thailand. *Vaccine* (2005) 23:2522–9. doi: 10.1016/j.vaccine.2004.10.028
118. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, et al. MOPH-TAVEG Investigators, Vaccination with ALVAC and AIDSVAX to Prevent HIV-1 Infection in Thailand. *N Engl J Med.* (2009) 361:2209–2220. doi: 10.1056/NEJMoa0908492
119. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, et al. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N Engl J Med.* (2012) 366:1275–86. doi: 10.1056/NEJMoa1113425
120. Yates NL, Liao HX, Fong Y, DeCamp A, Vandergrift NA, Williams WT. Vaccine-induced Env V1-V2 IgG3 correlates with lower HIV-1 infection risk and declines soon after vaccination. *Sci Transl Med.* (2014) 6:228ra39. doi: 10.1126/scitranslmed.3007730
121. Callaway E. Clues emerge to explain first successful HIV vaccine trial. *Nature* (2011). doi: 10.1038/news.2011.541
122. Bradley T, Pollara J, Santra S, Vandergrift N, Pittala S, Bailey-Kellogg C, et al. Pentavalent HIV-1 vaccine protects against simian-human immunodeficiency virus challenge. *Nat Commun.* (2017) 8:15711. doi: 10.1038/ncomms15711
123. Glienke W, Esser R, Priesner C, Suerth JD, Schambach A, Wels WS, et al. Advantages and applications of CAR-expressing natural killer cells. *Front Pharmacol.* (2015) 6:21. doi: 10.3389/fphar.2015.00021
124. Liu D, Tian S, Zhang K, Xiong W, Michel Lubaki N, Chen Z, et al. Chimeric antigen receptor (CAR)-modified natural killer cell-based immunotherapy and immunological synapse formation in cancer and HIV. *Protein Cell* (2017) 8:861–77. doi: 10.1007/s13238-017-0415-5
125. Zhen A, Kamata M, Rezek V, Rick J, Levin B, Kasparian S, et al. HIV-specific immunity derived from chimeric antigen receptor-engineered stem cells. *Mol Ther.* (2015) 23:1358–67. doi: 10.1038/mt.2015.102

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1.3 Problem statement

As mentioned before, there are several cohorts for the study of HESN individuals. Each of these cohorts represents an important source of information in the field of natural resistance to HIV. Despite the fact that serodiscordant couples are a common cohort for HESN study, there is a controversy on the real exposure of these individuals. Currently, most serodiscordant couples are recruited from transmitted disease clinics where positive individuals are under treatment while their partners receive counseling and medical accompaniment. This situation decreases risk behaviors, reducing the incidence, but also the number of sexually-exposed uninfected individuals for being recruited in the studies (14).

Although the extensive following of CSW cohorts has brought valuable information on natural resistance mechanisms, most CSW are women, generating a gap on the mechanisms that could be specific in men. Studying natural resistance mechanisms in men is necessary since this popultion is still carrying a disproportionate burden of HIV infection in many countries. For instance, in Colombia, men represent 74.6% of people living with HIV (1).

MSM represent an interesting cohort for the study of natural resistance mechanisms based on their social and biological characteristics that make them a very high-risk population, representing near to 69% of HIV positive men around the world (80). In Latin America, MSM represents 41% of people living with HIV; moreover, in some countries of Latin America, AIDS incidence is thirteen times higher among MSM than among heterosexual men (81). This high-risk tendency in the MSM population is conserved in different scenarios where it has been evaluated (82, 83).

Althought MSM represent a little percentage of the Colombian population, around 2%, they deserve special attention since 61.9% of the new infections are concentrated on them (5). This scenario is similar to other countries like E.E.U.U, where the number of MSM cohorts for the study of HIV resistance keeps rising in order to find immune and genetic mechanisms associated with a natural resistance against HIV (84). The Multicenter AIDS Cohort Study (MACS) is one of the largest cohorts for the study of HIV-1 infection in gay and bisexual men and include HESN

individuals; these studies have reported mechanisms associated with natural resistance to HIV infection and slow progression to AIDS on these individuals (50, 85).

There are biological and social factors that contribute to the high risk of acquiring HIV observed in MSM, which is 22 times higher compared with the general population (1). The nature of sexual intercourse between MSM represents a high-risk practice by itself. Receptive anal intercourse is one of the riskiest sexual behavior for HIV transmission. Receptive anal intercourse implies a risk of 138 infections per 10.000 exposures, while receptive penile-vaginal intercourse represents a risk of 8 infections per 10.000 exposures. In fact, anal intercourse represents a higher risk that sharing needles (63 infections per 10.000 exposures) (20).

The gastrointestinal mucosa contains the majority of $CD4^+$ T cells of the body, representing the largest reservoir and site of HIV replication (86). These tissues are densely populated also with dendritic cells and macrophages expressing CCR5 and CXCR4, making them susceptible to HIV infection (87). Besides, the epithelium of rectal mucosa is made by a single layer of columnar epithelium more prompt to laceration that stratified epithelium of the vagina, bringing to the virus easier access to a large population of activated lymphocytes residing in the GALT (86). Additionally, in this tissue, the passage of viral particles from the lumen to target cells is facilitated by dendritic cells projections that extend into the epithelial compartment (88).

Regarding the social factors, unprotected sex is one of the main factors associated with the high prevalence of HIV among MSM. UNAIDS reported than in 33 of the 87 countries studied, less than 60% of MSM reported condom use in the last anal sex encounter (19). Lack of knowledge of serological status also contributes to these statistics, primarily in Latin America. Studies conducted in developed countries show that the percentage of MSM that never tests for HIV is below 30% (89, 90), while in Latin America and Africa is 40% or higher (91, 92). These low levels of HIV testing are related to homophobic stigma, discrimination, and violence, which made MSM

less likely to access HIV diagnostic services (93). Finally, there are other factors, such as the high prevalence of illegal drug use and the high prevalence of STIs, which increase the risk of acquiring HIV in this population.

Some mechanisms described in CSW and serodiscordant couples have been described in MSM, as the quiescent phenotype in the mucosa (50). However, many others remain to be studied in this cohort, including the increased effector capacity of NK cells, which represents an important mechanism of natural resistance found in other HESN cohorts (58, 69).

Medellin is one of the cities with a higher prevalence of HIV among the MSM population, 20.7%, compared to 17% that is the general prevalence for MSM in the country (94). MSM from Medellin city reflects the high-risk situation of MSM around the world, and they can be an important source of information about the role of NK cells in the natural resistance phenomenon. So far, there are no studies on resistance mechanisms in MSM in Colombia. Besides, there is no information about increased NK activity or the frequency of memory NK cells and their possible implication in this phenomenon in MSM.

2. OBJECTIVES

2.1 General objective

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

2.2 Specific objectives

- To determine the phenotype and frequency of NK cells of MSM individuals including the memory NK cell phenotype.
- To evaluate the natural cytotoxicity of NK cells obtained from peripheral blood of MSM in response to co-culture with the K562 cell line.
- To quantify the expression of Granzyme B, MIP-1β, IFN-ɣ, and CD107a as markers of NK cell activation, obtained from MSM, in response to the stimulation with an HIV-1 infected cell line

- To determine the relative expression of RANTES, MIP-1β, MIP-1α, Perforin, Granzyme B, TNF-α and IFN-ɣ, associated with the antiviral activity of NK cells, from mRNA obtained from PBMC.
- To establish the frequency of HLA/KIR alleles combination associated with a protective phenotype against HIV-1 infection.

2.3 Hypothesis and research question

Based on the evidence from previous studies, conducted in different HESN cohorts, we hypothesize that there is an increase in the effector activity of NK cells from MSM at high-risk of HIV infection, compared to low-risk MSM, reflected in an increase of one or several of the following parameters i) production of effector molecules; ii) cytotoxic activity of NK cells; iii) expression of molecules associated with NK activity; iv) frequency of NK cells with memory phenotype ($CD56^{dim}CD57^{+}NKG2C^{high}$), and v) the frequency of HLA/KIR phenotype associated with protection against HIV infection

Our study pretends to glimpse data about what is the role of NK cells in the phenomenon of natural resistance to HIV-1 infection, observed in high-risk MSM from Medellin.

3. MATERIALS AND METHODS

3.1 *Study population*

Forty-two 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 unprotected sexual intercourse reported (high-risk MSM), and ii) MSM at lower risk of infection: MSM with less than 4 different sexual partners in the last 3 months with unprotected sexual intercourse reported (low-risk MSM). MSM younger than 18 years of age, positive for HIV 1/2 rapid test (SD BIOLINE, Abbott), positive proviral DNA PCR or homozygous for *CCR5* Δ32 mutation were excluded (***Supplementary figure 1***).

Signed informed consent and a questionnaire for risk behaviors were obtained for each individual after an explanation of the study. Later, 50mL of peripheral blood was taken with a disposable syringe. The ethics committee from the Universidad de Antioquia approved the development of the study.

3.2 PCR for Δ32 mutation and proviral DNA

DNA was extracted from PBMCs with a phenol-chloroform protocol. Briefly, unfrozen PBMC were incubated with 1 mL of a white blood cells lysis buffer, containing Tris HCl 1M (Invitrogen, Waltham, MA, USA), EDTA 0.5M (PanReac AppliChem, Darmstadt, Germany), NaCl 5M (Merck, Kenilworth, NJ, USA) and SDS 0.1% (PanReac AppliChem), along with 7 µL proteinase K (Thermo Scientific, Wilmington, DE, USA), at 56ºC overnight. After the incubation, 1 mL of phenol/chloroform pH 6.7/8.0 (AMRESCO, Solon, OH, USA) was added to the samples, followed by a centrifugation step at 2450 x g for 15 min. The aqueous phase was transferred to a new vial, where 1 mL of ice-cold isopropanol (Merck, Kenilworth, NJ, USA) was added. The samples were kept at -20ºC for DNA precipitation, followed by a centrifugation step at 18000 x g; the supernatant was carefully discarded, and 1 mL of 70% ethanol (Sigma-Aldrich, St. Louis, MO, USA) was added to further spin the sample at 6800 x g for 5 min. The ethanol supernatant was removed and the DNA was finally suspended in NaOH 8mM and incubated at 56ºC for 1 hour. DNA quantification was performed in a NanoDropTM 1000 Spectrophotometer (Thermo Scientific), and samples were stored at -20ºC until they were used.

The Δ32 mutation at the CCR5 gene was detected by PCR with the following protocol: 95°C 3 min, 30 cycles of amplification at 95°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec, and a final extension step 72°C for 5 min. The PCR product was observed by electrophoresis in a 2% agarose gel. For wild-type genotype (CCR5/CCR5), a 225bp PCR product was obtained, a product of 193 bp indicated a mutant homozygous genotype (Δ32/Δ32). The presence of both bands indicates a heterozygous genotype (CCR5/Δ32). Primers used for Δ32 PCR were: Δ32 Fwd: 5'-ACCAGATCTCAAAAAGAAGGTCT-3', Δ32Rv: 5'-CATGATGGTGAAGATAAGCCTCACA-3'.

To determine the presence of proviral DNA the *env* gene was amplified by nested PCR with the following protocol: 3 cycles at 94°C for 1 min, 64°C for 1 min, 72°C for 1 min, 37 cycles of amplification at 94°C for 15 sec, 64°C for 45 sec, 72°C for 1 min, and finally 72°C x 5 min. The second PCR amplified the HIV-1 *env* C2V3C3 region with the following protocol: 3 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 37 cycles of amplification at 94°C for 15 sec, 55°C for 45 sec, 72°C for 1 min and finally 72°C x 5 min. The PCR product was observed by electrophoresis in a 1.5% agarose gel. A product of 562bp indicates the presence of integrated HIV DNA. The primers sequences are in **Supplementary Table 1**

3.3 Determination of anti HCMV IgG titers

Titers of anti-IgG antibodies specific for HCMV were determined using a Human Anti-Cytomegalovirus IgG ELISA Kit (CMV) (Abcam, Cambridge) according to the manufacturer's instructions.

3.4 Frequency and phenotype of NK cells

Aliquots of whole blood specimens (170µL) were stained using the following monoclonal antibodies (Mabs): CD45 (HI30), CD56 (CMSSB), CD3 (UCHT1), CD57 (TB01; All Thermo Scientific, Wilmington, DE, USA); NKG2C (134591; R&D Systems) and CD16 (3G8; BD Biosciences, San Jose, CA, USA) during 25 min in dark. After staining, the sample was treated with a lysing solution (BD Biosciences) according to the manufacturer's instructions to eliminate red blood cells. Finally, cells were washed twice with Phosphate-Buffered Saline (PBS) (Lonza, Rockland, ME, USA) and were suspended in paraformaldehyde 2%. Cells were acquired using LS Fortessa (BD Biosciences) and data were analyzed using FlowJo version 10.5.3 (FlowJo, LLC, Oregon, USA).

3.5 Natural cytotoxicity assays

The PBMCs were isolated through a density gradient with Ficoll-Histopaque (Sigma-Aldrich) by centrifugation at 400 x g for 30 min; then PBMCs were washed 3 times with PBS (Lonza) to eliminate platelets and debris. After, cells were counted and frozen until they were used.

Cells were thawed and let in culture for 24h before each experiment. For natural cytotoxicity assays, K562 were used as target cells, because of the absence of classical HLA I molecules expression. Before the culture, $1x10^6$ K562 were stained with eFluor670 (Thermo Scientific) 0.1mM in 1mL of PBS for 10min at 37°C (Lonza). Then, PBMCs were co-cultured with K562 cells in round-bottom tubes at 10:1 relation ($1x10^6$ PBMCs:$1x10^5$ K562) in 200uL of RPMI with 10% of fetal bovine serum (FBS) (Gibco, Grand Island, NY) for 4h at 37°C with 5% CO2.

After incubation, cells were stained with propidium iodide (PI) (Thermo Scientific) and DIOC-6 (Thermo Scientific) to evaluate the integrity of the cell and mithochondrial membrane respectively. K562 were cultured in the absence of PBMCs as a spontaneous apoptosis control. For every assay, spontaneous apoptosis should be less than 15%; the percentage of cytotoxicity was adjusted based on this data.

3.6 NK cell activation assays

K562 and H9HTLVIII cell lines were used as target cells; H9HTLVIII cells were used to evaluate the activation of NK cells against HIV-1 infected cells. Both cell lines were cultured in RPMI (Sigma-Aldrich) supplemented with 10% of FBS (Gibco) at the density of $1x10^6$ per mL until the co culture.

Fresh PBMCs (at density of $5x10^6$cells/mL) were stimulated with 25ng/mL of IL-15 (Thermo Scientific) in 6 well plates with 2mL of RPMI (Sigma-Aldrich) supplemented with 10% of FBS (Gibco) overnight. Then, PBMCs were cultured in round-bottom tubes at 10:1 relation with target cells ($1x10^6$ PBMC:$1x10^5$ target cells) in 300uL of RPMI (Sigma-Aldrich, St. Louis, MO, USA) with 10% of FBS (Gibco), 6ug/mL of Brefeldin A (Thermo Scientific), 2mM of Monensin (Thermo Scientific) and 1uL of Anti CD107a (BD Biosciences) for 4h at 37°C, 5%CO_2.

Before staining, cells were incubated with 100uL of IgG (20mg/mL) to block Fc receptors. After Fc receptor blocking, cells were stained with Mabs against CD45 (HI30; Thermo Scientific), CD56 (CMSS; Thermo Scientific,) and CD16 (3G8; BD Biosciences) during 20 min in the dark. Then, cells were permeabilized with

Foxp3/Transcription Factor Staining Buffer Set (Thermo Scientific) according to the manufacturer´s instructions. After, cells were stained with anti CD3 (UCHT1; All Thermo Scientific), IFN-ɣ (4S.B3, Biolegend), Granzyme B (BD Biosciences) and MIP-1β (BD Biosciences) for 25 min in dark. Finally, cells were suspended in 350uL of paraformaldehyde 2% and they were acquired using LS Fortessa (BD Biosciences). Data were analyzed using FlowJo version 10.5.3 (FlowJo, LLC).

3.7 Effector molecules quantification by CBA

Supernatants of NK cell activation assays were collected and stored at -80°C until they were used. Supernatants were unthawed at 4°C, just before the Cytometric Bead Array (CBA) assay. For CBA, flex set for MIP-1α, RANTES, TNF-α and IFN-ɣ (BD Biosciences) were included.

CBA was done according to the manufacturer´s instructions. After procedure, beads complex were acquired using LS Fortessa (BD Biosciences). Data were analyzed using FlowJo version 10.5.3 (FlowJo, LLC).

3.8 mRNA quantification by real-time RT-PCR

Total RNA was purified from PBMCs using a commercial kit (Direct-zol RNA Miniprep kit, Zymo Research) according to the manufacturer´s instructions. The RNA was retrotranscribed to cDNA using a high capacity cDNA reverse transcription kit (Thermo Scientific). PCR reactions were performed using the Maxima SYBR Green qPCR master mix kit (Fermentas, France). Real-time RT-PCR was performed in a QuantStudio 5 Real-Time PCR System (Thermo Scientific). The data are expressed as relative units of each gene normalized against the constitutive gene PGK (Phosphoglycerate kinase) using the formula $1.8^{-[\Delta Ct]}$, where 1.8 corresponds to the mean PCR efficiency of 80%. The primers sequence and PCR conditions are described in **Supplementary Table 2.**

3. 9 HLA and KIR typing

HLA and KIR genotyping was performed in collaboration with the Hospital San Vicente Fundación. HLA genotyping was done using the DNA hybridization assay LifeCodes HLA-SSO Typing (Immucor, Stamford, CT, USA) with SSO probes

atached to multicolored beads; the bead complexes were detected with a Luminex 100 Flow cytometer. KIR alleles were genotyped using RSSOKIR (One-Lambda, Canoga Park/CA) a multiplex polymerase chain reaction sequence-specific primer reaction.

3.10 Statistical analysis

To compare data from high-risk MSM vs. low-risk MSM, Mann–Whitney U or t-student test were done, depending on the bivariate normality assumption according to the Shapiro-Wilk normality test. Correlation analyses were based on Spearman correlation coefficient calculation. A p-value <0.05 was considered statistically significant. The statistical tests were performed using the GraphPad Prism Software version 7.02.

4. RESULTS

4.1 MSM socio-demographic data

Forty-two MSM who fulfilled inclusion criteria were enrolled in the study. Some socio-demographic data are summarized in ***Table 1.*** High-risk MSM has a median number of sexual partners in a lifetime of 1200 compared to 29 of low-risk MSM, showing a higher sexual exposure, not only in the previous 3 months to the study, as inclusion criteria, but also during all active sexual life. History of HIV-positive sexual partners, sexually transmitted disease (STD) (p=0.003) and the heterozygous phenotype for *CCR5* ∆32 mutation was frequent in high-risk MSM. The percentage of condom use was near 50% in both groups. The age of onset of sexual intercourse was similar between both groups; however, high-risk MSM were older and showed a higher duration of high-risk sexual activity compared to low-risk MSM.

Table 1. Socio-demographic data of study participants

	High-risk MSM	*Low-risk MSM*	*p*
n	16	26	
Age, median (Q1-Q3)	29.5 (26.2-36.7)	25.5 (20.7-29.7)	0.0127[a]
Sexual partners in the last 3 months, median (Q1-Q3)	27(21-36)	2 (1-4)	<0.0001[a]
Sexual partners in a lifetime, median (Q1-Q3)	1200 (571-2954)	29 (11-100)	<0.0001[a]
% of unprotected sex in the last 3 months, median (Q1-Q3)	46.5 (12.7-74.7)	48 (0-91)	0.984[a]
Frequency of STD	87.5%	38.4%	0.003[b]
Frequency of HIV positive partners	62.5%	42.3%	0.340[b]
Frecuency of CCR5 Δ 32 heterozygous	18.7%	3.85%	0.146[b]
Age of onset of sexual intercourse , median (Q1-Q3)	15 (13.2-17.7)	17 (14-20.2)	0.172[a]
Duration of sexual activity (years), median (Q1-Q3)	13.5 (11.2-20.5)	9 (5-12.2)	0.001[a]

a Mann-Whitney test
b Fisher´s exact test
STD: Sexually transmitted disease
CCR5: C-C chemokine receptor type 5

The most common STDs were gonorrhea and syphilis in all study population (***Figure 1A***). When STD frequency was evaluated based on the nature of the causing agent, the results showed that STD with viral origins like herpes and condyloma were more frequent in low-risk MSM. (***Figure 1B***).

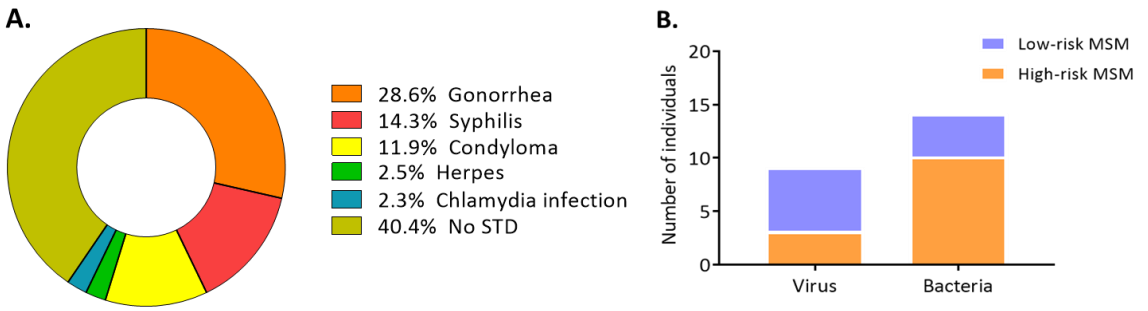


Figure 1. Lower frequency of viral STD in high-risk MSM. A. Distribution of STD among all study participants (STD history was auto-reported by each individual in the questionnaire). **B.** Distribution of STD according to the nature of causing agent (viral or bacterial) between both groups.

4.2 High-risk MSM exhibit a more mature phenotype in NK cells compartment compared to low-risk MSM

The frequency and phenotype of NK cells in peripheral blood of MSM were analyzed by flow cytometry. The two main NK cell subpopulations $CD56^{bright}$ and $CD56^{dim}$, as well as terminally differentiated NK cells, identified by the expression of the maturation marker CD57, were analyzed. Memory NK cells, terminally differentiated NK cells with high expression of activating receptor NKG2C, were also included in the analysis (***Figure 2A***).

Although there were no differences in the percentage of $CD56^{bright}$ NK cells (1.1±0.4 vs 1.4± 0.6 p=0.09) (***Figure 2B***) between both groups, the percentage of $CD56^{dim}$ NK cells was higher in high-risk MSM compared to low-risk MSM (50±17.1 vs 38.4± 8.3 p=0.041) (***Figure 2C***). No significant differences were observed in the frequency of $CD56^{dim}$ subpopulations, $CD56^{dim}CD16^{-}$ and $CD56^{dim}CD16^{+}$(***Figure 2D, E***). The frequency of terminally differentiated NK cells was also higher in high-risk MSM (76.2±8.7 vs 59± 15 p=0.001) (***Figure 2F***).

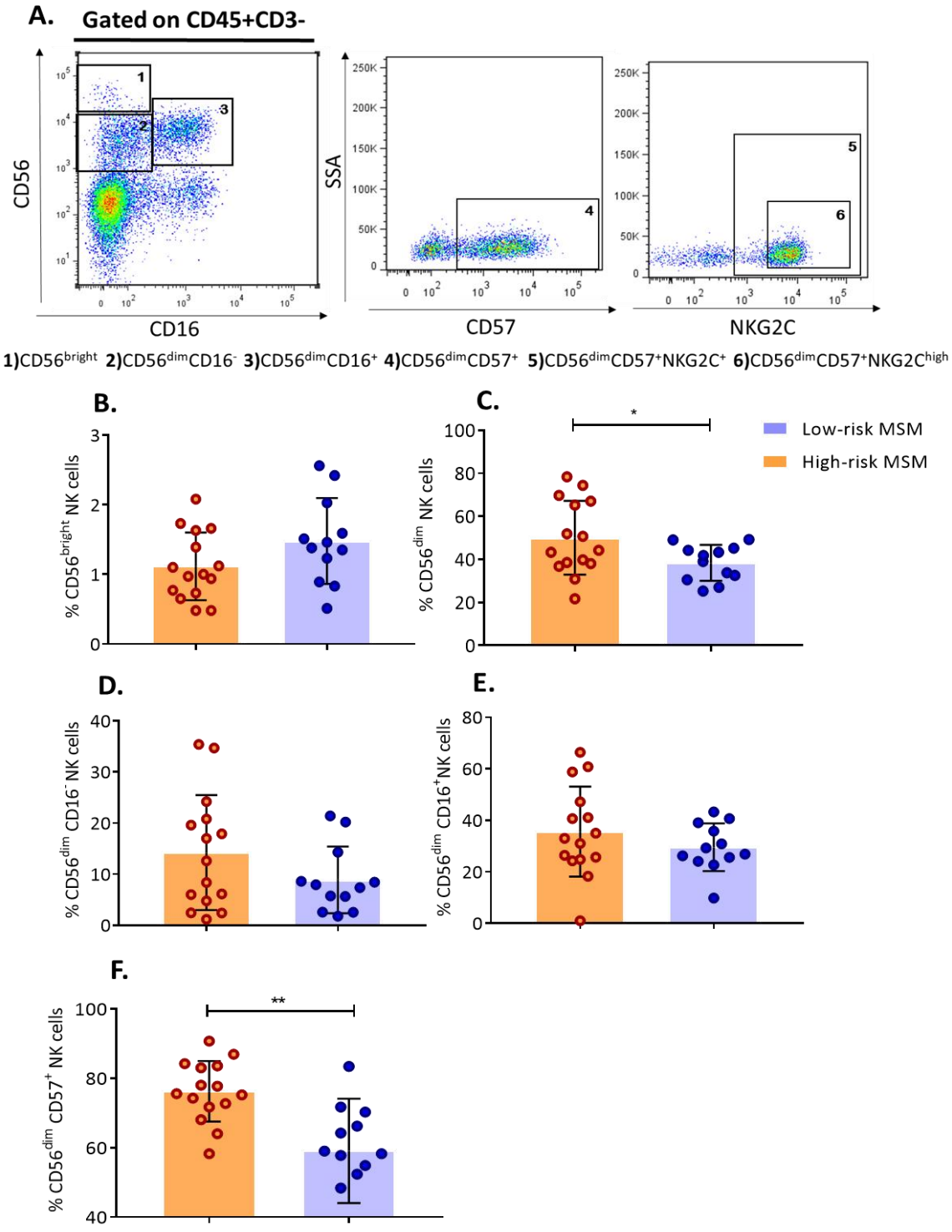


Figure 2. High-risk MSM has a higher frequency of CD56^{dim} cells and terminally differentiated NK cells. A. Representative gating strategy identifying NK cell subpopulations. NK cells were selected on CD45+CD3- cells from peripheral blood;

after, according CD56 and CD16 expression three NK cells subpopulation were defined: **1)** $CD56^{bright}$, **2)** $CD56^{dim}CD16^{-}$ and **3)** $CD56^{dim}CD16^{+}$. On $CD56^{dim}$ subpopulation (2+3), the cells expressing CD57 marker were defined as terminally differentiated NK cells: **4)** $CD56^{dim}CD57^{+}$. On the last subpopulation, two gates were done **5)** $CD56^{dim}CD57^{+}NKG2C^{+}$, and **6)** $CD56^{dim}CD57^{+}NKG2C^{high}$, based on NKG2C expression. $CD56^{dim}CD57^{+}NKG2C^{high}$ NK cells were defined as Memory NK cells. **B, C, D, E, and F.** Frequencies of NK cell subpopulations in peripheral blood; the results are shown as mean± SD, n: 15;12. Statistical evaluations were made with Unpaired t-test (**B, C, E, F) or** Mann-Whitney U **(D)**. *p<0.05, and **p<0.01.

It has been proposed that NK cells subpopulations correspond to different maturation stages. $CD56^{bright}$ the most immature, and $CD56^{dim}CD16^{+}$ the most mature stage, the last one, is the cellular compartment where most of the terminally differentiated NK cells are located. The CD57 maturation marker is positive regulated during the maturation process and it allows to identify of those maturation stages as shown in ***Figure 3A***. The median fluorescence intensity (MFI) of CD57 in NK cells subpopulations was evaluated to compare the maturation of NK cells in each subpopulation between groups.

Results indicate that $CD56^{bright}$ (2570±1633 vs 1230±880 p=0.010), $CD56^{dim}CD16^{-}$ (3558±1826 vs 1419±1051 p=0.0003), and $CD56^{dim}CD16^{+}$(5619±2498 vs 2469± 1598 p=0.0009) (***Figure 3B***) cells of high-risk MSM have a higher MFI of CD57. These results indicate a more mature phenotype of NK cells in high-risk MSM when compared with low-risk MSM.

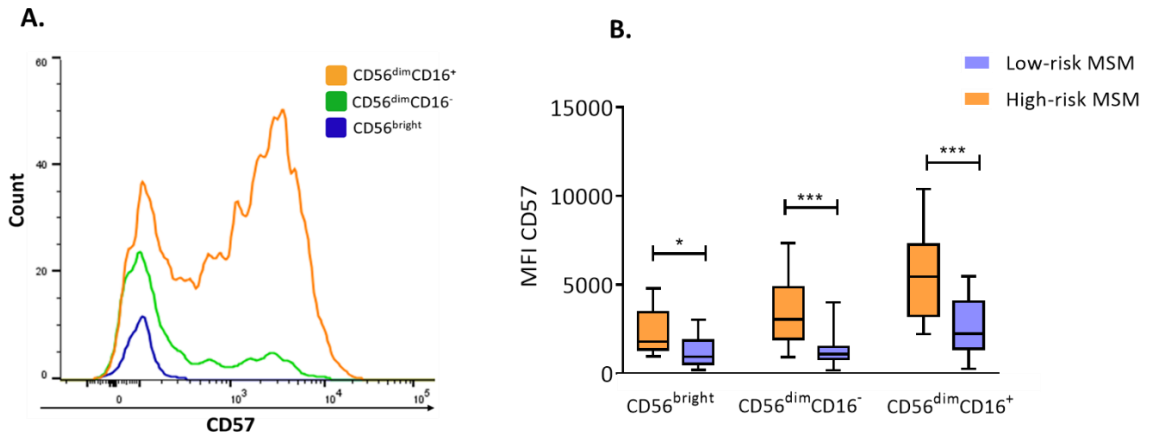


Figure 3. NK cells from high-risk MSM exhibit a more mature phenotype than low-risk MSM. A. Histogram representing the CD57 expression on NK cell subpopulations. $CD56^{bright}$, the immature population, has little or no expression of CD57. $CD56^{dim}CD16^{+}$ cells showed a higher frequency and intensity in the expression of this marker according to their maturation stage, while $CD56^{dim}CD16^{-}$ show an intermediate expression **B.** MFI of CD57 on NK cells subpopulations. Line inside the box indicates mean and whiskers min to max value. n:15,12. Statistical evaluations were made with Mann-Whitney U. *p<0.05, **p<0.01, and ***p<0.001.

4.3 Memory NK cells are more frequent in high-risk MSM than in low-risk MSM

Accumulating evidence indicates there is an NK cell subpopulation with certain adaptive characteristics. Memory NK cells are terminally differentiated NK cells that under certain stimuli, frequently associated with HCMV infection, suffer some phenotypic and functional changes. These changes include the acquisition of memory features and the up-regulation of NKG2C expression, which represents the key activation receptor of memory NK cells.

The frequency of memory NK cells ($CD56^{dim}CD57^{+}NKG2C^{high}$), as well as MFI of NKG2C, was evaluated in both groups. The results show that high-risk MSM has not only a higher frequency of these cells compared to low-risk MSM (34.3±19.7 vs. 12.4±15.2 p=0.006) (***Figure 4A***), but also indicate a higher density of NKG2C

expression on $CD56^{dim}$ NK cells (837.4±455.5 vs 334.2±312.6 p=0.0026) (***Figure 4B***).

The frequency of memory NK cells has been related to the aging process along with the exposition to HCMV. Previously, the levels of anti HCMV IgG has been related to higher numbers of memory NK cells. For that reason, the levels of anti HCMV IgG were evaluated in order to explain the higher frequency of memory NK cells of high-risk MSM. High-risk MSM exhibit titers of anti HCMV IgG ranging from 176.2 to 1845 and low-risk MSM from 79 to 814.5 UI/mL indicating there is no difference between both groups (p=0.217).

As shown in sociodemographic data, high-risk MSM were older than low-risk MSM; for that reason, the correlation between the frequency of memory NK cells and the age was evaluated in study participants. There was no correlation between this both variants (r=0.0137, p=0.9457) (***Figure 4C***). However, there was a strong positive correlation between the frequency of memory NK cells and the number of sexual partners in a lifetime (r0.6219, p=0.0007) (***Figure 4D***), but no with sexual partners in the last three months (data not show). This data suggests that the magnitude of sexual exposure, measured as the number of sexual partners, could be the trigger for memory NK cells expansion rather than aging.

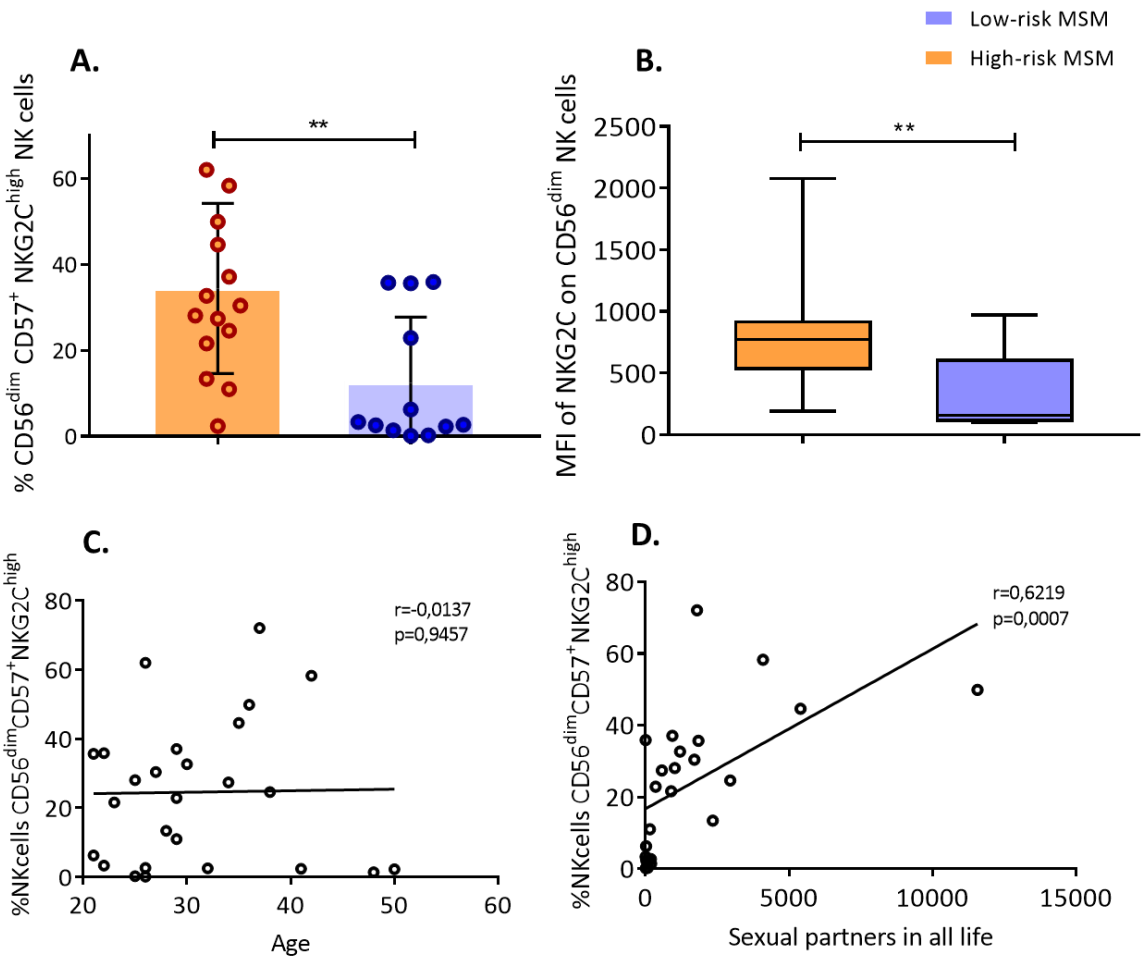


Figure 4. Memory NK cells are more frequent in high-risk MSM. A. Frequency of memory NK cells. **B**. MFI of NKG2C on CD56^{dim} NK cells. **C, D**. Correlation of memory NK cells frequency with age and sexual partners in all life respectively. Statistical evaluations were made with Mann-Whitney U or Spearman´s correlation test. *p<0.05, **p<0.01

A strong correlation was found between the number of sexual partners in a lifetime and the density of NKG2C expression on CD56^{dim} NK cells (r=0.7545, p<0.0001) (***Figure 5A)***, this at the same time correlates with the frequency of memory NK cells (r=0.8023, p<0.0001) (***Figure 5B)***. It is confirming the strong nexus that exist between sexual exposure and the frequency of memory NK cells found in peripheral blood of these individuals.

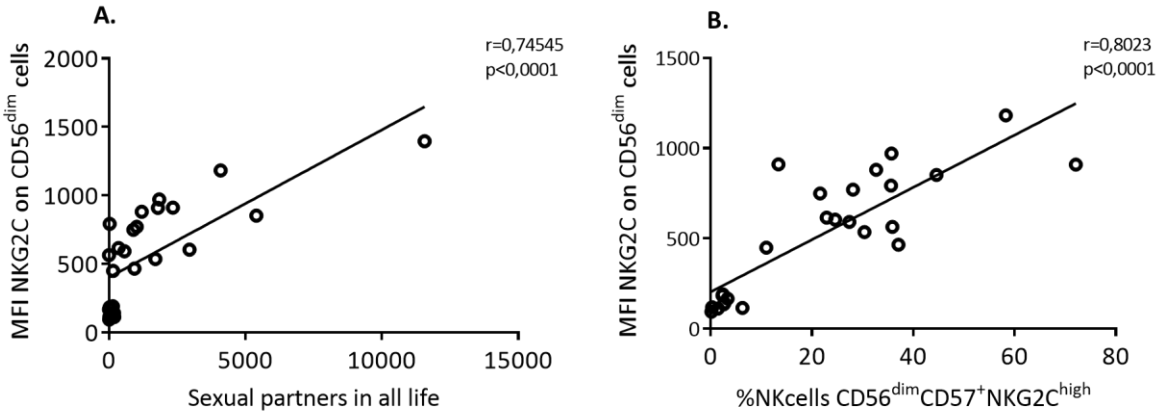


Figure 5. The magnitude of sexual exposure is strongly correlated with the density of NKG2C expression A. Correlation of MFI of NKG2C on $CD56^{dim}$ NK cells and sexual partners in a lifetime. **B**. Correlation of MFI of NKG2C on $CD56^{dim}$ NK cells and the frequency of memory NK cells. Statistical evaluations were made with Spearman´s correlation test. *p<0.05, and **p<0.01.

4.4 Cytotoxic activity of NK cells is higher in high-risk MSM than in low-risk MSM

A higher cytotoxic capacity of NK cells has been reported in several cohorts for the study of HESN individuals and has also been related to protection against HIV infection. In this study, the cytotoxic capacity of NK cells in both groups of MSM was evaluated by co-cultures of PBMCs with the K562 cell line. A representative scheme of the cytotoxicity assessment is shown in ***Figure 6A***.

PBMCs of high-risk MSM showed a higher cytotoxic capacity than low-risk MSM against tumoral cell line K562 (44.2±18.7 vs 27.9±15.5 p=0.002) (***Figure 6B***). The cytotoxic capacity of NK cells and $CD8^+$ T cells has been previously related to the expression of CD57; for this reason, the correlation between the frequency of cells expressing this marker and the cytotoxic capacity of NK cells in K562 co-culture was evaluated. There was a positive correlation between the cytotoxic capacity and $CD56^{dim}CD57^+$ frequency (r= 0.497, p= 0.009) in the K562 assay (***Figure 6C***), supporting the relation between CD57 expression and the cytotoxic capacity.

A correlation between MFI of CD57 and cytotoxicity was found in CD56^{dim}CD16^{-} NK cells (r=0.458, p=0.021), but no with other subpopulation (***Figure 6D***).

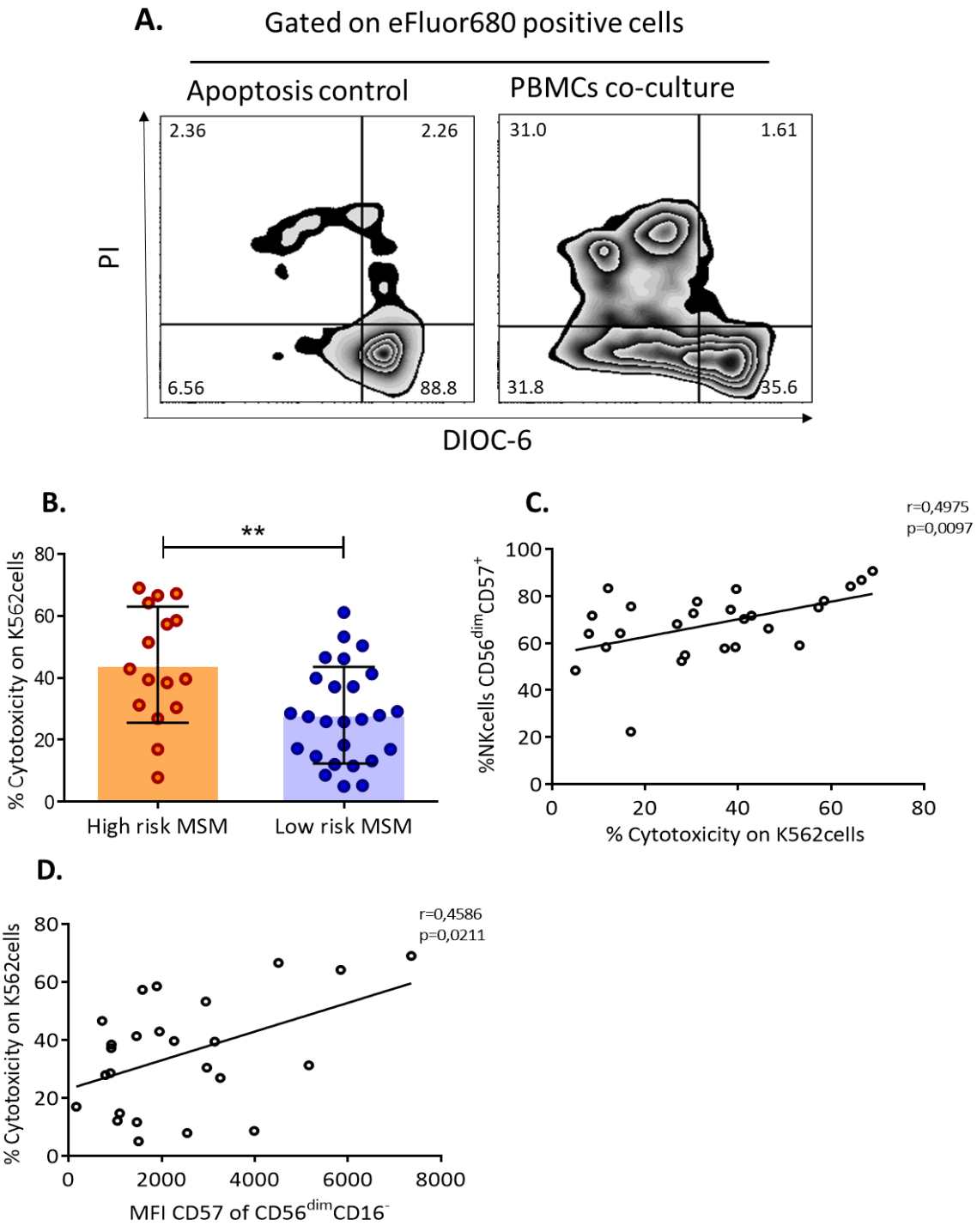


Figure 6. PBMCs from high-risk MSM exhibit a higher cytotoxic activity against K562 cells A. Representative gate of target cells (K562) selected by eFluor670 expression after 4h of culture. On the left the apoptosis control with target cells alone, on the right, the co-culture with PBMCs. **B.** The cytotoxic capacity of NK cells over K562 cells. The percentage of cytotoxicity is expressed as the percentage of cell death corrected with apoptosis control (live target cells are DIOC-6^{+}/PI^{-}). The results are shown as mean± SD, n:16,26. Statistical evaluations were made with unpaired t-test. **C.** Correlation of cytotoxicity with terminally differentiated NK cells. Pearson´s correlation test**. D.** Correlation of cytotoxicity with MFI of CD57 in$CD56^{dim}CD16^{-}$ subpopulation. Pearson´s correlation test. *p<0.05, and **p<0.01.

4.5 High-risk MSM exhibit higher frequency of IFN-γ-expressing NK cells and higher levels of MIP-1α after K562 co-culture

In this assay, PBMCs from MSM were co-cultured with H9HTLVIII and K562 cells during 4h. Then, granzyme B, CD107a, MIP-1β, and IFN-ɣ were evaluated as activation markers, by flow cytometry. A representative scheme of the cytotoxicity assessment is shown in ***Figure 7.***

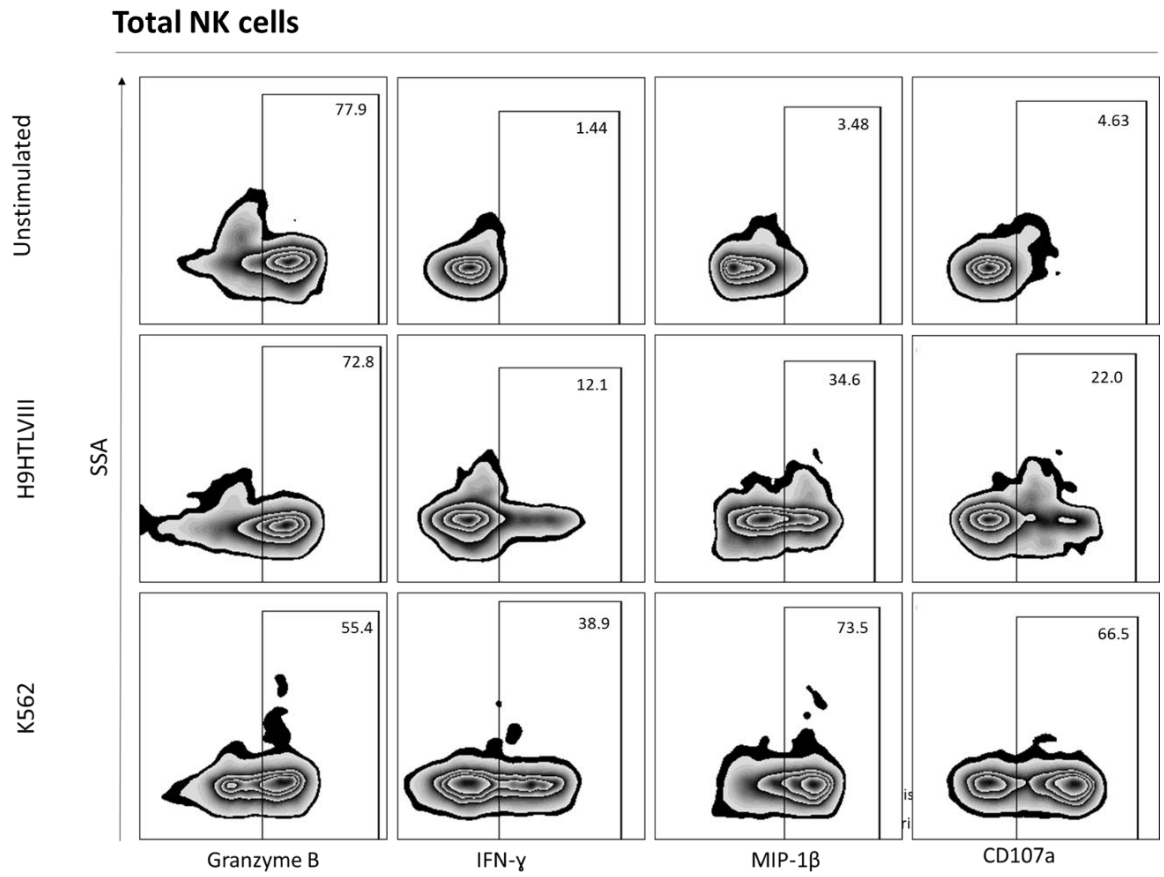


Figure 7. Gating strategies to NK cells activation markers with H9HTLVIII and K562 stimuli. Expression of activation markers in total NK cells before the stimulus and after 4h of H9HTLVIII and K562 co-culture.

There were no differences in the percentage of positive cells for effector molecules between both groups after H9HTLVIII stimulus (***Figure 8A***). However, in response to K562, high-risk MSM had a higher frequency of NK cells positive for IFN-ɣ at 4h post-culture (42.5±6.8 vs 32.6± 14.6 p=0.039) (***Figure 8B***).

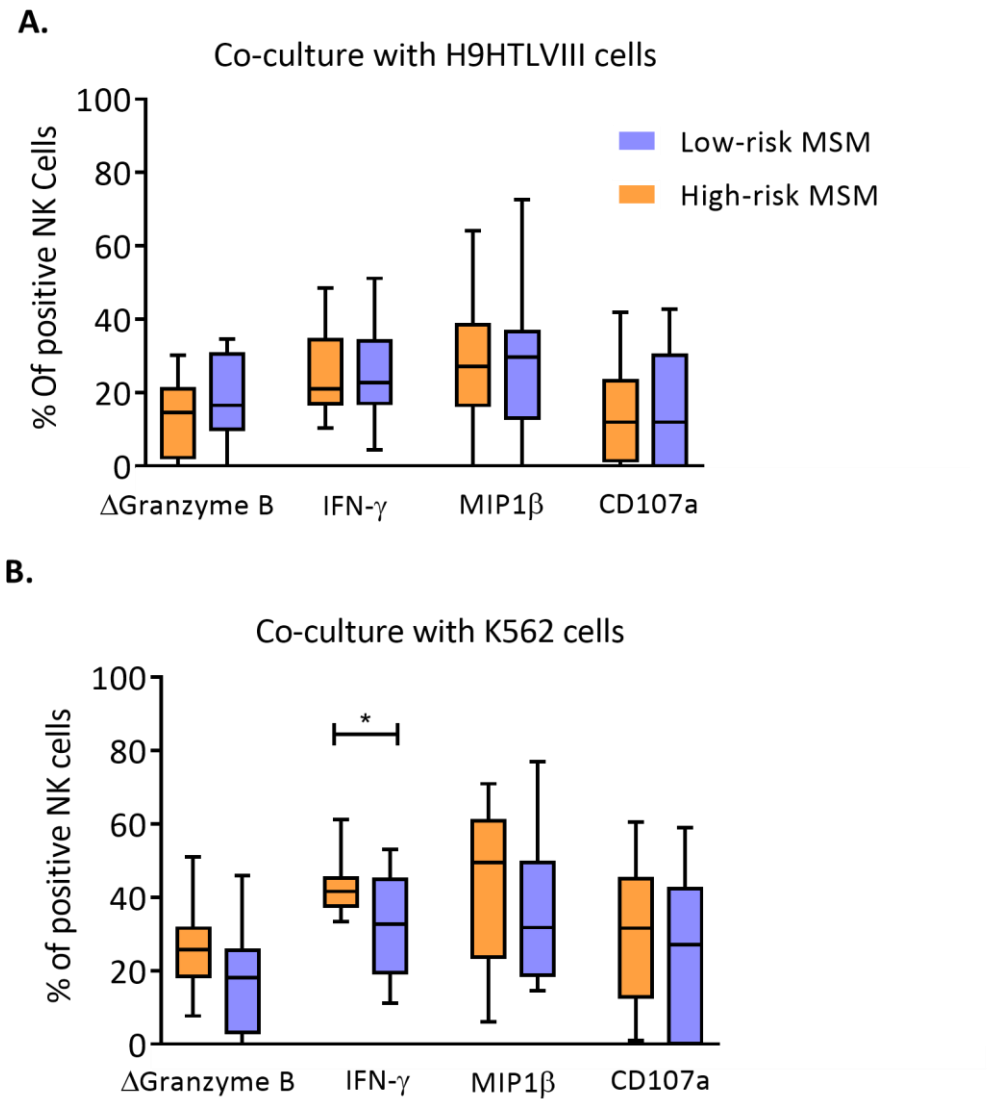


Figure 8. High-risk MSM shows a higher percentage of IFN-ɣ positive NK cells after K562 co-culture. A, B. Frequency of positive NK cells for effector molecules after 4h co-culture. Granzyme B is shown as ΔGranzyme B (ΔGranzyme B = % Granzyme B positive cells in unstimulated condition - % Granzyme B positive cells after H9HTLVIII or K562 stimuli). The subtraction represents the percentage of NK cells that degranulate Granzyme B in response to the stimulus. Line inside de box indicated mean and whiskers min to max value. n:15,10. Statistical evaluations were made with unpaired t-test. *p< 0.05.

Other effector molecules (MIP-1α, TNF-α, and RANTES) were evaluated inK562 co-culture supernatants by CBA. High-risk MSM showed higher MIP-1α production compared to low-risk MSM (616.4 vs 46.1 p=0.005) (***Figure 9A***). IFN-ɣ production was detected in 18.7% of high-risk MSM supernatants but was not detected in any low-risk supernatant (data not shown).

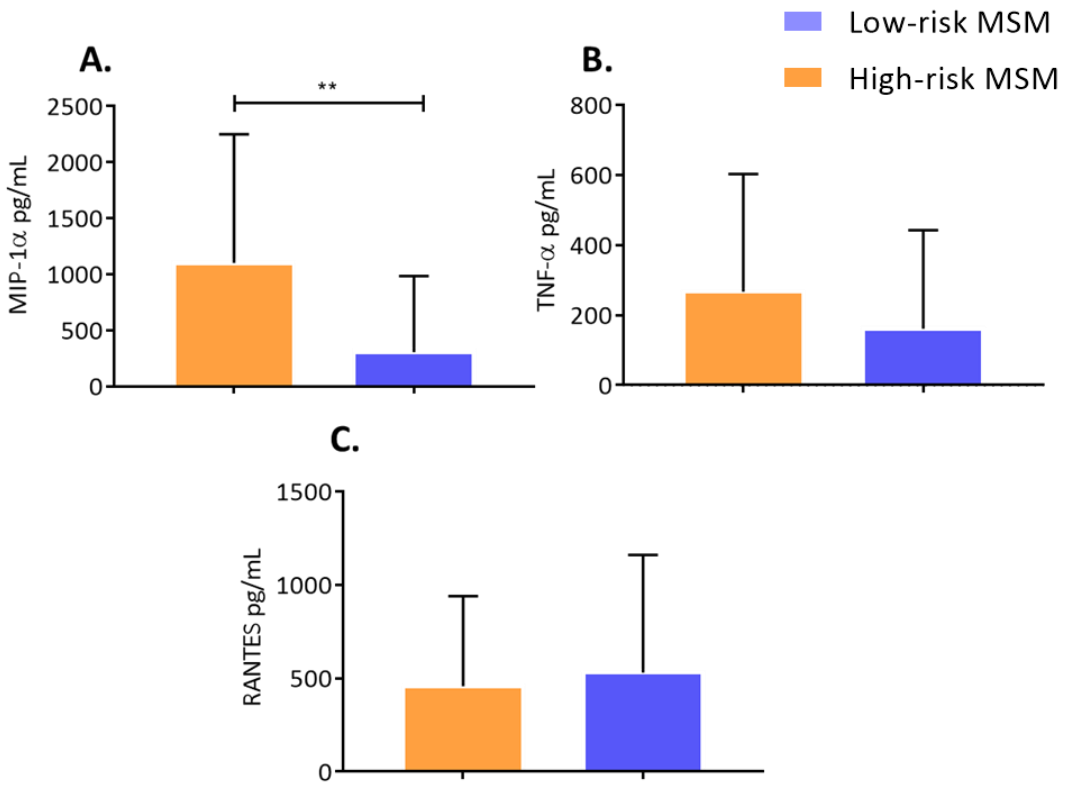


Figure 9. High-risk MSM produced higher amounts of MIP-1α after the K562 co-culture. A, B, and C. Concentration of effector molecules in the supernatant after 4h of PBMCs and K562 co-culture, soluble factors were measured by CBA and are expressed as pg/mL. Statistical evaluations were made with Mann-Whiney test. *p<0.05, and **p<0.01.

4.6 High-risk MSM showed distinct functional profile of NK cell than low-risk MSM

After the evaluation of single molecule production, the functional profile of NK cells was evaluated with the same stimulus, H9HTLVIII, and K562 cell lines. The measured molecules included the expression of CD107a, as degranulation marker, MIP-1β, and IFN-ɣ.

The results of the functional profile analysis showed a different NK cell population between both groups, which is characterized by $CD107a^{+}$/IFN-$ɣ^{+}$/MIP-$1β^{-}$ profile. These cells were more frequent in high-risk MSM compared with low-risk MSM with both H9HTLVIII (p=0.012) (***Figure 10A***) and K562 (p= 0.043) (***Figure 10B***) stimulus.

After, the MFI of IFN-ɣ in NK cells with different functional profiles was determined. The MFI of IFN-ɣ in NK cells with $CD107a^{+}$/IFN-$ɣ^{+}$/MIP-$1β^{-}$ profile, was higher in high-risk MSM compared to low-risk MSM (3499±1363 vs 2546±737 p=0.035) (***Figure 11B***). Differences in the MFI of IFN-ɣ was not observed in NK cells with other functional profiles between groups (***Figure 11A, C***). These results showed that high-risk MSM exhibit not only higher frequency but also major IFN-ɣ production in NK cells with this functional profile, which could explain their better functional activity.

.

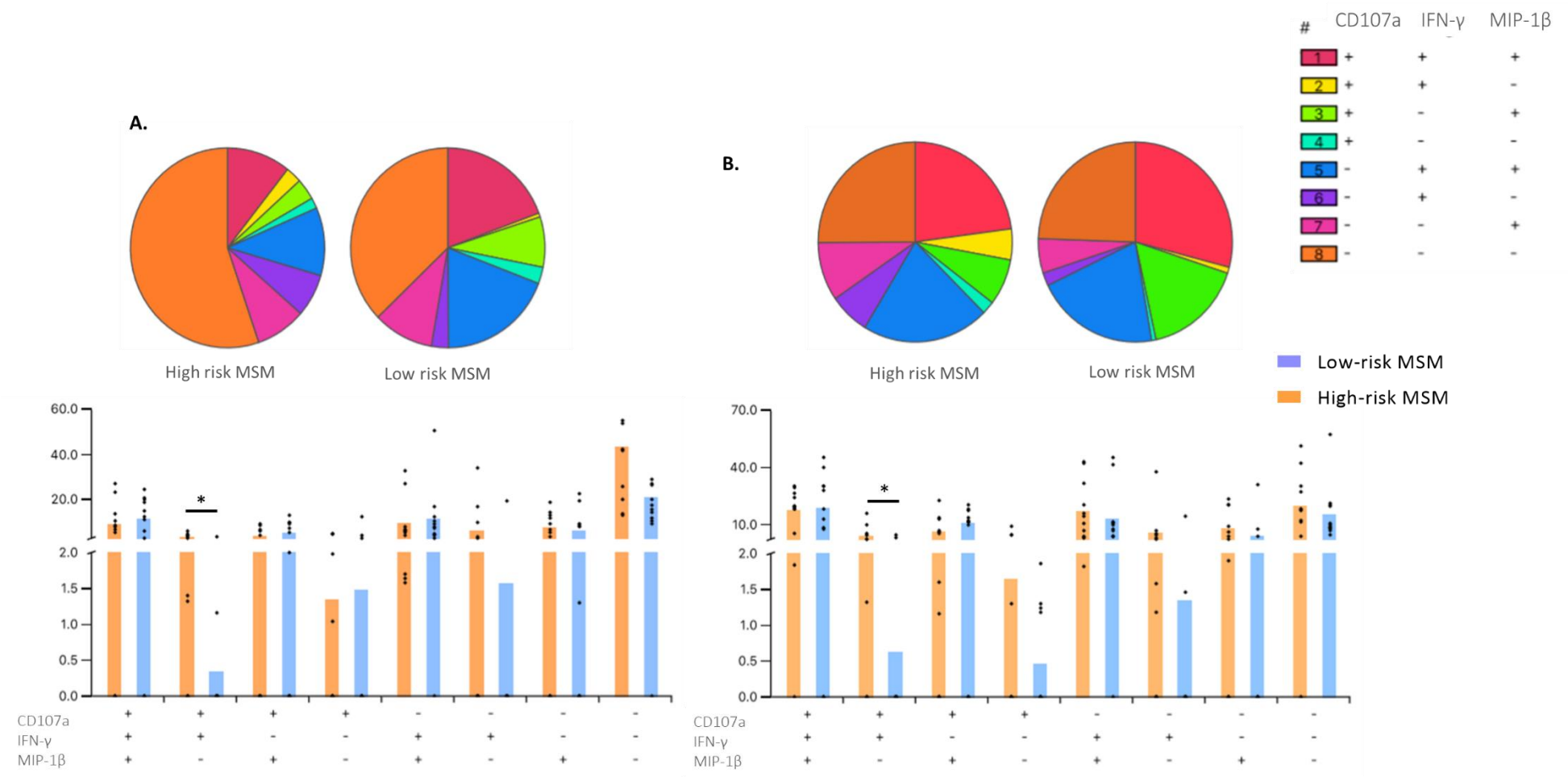


Figure 10. High-risk MSM exhibit a higher frequency of NK cells with CD107a^{+}/IFN-ɣ$^{+}$/MIP-1β^{-} profile A. Functional profile analysis of NK cells after co-culture with H9HTLVIII cell line **B.** Functional profile analysis of NK cells after co-culture with K562 cell line. The results are presented as mean. n:15,10. Statistical evaluations were made with unpaired t-test. *p< 0.05.

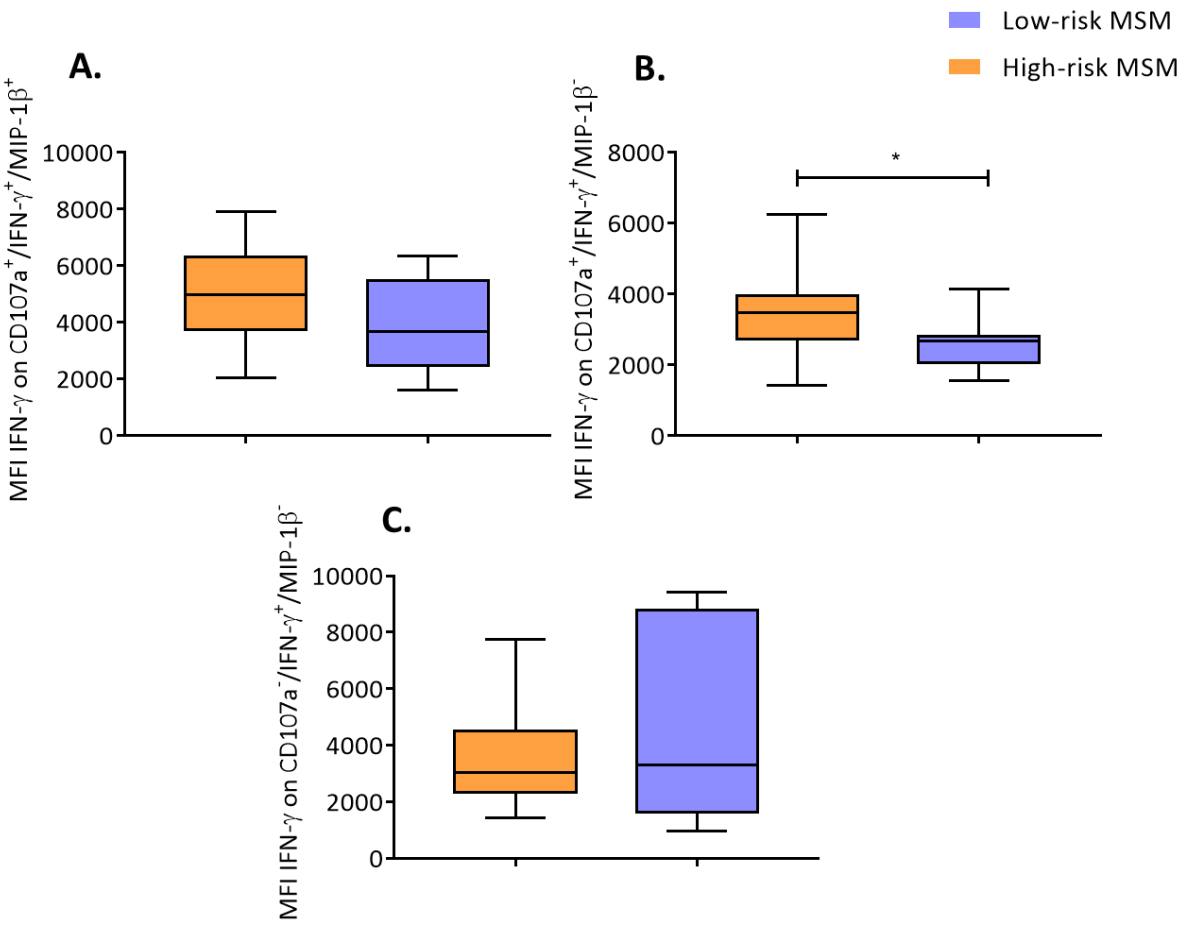


Figure 11. NK cells with CD107a+/IFN-ɣ+/MIP-1β- profile had higher MFI of IFN-ɣ in high-risk MSM. A, B, C. MFI of IFN-ɣ in $CD107a^{+}$/IFN-$ɣ^{+}$/MIP-$1β^{+}$, $CD107a^{+}$/IFN-$ɣ^{+}$/MIP-$1β^{-}$ and $CD107a^{-}$/IFN-$ɣ^{+}$/MIP-$1β^{-}$ NK cells respectively. Line inside de box indicated mean and whiskers min to max value. n:15,10. Statistical evaluations were made with unpaired t-test. *p< 0.05.

.

4.7 Basal mRNA of IFN-ɣ is higher in high-risk MSM than low-risk MSM

The relative expression of molecules associated with the effector function of NK cells was evaluated. Cytotoxic molecules (granzyme and perforin), β-chemokines (MIP-1α, MIP-1β, and RANTES), IFN-ɣ and TNF-α were evaluated together with PGK as a housekeeping gene.

mRNA levels of IFN-ɣ in PBMCs were higher in high-risk MSM that in low-risk MSM at basal state (0.07±0.06 and 0.04±0.03 p=0.040) (***Figure 12A***). IFN-ɣ transcripts levels were correlated with the frequency of memory-like NK cells (r=0.405, p=0.039) (***Figure 12B***).

No differences between groups were found when the relative expression of other molecules was evaluated (***Figure 12 C- H)***.

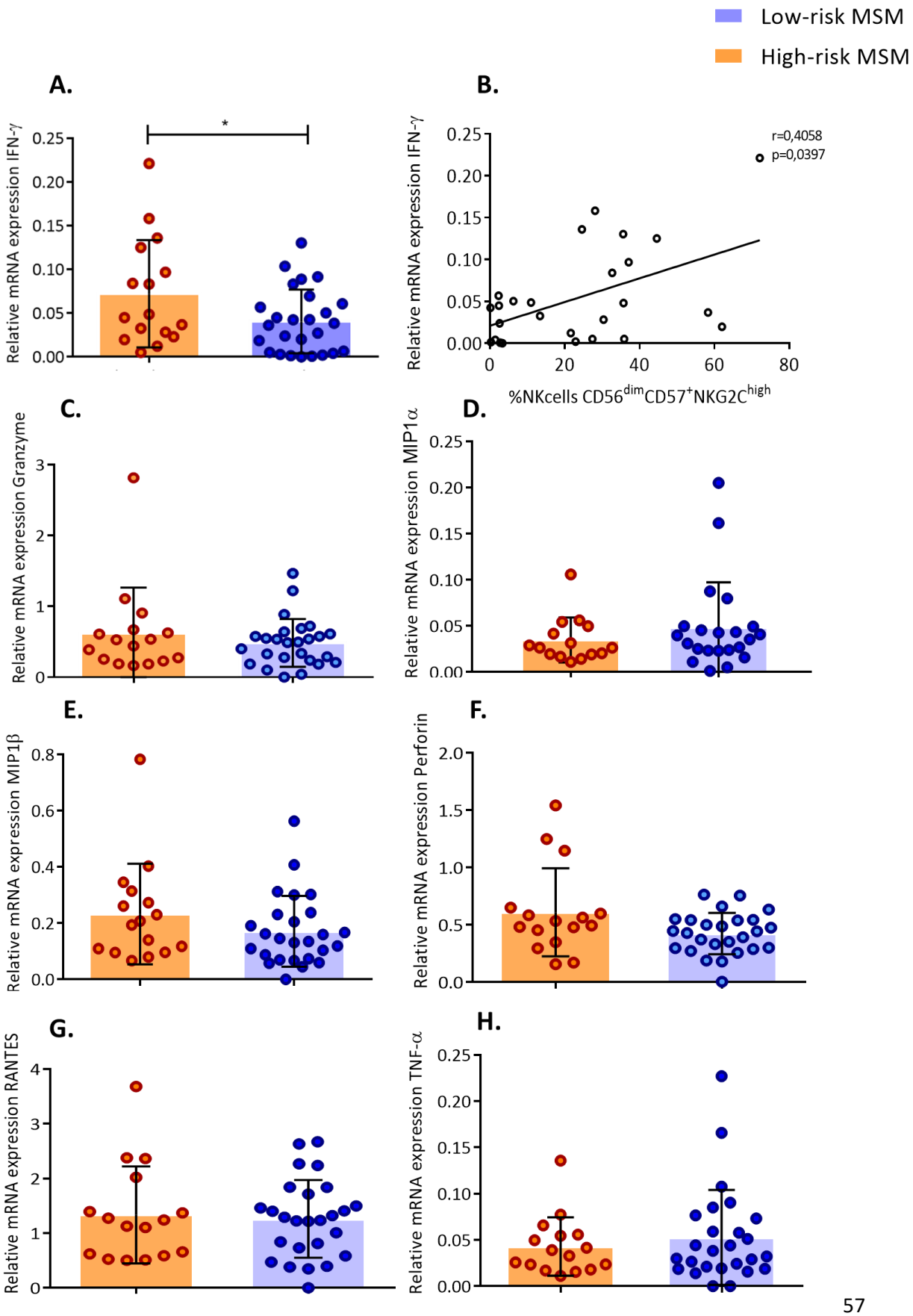

Low-risk MSM
High-risk MSM
A.
Relative mRNA expression IFN-γ
*
B.
Relative mRNA expression IFN-γ
r=0,4058
p=0,0397
%NKcells CD56dimCD57+NKG2Chigh
C.
Relative mRNA expression Granzyme
D.
Relative mRNA expression MIP1α
E.
Relative mRNA expression MIP1β
F.
Relative mRNA expression Perforin
G.
Relative mRNA expression RANTES
H.
Relative mRNA expression TNF-α

Figure 12. High-risk MSM has higher levels of IFN-ɣ mRNA at basal state. A. Relative mRNA expression of IFN-ɣ. n:16,26. Statistical evaluations were made with unpaired t-test. Results are shown as mean± SD, *p<0.05. **B.** Correlation of Relative mRNA expression of IFN-ɣ with the frequency of memory-like NK cells. Spearman´s correlation test. **C, D, E, F, G and H.** Relative mRNA expression of the other molecules evaluated, the results are shown as mean± SD. n:16,26. Statistical evaluations were made with unpaired t-test

4.8 KIR/HLA protective combination frequency

Finally, the frequency of HLA-B and KIR receptor alleles was determined (***Supplementary figures 2 and 3***). The combination of certain HLA alleles expressed at the same time with some KIR alleles has been associated with resistance to HIV infection. The frequency of protective combination KIR3DL1/DS1-HLA-B*Bw4 in homozygous state was established.

HLA-B alleles belonging to Bw4 act as KIR3DL1 ligand. Bw4 alleles were more frequent in high-risk MSM in both, heterozygous and homozygous states. Inhibitor KIR3DL1 receptor was also more frequent in high-risk MSM compared to low-risk MSM. The protective phenotype KIR3DL1/DS1-HLA-B*Bw4/Bw4 was present in 30.7% of high-risk MSM while this phenotype was present only in 8% of low-risk MSM. The HLA-B alleles HLA-B*18 and HLA-B*39 were enriched in high-risk MSMS groupThere were no differences between groups when individual KIR alleles were compared.

Table 2. Frequency of KIR/HLA alleles associated with protection*.

	High-risk MSM n=13 *% (n)*	Low-risk MSM n=25 *% (n)*	*OR*	*95% CI*	*p*
KIR3DS1	23 *(3)*	44 *(11)*	0.381	0.096 – 1.796	ns
KIR3DL1	92.3 *(12)*	88 *(22)*	1.636	0.219 -22.83	ns
KIR3DL1/DS1	92.3 *(12)*	96 *(24)*	0.5	0.025 -10.23	ns
HLA-B* Bw4	61.5 *(8)*	25 *(13)*	1.477	0.356 – 5.732	ns
HLA-B*Bw4/Bw4	30.7 *(4)*	8 *(2)*	5.111	0.953-29.05	ns
3DL1/DS1-B*Bw4/-	53.8 *(7)*	48 *(12)*	1.264	0.298-4.275	ns
3DL1/DS1-B*Bw4/Bw4	30.7 *(4)*	8 *(2)*	5.111	0.953-29.05	ns

*The frequency of each phenotype is shown as a percentage and OR indicating how many times is more probable to find that condition in high-risk MSM group, column *p* correspond to Fisher's exact test

5. DISCUSSION

NK cells have a crucial role in controlling HIV-1 infection, by not only eliminating infected cells through cytotoxic mechanisms, but also producing effector molecules involved in the induction of adaptive immune response, and viral inhibition by receptor blocking, among others. The studies herein provide information on combinatorial mechanisms mediated by NK cells, protecting against HIV infection in some high-risk populations. These data revealed important characteristics in NK cells phenotype and functional capacity, which could correlate with natural protection against HIV infection exhibited by high-risk MSM. In fact, it is the first report of increased NK cell activity in the MSM population.

The frequency of sexual partners in our high-risk MSM group overcome the numbers reported in previous studies of high-risk MSM, ranging from 3 to 6 different sexual partners in the past three months (95, 96). That frequency is considered a high-risk practice; indicating, that our high-risk population has similar or even higher exposure

than other MSM cohorts. The median of sexual partners in a lifetime indicates conserved high-risk practices during all sexual life. This fact, along with the high prevalence of STD and the fact that near to 60% of our high-risk group reported having at least one HIV positive sexual partner, reflects the magnitude of sexual exposure and immunological experience of this population.

One dilemma in high-risk sexual cohort studies is the selection of control subjects. Many studies include low risk (non-MSM) individuals as controls; however, it is important to mention that including this kind of controls does not allow to evaluate factors as repeated anal intercourse, allo-exposure (due to sperm contact), and STDs, that most likely affect baseline immunological parameters (14). In this study, low-risk MSM represents individuals from the same MSM community (considered globally as a high-risk population), with less infectious pressure, insufficient to be considered HIV resistant based on the previously mentioned factors.

Demographic data revealed that the frequency of *CCR5 Δ32* mutation in heterozygous sate in our high-risk population was similar to the frequency found in other MSM cohorts from United States (12.9%) and Italy (20%) (97, 98). These frequencies are higher than frequencies reported for general population (near to 2%) (99) and have been related to a certain degree of protection in HESN cohorts (97, 98) but no in general population (100). In 1998 *Paxton et al*. reported that $CD4^+$ T cells with reduced CCR5 expression from HESN individuals, exhibit a decreased susceptibility to HIV infection *in vitro* (101). In 2005 *Thomas et al.* reported that the time that MSM remain seronegative despite high-risk sexual behaviors was negative correlated to the expression of CCR5 on CD4+ T cells and monocytes (102). Together, this data suggests that the high frequency of *CCR5* Δ32 mutation in a heterozygous state, can contribute to the fact that high-risk MSM remains seronegative despite long periods of high-risk sexual behaviors. Especially if considered that *CCR5* Δ32 mutation in heterozygous state is one of the main mutations related to a reduced receptor density (103).

Evaluation of NK cells phenotype revealed higher frequencies of mature, terminally differentiated and memory NK cells in high-risk MSM. This population also showed

higher expression of the maturation marker CD57 in all NK cells subpopulations. It has been reported that NK cell maturation is a highly age-dependent process, where young people have high frequencies of $CD56^{bright}$ cells compared to older people that shows a higher frequency of $CD56^{dim}$ cells expressing maturation markers as CD57 (104, 105). However, it is not clear if this phenomenon is explained by an intrinsic ageing process or by a cumulative exposure throughout the lifetime. *Goodier et al*. reported that in a Gambian population with high-frequency of HCMV infection, children reached the percentage of terminally differentiated NK cells ($CD56^{dim}CD57^{+}NKG2C^{+}$) of an adult (near to 70%) at the age of six, while European population barely reached these numbers at adult age (near to 50%) (106). A similar phenomenon has been reported in transplant recipients, where $CD57^{+}NKG2C^{+}$ NK cells are detected within 3 months in patients who reactivated HCMV infection after transplantation; while these cells can take more than 1 year to emerge in patients who did not reactivate the infection (107). The expansion of $CD57^{+}NKG2C^{+}$ NK cells has been also reported in individuals infected with Hantavirus, Hepatitis B and C virus and Chikungunya virus (108-110). It is suggesting that exposure to infection is a significant determinant of NK cell maturation rates.

Some of the known factors explaining differences in mature and memory NK cells frequency between high-risk and low-risk MSM were evaluated. IgG titers against HCMV have been found to be positive correlated with the frequency of memory NK cells in HESN individuals (78); however, there was no difference in the HCMV IgG titers between our groups. High-risk MSM were older than low-risk MSM, though, there was no correlation between $CD56^{dim}CD57^{+}$ or $CD56^{dim}CD57^{+}NKG2C^{high}$ frequencies and age. Nevertheless, a strong correlation between $CD56^{dim}CD57^{+}NKG2C^{high}$ frequency and the number of sexual partners in the lifetime was found. The number of sexual partners was also strongly correlated with the MFI of NKG2C, the main activating receptor of memory NK cells, in the $CD56^{dim}$ population. These results suggest that the magnitude of exposure, measured as the number of sexual partners, instead of aging or HCMV infection, may be implicated in the expansion of memory NK cells observed in this population.

In NK cells, a more mature phenotype has been related to a better cytotoxic capacity (111, 112). In our study, high-risk MSM have indeed higher cytotoxicity against tumor cell line K562 compared to low-risk MSM. In fact, mature NK cells were positively correlated with the cytotoxic capacity, in both, frequency and density CD57 expression, which was correlated with cytotoxic capacity particularly in $CD56^{dim}CD16^{-}$ NK cells subpopulation, that are better responders against tumors (113).

The functional capacity of NK cells was also distinct in high-risk MSM. They exhibited a higher frequency of IFN-γ^{+} NK cells, and higher production of MIP-1α was also detected in supernatants of high-risk MSM co-cultures. High percentages of IFN -γ^{+} NK cells and β-chemokines production has been related to HIV protection in other HESN cohorts, including serodiscordant couples, UDIs and babies born to HIV-positive mothers (69-71); likewise, it has been related to delay progression to AIDS in LTNPs (114).

Functional profile analysis revealed the presence of an NK cell population with $CD107a^{+}$/IFN-γ^{+}/MIP-1β functional profile, which seems to be specialized in IFN-γ production. This population was more frequent in high-risk MSM and it exhibited a higher IFN-γ production capacity, compared to the same population in low-risk MSM. NK cells with $CD107a^{+}$/IFN-γ^{+} functional profile has been associated with better control of HIV infection (114,115). In 2013, *Jiang et al*. reported that LTNPs had higher frequencies of these cells compared to typical progressors and healthy controls (114). The higher frequency of NK cells with this functional profile has been also associated with lower viral load and lower CD4+ T cells slope (114, Chung, 2011 #1364). Our findings suggest that the high number of NK cells with $CD107a^{+}$/IFN-γ^{+} functional profile found in high-risk MSM plays a role in natural resistance against HIV.

Higher mRNA levels of IFN-γ were found in high-risk MSM. mRNA levels of IFN-γ were positively correlated with the number of memory NK cells. Along with phenotypic changes, memory NK cells suffer a series of epigenetic changes

including demethylation in the *IFNG* gene, resulting in an enhanced capacity of IFN-ɣ production (116), that was consistently observed in our functional analysis.

IFN-ɣ is involved in chronic immune activation that leads to immune exhaustion during the chronic phase, but it is required to establish an adequate antiviral response, early during the infection. There are several IFN-ɣ-induced proteins with antiviral activity, which can inhibit the synthesis of viral proteins, edit viral sequences, degraded RNA and impair transport of viral nucleocapsid to the nucleus that can explain why higher levels of IFN-ɣ are beneficial and related to protection in HESN individuals (117, 118).

This is the first report of high cytotoxic capacity as well as higher IFN-ɣ and MIP-1α production in high-risk MSM, and revealed these mechanisms are present in different cohorts of HESN individuals. However, a more mature phenotype and higher frequency of memory NK cells is a poorly reported feature in HESN individuals.

Higher frequencies of fully mature and memory NK cells has only been reported in a cohort serodiscordant couples from Brazil, with NK cells showing increased CD107a and IFN-ɣ expression (78). Thus, higher frequencies of memory NK cells present also in our group of study let to think these cells could be implicated in natural resistance to HIV infection.

Studies conducted on HIV positive individuals revealed a protective role of memory NK cells during HIV infection. In 2017, *Gondois-Rey et al.* reported that memory NK cells contributed to the control of HIV viremia during primary infection. In this study, high-risk MSM exhibited not only a more mature phenotype but also a higher frequency of memory NK cells, parameters that were correlated with lower HIV viremia, higher CD4+ T cell counts and a most rapid decrease of viremia after the instauration of antiretroviral treatment in infected patients (79). These results suggest memory NK cells can contribute not only to slow progression but also with resistance to HIV infection.

It has been extensively reported that NK cells need priming to acquire functional competence. In fact, NK cells from specific pathogen-free mice exhibit poor effector functions. For acquiring adequate cytotoxicity, NK cells require cytokines produced by APCs , such as type I IFN-, IL-12, IL-15, and IL-18, (119-121). Although the intensity and time of the proinflammatory stimulus necessary to induce NK cell priming is unknown, experimental models of persistent viral infections have shown that lower but persistent levels of proinflammatory cytokines induce NK cell activation for long periods, where NK cells display enhanced cytotoxicity, higher expression of granzyme B and IFN-ɣ production (122, 123). Similar results have been observed after *bacillus Calmette-Guérin* (BCG), yellow fever virus and influenza vaccination. In both cases, proinflammatory stimuli primes NK cells, resulting in increased cytokine production following *ex vivo* restimulation with either, challenging or other unrelated pathogen (124-126). High sexual exposition of high-risk MSM may be driving the required environment for the priming process, explaining the enhanced citotoxity and IFN- ɣ production found in experienced NK cells population.

Although proinflammatory stimulus may explain some long-term adaptations in conventional NK cells, different adaptations occur in the case of memory NK cells. Memory NK cells response to a proinflammatory stimulus is low or absent due to low expression of cytokine receptors. This has been probed after influenza or yellow fever vaccination that fail to induce response on memory NK cells (125). However, some infections like hantavirus, chikungunya an HIV are effective in activating or expanding the memory NK cells pool generated after HCMV infection (108, 109, 127). Although the mechanism underlining this phenomenon is unclear, some of these responses have been attributable to elevated HLA-E expression (108).

Upregulation of HLA-E, the ligand of NKG2C, is a common feature found in several viral infections like dengue, Hantavirus, and HIV, among others (108, 128, 129). Engagement of NKG2C, the signature activating receptor of memory NK cells, leads to polyfunctional responses characterized by degranulation of cytolytic molecules as well as TNF-α and IFN-ɣ (109). Thereby, different from other NK cells activating

receptors, cross-linking of NKG2C alone, is sufficient to drive IFN-ɣ production in memory NK cells (130), contradicting previously described minimal requirements of NK cells activation (131). When the frequency of STD was evaluated according to the nature of the causing agent, high-risk MSM showed lower frequencies of viral STD but no bacterial. The reduced frequency of viral STD in high-risk MSM could be potentially explained by this phenomenon, where memory NK cells, activated trough NKG2C by HLA-E, whose expression is conserved and even upregulated in several viral infections, induces a strong antiviral response mediated by memory NK cells that can protect these individuals against a variety of viral infections including HIV.

In both cases, higher frequencies of experienced conventional NK cells and memory NK cells indicate that exists a well-trained and powerful army of effector cells that can mediate a quick and strong response against a variety of pathogens. This phenomenon has been also reported in cancer, where patients, undergoing human stem cells transplantation, have reduced relapse when donor or recipient are seropositive to HCMV before transplantation due to memory NK cells expansion (132, 133). In line with these results, in 2009 *Nguyen et al.* reported that cross-reactive recognition of HLA-E leukemic blast by memory NK cells could contribute to the eradication of minimal residual disease (134). A similar phenomenon can be observed in our cohort of high-risk MSM were fully mature and memory NK cells might be playing an important role in the context of natural resistance to HIV infection. Either by memory responses, generated after HIV contact or by heterologous responses, generated by high exposure to other pathogens, NK cells might mediate an adequate control, avoiding the establishment of HIV infection.

In addition to particular phenotypic and functional features, some genetic characteristics were found to be different between groups. High-risk MSM showed higher frequencies of HLA-B*18 and HLA-B*39 alleles compared to low-risk MSM. Besides, the protective phenotype of KIR3DL1/S1 in combination with HLA-Bw4 in homozygous state was found in 30.7% of high-risk MSM compared to 8% in low-risk MSM. HLA-B*18 allele has been associated with protection in different cohorts of HIV-exposed individuals. In babies born to seropositive mothers, the frequencies of

the HLA-B*18 allele were associated with a significantly lower risk of early HIV-1 transmission via breastfeeding (135). HLA-B*18 has been also related to HIV protection in serodiscordant couples (136). Although with less frequency, HLA-B*39 allele has been related to HIV protection in some studies (137, 138). However, there are some contradictory results, most likely associated with subtypes of this allele, such as HLA-B*39:02 and HLA-B*39:01, which has been related to protection and risk, respectively (137).

NK cells recognize self-HLA trough KIR receptors, generating an inhibitory signal preventing autoagression. Down-regulation of HLA molecules at the cell membrane, a common phenomenon in viral infections, allows NK cells activation by “missing-self recognition” (139).

HLA alleles that contain the public Bw4 epitope act as KIR ligands, while alleles with Bw6 epitope do not. KIR3DL1/S1^{+} NK cells educated in the context of Bw4 alleles exhibit stronger capacity to kill HIV-1-infected cells, and, consequently, patients with KIR3DL1/S1^{+} NK cells experience better clinical outcomes during the course of the infection (140-143). Boudreau *et al.* reported that KIR3DL1 and Bw4-80I (Bw4 alleles with an isoleucine at position 80) partnerships endow NK cells with the greatest reactivity against HLA negative targets; whereas NK cells exhibiting KIR3DL1 in combination with HLA-Bw4 no 80I, demonstrated intermediate responsiveness; and Bw4^{-}/KIR3DL1+ NK cells are poorly responsive (141). Differences in NK cells effector capacity are explained by the inhibition capacity of each KIR ligand; studies carried out in the context of these alleles, expressed together, showed that Bw4 alleles (particularly 80I) have high inhibition ability on NK cells.

During HIV infection, the nef protein induces potential target for KIR3DL1^{+} NK cells by down-regulating HLA-B expression, due to the absence of a strong inhibitory signal (144). These findings reveal that the degree of NK cells inhibition, mediated by certain HLA, might predict the degree of licensing and functional responsiveness of NK cells in the absence of that signal. Regarding KIR3DS1 receptor ligand has been described that it does not engage Bw4 molecules on neighboring cells;

however, specific peptides, including those from HIV, may facilitate engagement of KIR3DS1 by Bw4-80I (145).

The evidence support why the expression of HLA-Bw4 alleles in combination with KIR3DL1/S1 can be associated with resistance to both progression and HIV infection in different HESN cohorts including this one (60-62).

4. CONCLUDING REMARKS

Increased NK cells effector capacity observed in high-risk MSM was related to the mature phenotype exhibited by this population. High sexual exposition may be driving the changes observed in this population resulting in higher numbers of fully mature trained NK cells and memory NK cells.

Fully mature NK cells and memory NK represents an army of well-trained cells that can respond quick and strong against a variety of pathogens well responding to proinflammatory state or particular conditions as up-regulation of HLA-E, conserved feature among viral infections and tumors. These modifications generated in the NK cell compartment can explain why these individuals remain seronegative despite long periods of high-risk sexual behavior and open an interrogate about how NK cells can be primed and trained to induce this mature phenotype that seems to be protective against a variety of pathogens.

5. LIMITATIONS AND FUTURE PERSPECTIVES

- It would have been interesting to evaluate other mechanisms of killing mediated by NK cells as ADCC. Although this mechanism would not explain the natural resistance to HIV-1 infection, due to the absence of HIV-specific antibodies in MSM population. ADCC has shown to be an important immune mechanism in HIV infection, particularly after the RV144 assay where ADCC was one of the differential features between individuals who became infected and those who did not. It would reveal if changes observed in high-risk MSM like the more mature phenotype and higher memory NK cells frequency would be affecting other functional ways.

- The evaluation of functional features was limited to the stimulus with K562 cells. Although K562 cells represent a stimulus widely used for functional assays, it is not possible to evaluate the effect of NK cell education via HLA/KIR or HIV-specific response with this particular stimulus. For that reason, a functional in vitro assay with autologous $CD4^+$ T cells infected with HIV would be interesting to include both parameters in the analysis. The evaluation of cytotoxic capacity in co culture with autologous CD4+T cells allow to evaluate the effect of HLA-B*Bw4 in NK cells education. Besides that will be a more realistic scenario to study the response of NK cells against HIV infected cells.
- All results presented in this study were obtained from peripheral blood samples. To Include the GALT samples would bring valuable information about phenotypic and functional features of NK cells present in the tissue that represents the main point of entry of HIV in this particular population.
- Genotyping only HLA-B alleles result in a limited knowledge about how HLA/KIR interaction can be involved in NK cells functional capacity. This study has no information about HLA-A or HLA-C alleles present in the population, which could be implicated in natural resistance to HIV infection. Besides limit the knowledge of real frequency of protective phenotype KIR3DL1/S1-Bw4, because there are also some HLA-A alleles that belong to Bw4 group. Knowing the complete haplotype of this population would lead to a better knowledge of resistance associated alleles and combinations that can be related to the fact these individuals remain seronegative.

6. REFERENCES

1. UNAIDS. Global HIV & AIDS statistics — 2018 fact sheet 2018 [Available from: http://www.unaids.org/en/resources/fact-sheet.
2. Social MdSyP. Cuenta de Alto Costo- Fondo Colombiano de Enfermedades de Alto Costo 2018 [Available from: http://www.cuentadealtocosto.org/.
3. AIDS. COMMUNITIES AT THE CENTRE THE RESPONSE TO HIV IN LATIN AMERICA. 2019.

4. (UNFPA) FdpdlNU. COMPORTAMIENTO SEXUAL Y PREVALENCIA DE VIH EN HOMBRES QUE TIENEN RELACIONES SEXUALES CON HOMBRES EN SIETE CIUDADES DE COLOMBIA In: Social MdSyP, editor. 2011.
5. Ministerio de Salud y Protección S. Informe GARPR 2014 - Seguimiento de la declaración de compromiso sobre el VIH/SIDA. Ministerio de Salud y Protección Social República de Colombia. 2014:1-71.
6. Shaw GM, Hunter E. HIV transmission. Cold Spring Harb Perspect Med. 2012;2(11).
7. Lawn SD, Butera ST, Folks TM. Contribution of immune activation to the pathogenesis and transmission of human immunodeficiency virus type 1 infection. Clin Microbiol Rev. 2001;14(4):753-77, table of contents.
8. Hong HS, Eberhard JM, Keudel P, Bollmann BA, Ahmad F, Ballmaier M, et al. Phenotypically and functionally distinct subsets contribute to the expansion of CD56-/CD16+ natural killer cells in HIV infection. AIDS (London, England). 2010;24(12):1823-34.
9. Hong HS, Eberhard JM, Keudel P, Bollmann BA, Ballmaier M, Bhatnagar N, et al. HIV infection is associated with a preferential decline in less-differentiated CD56dim CD16+ NK cells. Journal of virology. 2010;84(2):1183-8.
10. Peretz Y, He Z, Shi Y, Yassine-Diab B, Goulet JP, Bordi R, et al. CD160 and PD-1 co-expression on HIV-specific CD8 T cells defines a subset with advanced dysfunction. PLoS Pathog. 2012;8(8):e1002840.
11. Levy JA. HIV pathogenesis: 25 years of progress and persistent challenges. AIDS. 2009;23(2):147-60.
12. Gaardbo JC, Hartling HJ, Ronit A, Thorsteinsson K, Madsen HO, Springborg K, et al. Different immunological phenotypes associated with preserved CD4+ T cell counts in HIV-infected controllers and viremic long term non-progressors. PLoS One. 2013;8(5):e63744.
13. Chun TW, Shawn Justement J, Murray D, Kim CJ, Blazkova J, Hallahan CW, et al. Effect of antiretroviral therapy on HIV reservoirs in elite controllers. J Infect Dis. 2013;208(9):1443-7.
14. Horton RE, McLaren PJ, Fowke K, Kimani J, Ball TB. Cohorts for the Study of HIV-1–Exposed but Uninfected Individuals: Benefits and Limitations. The Journal of Infectious Diseases. 2010;202(S3):S377-S81.
15. Fenizia C, Rossignol JF, Clerici M, Biasin M. Genetic and immune determinants of immune activation in HIV-exposed seronegative individuals and their role in protection against HIV infection. Infect Genet Evol. 2018;66:325-34.
16. Ranki A, Mattinen S, Yarchoan R, Broder S, Ghrayeb J, Lahdevirta J, et al. T-cell response towards HIV in infected individuals with and without zidovudine therapy, and in HIV-exposed sexual partners. AIDS. 1989;3(2):63-9.
17. Fowke KR, Nagelkerke NJ, Kimani J, Simonsen JN, Anzala AO, Bwayo JJ, et al. Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. Lancet. 1996;348(9038):1347-51.
18. NAIROBI CITY COUNTY HIV & AIDS STRATEGIC PLAN 2018 [Available from: https://nacc.or.ke/mdocs-posts/nairobi-county-hiv-aids-strategic-plan/.
19. UNAIDS DATA 2018 [Internet]. 2018. Available from: http://www.unaids.org/sites/default/files/media_asset/unaids-data-2018_en.pdf.
20. Patel P, Borkowf CB, Brooks JT, Lasry A, Lansky A, Mermin J. Estimating per-act HIV transmission risk: a systematic review. AIDS. 2014;28(10):1509-19.
21. Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell. 1996;86(3):367-77.
22. Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5

structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. Science. 1996;273(5283):1856-62.
23. Galvani AP, Novembre J. The evolutionary history of the CCR5-Delta32 HIV-resistance mutation. Microbes Infect. 2005;7(2):302-9.
24. Diaz FJ, Vega JA, Patino PJ, Bedoya G, Nagles J, Villegas C, et al. Frequency of CCR5 delta-32 mutation in human immunodeficiency virus (HIV)-seropositive and HIV-exposed seronegative individuals and in general population of Medellin, Colombia. Mem Inst Oswaldo Cruz. 2000;95(2):237-42.
25. Kaul R, Plummer FA, Kimani J, Dong T, Kiama P, Rostron T, et al. HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi. J Immunol. 2000;164(3):1602-11.
26. Kuhn L, Meddows-Taylor S, Gray G, Tiemessen C. Human immunodeficiency virus (HIV)-specific cellular immune responses in newborns exposed to HIV in utero. Clin Infect Dis. 2002;34(2):267-76.
27. Pinto LA, Sullivan J, Berzofsky JA, Clerici M, Kessler HA, Landay AL, et al. ENV-specific cytotoxic T lymphocyte responses in HIV seronegative health care workers occupationally exposed to HIV-contaminated body fluids. The Journal of clinical investigation. 1995;96(2):867-76.
28. Kaul R, Trabattoni D, Bwayo JJ, Arienti D, Zagliani A, Mwangi FM, et al. HIV-1-specific mucosal IgA in a cohort of HIV-1-resistant Kenyan sex workers. AIDS. 1999;13(1):23-9.
29. Devito C, Hinkula J, Kaul R, Lopalco L, Bwayo JJ, Plummer F, et al. Mucosal and plasma IgA from HIV-exposed seronegative individuals neutralize a primary HIV-1 isolate. AIDS. 2000;14(13):1917-20.
30. Watkins JD, Sholukh AM, Mukhtar MM, Siddappa NB, Lakhashe SK, Kim M, et al. Anti-HIV IgA isotypes: differential virion capture and inhibition of transcytosis are linked to prevention of mucosal R5 SHIV transmission. AIDS. 2013;27(9):F13-20.
31. DeVico AL, Gallo RC. Control of HIV-1 infection by soluble factors of the immune response. Nat Rev Microbiol. 2004;2(5):401-13.
32. Zapata W, Aguilar-Jimenez W, Feng Z, Weinberg A, Russo A, Potenza N, et al. Identification of innate immune antiretroviral factors during in vivo and in vitro exposure to HIV-1. Microbes Infect. 2016;18(3):211-9.
33. Quinones-Mateu ME, Lederman MM, Feng Z, Chakraborty B, Weber J, Rangel HR, et al. Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication. AIDS. 2003;17(16):F39-48.
34. Zapata W, Rodriguez B, Weber J, Estrada H, Quinones-Mateu M, Zimermman P, et al. Increased Levels of Human Beta-Defensins mRNA in Sexually HIV-1 Exposed But Uninfected Individuals. Current HIV Research. 2008;6(6):531-8.
35. Pace BT, Lackner AA, Porter E, Pahar B. The Role of Defensins in HIV Pathogenesis. Mediators Inflamm. 2017;2017:5186904.
36. Tugizov SM, Herrera R, Veluppillai P, Greenspan D, Soros V, Greene WC, et al. HIV is inactivated after transepithelial migration via adult oral epithelial cells but not fetal epithelial cells. Virology. 2011;409(2):211-22.
37. Biasin M, Piacentini L, Lo Caputo S, Kanari Y, Magri G, Trabattoni D, et al. Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G: a possible role in the resistance to HIV of HIV-exposed seronegative individuals. J Infect Dis. 2007;195(7):960-4.
38. Cagliani R, Riva S, Fumagalli M, Biasin M, Caputo SL, Mazzotta F, et al. A positively selected APOBEC3H haplotype is associated with natural resistance to HIV-1 infection. Evolution. 2011;65(11):3311-22.

39. Mussil B, Sauermann U, Motzkus D, Stahl-Hennig C, Sopper S. Increased APOBEC3G and APOBEC3F expression is associated with low viral load and prolonged survival in simian immunodeficiency virus infected rhesus monkeys. Retrovirology. 2011;8:77.
40. Farquhar C, VanCott TC, Mbori-Ngacha DA, Horani L, Bosire RK, Kreiss JK, et al. Salivary secretory leukocyte protease inhibitor is associated with reduced transmission of human immunodeficiency virus type 1 through breast milk. J Infect Dis. 2002;186(8):1173-6.
41. Pillay K, Coutsoudis A, Agadzi-Naqvi AK, Kuhn L, Coovadia HM, Janoff EN. Secretory leukocyte protease inhibitor in vaginal fluids and perinatal human immunodeficiency virus type 1 transmission. J Infect Dis. 2001;183(4):653-6.
42. Anokhin VV, Bakhteeva LB, Khasanova GR, Khaiboullina SF, Martynova EV, Tillett RL, et al. Previously Unidentified Single Nucleotide Polymorphisms in HIV/AIDS Cases Associate with Clinical Parameters and Disease Progression. Biomed Res Int. 2016;2016:2742648.
43. Gonzalez SM, Taborda NA, Feria MG, Arcia D, Aguilar-Jimenez W, Zapata W, et al. High Expression of Antiviral Proteins in Mucosa from Individuals Exhibiting Resistance to Human Immunodeficiency Virus. PLoS One. 2015;10(6):e0131139.
44. Drannik AG, Nag K, Yao XD, Henrick BM, Ball TB, Plummer FA, et al. Anti-HIV-1 activity of elafin depends on its nuclear localization and altered innate immune activation in female genital epithelial cells. PLoS One. 2012;7(12):e52738.
45. Bedoya VI, Boasso A, Hardy AW, Rybak S, Shearer GM, Rugeles MT. Ribonucleases in HIV type 1 inhibition: effect of recombinant RNases on infection of primary T cells and immune activation-induced RNase gene and protein expression. AIDS Res Hum Retroviruses. 2006;22(9):897-907.
46. Zapata W, Aguilar-Jiménez W, Feng Z, Weinberg A, Russo A, Potenza N, et al. Identification of innate immune antiretroviral factors during in vivo and in vitro exposure to HIV-1. Microbes and Infection. 2016;18(3):211-9.
47. Appay V, Kelleher AD. Immune activation and immune aging in HIV infection. Current Opinion in HIV and AIDS. 2016;11(2):242-9.
48. Appay V, Sauce D. Immune activation and inflammation in HIV-1 infection: causes and consequences. The Journal of Pathology. 2008;214(2):231-41.
49. Card CM, Blake Ball T, Fowke KR. Immune quiescence: a model of protection against HIV infection. 2013.
50. Fulcher JA, Romas L, Hoffman JC, Elliott J, Saunders T, Burgener AD, et al. Highly Human Immunodeficiency Virus-Exposed Seronegative Men Have Lower Mucosal Innate Immune Reactivity. AIDS research and human retroviruses. 2017;33(8):788-95.
51. Yao XD, Omange RW, Henrick BM, Lester RT, Kimani J, Ball TB, et al. Acting locally: innate mucosal immunity in resistance to HIV-1 infection in Kenyan commercial sex workers. Mucosal Immunology. 2014;7(2):268-79.
52. Taborda NA, Hernández JC, Lajoie J, Juno JA, Kimani J, Rugeles MT, et al. Short Communication: Low Expression of Activation and Inhibitory Molecules on NK Cells and CD4(+) T Cells Is Associated with Viral Control. AIDS research and human retroviruses. 2015;31(6):636-40.
53. Card Catherine M, McLaren Paul J, Wachihi C, Kimani J, Plummer Francis A, Fowke Keith R. Decreased Immune Activation in Resistance to HIV-1 Infection Is Associated with an Elevated Frequency of CD4 ⁺ CD25 ⁺ FOXP3 ⁺ Regulatory T Cells. The Journal of Infectious Diseases. 2009;199(9):1318-22.
54. Abdulhaqq SA, Zorrilla C, Kang G, Yin X, Tamayo V, Seaton KE, et al. HIV-1-negative female sex workers sustain high cervical IFNε, low immune activation and low expression of HIV-1-required host genes. Mucosal Immunology. 2016;9(4):1027-38.

55. McLaren Paul J, Ball TB, Wachihi C, Jaoko W, Kelvin David J, Danesh A, et al. HIV-Exposed Seronegative Commercial Sex Workers Show a Quiescent Phenotype in the CD4 ⁺ T Cell Compartment and Reduced Expression of HIV-Dependent Host Factors. The Journal of Infectious Diseases. 2010;202(S3):S339-S44.
56. Songok EM, Luo M, Liang B, McLaren P, Kaefer N, Apidi W, et al. Microarray analysis of HIV resistant female sex workers reveal a gene expression signature pattern reminiscent of a lowered immune activation state. PLoS One. 2012;7(1):e30048.
57. Biasin M, Caputo Sergio L, Speciale L, Colombo F, Racioppi L, Zagliani A, et al. Mucosal and Systemic Immune Activation Is Present in Human Immunodeficiency Virus–Exposed Seronegative Women. The Journal of Infectious Diseases. 2000;182(5):1365-74.
58. Tomescu C, Seaton KE, Smith P, Taylor M, Tomaras GD, Metzger DS, et al. Innate activation of MDC and NK cells in high-risk HIV-1-exposed seronegative IV-drug users who share needles when compared with low-risk nonsharing IV-drug user controls. Journal of acquired immune deficiency syndromes (1999). 2015;68(3):264-73.
59. Kuebler PJ, Mehrotra ML, Shaw BI, Leadabrand KS, Milush JM, York VA, et al. Persistent HIV Type 1 Seronegative Status Is Associated With Lower CD8+ T-Cell Activation. J Infect Dis. 2016;213(4):569-73.
60. Flores-Villanueva PO, Yunis EJ, Delgado JC, Vittinghoff E, Buchbinder S, Leung JY, et al. Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity. Proc Natl Acad Sci U S A. 2001;98(9):5140-5.
61. Jiang Y, Chen O, Cui C, Zhao B, Han X, Zhang Z, et al. KIR3DS1/L1 and HLA-Bw4-80I are associated with HIV disease progression among HIV typical progressors and long-term nonprogressors. BMC Infect Dis. 2013;13:405.
62. Habegger de Sorrentino A, Sinchi JL, Marinic K, Lopez R, Iliovich E. KIR-HLA-A and B alleles of the Bw4 epitope against HIV infection in discordant heterosexual couples in Chaco Argentina. Immunology. 2013;140(2):273-9.
63. Boulet S, Kleyman M, Kim JY, Kamya P, Sharafi S, Simic N, et al. A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection. AIDS (London, England). 2008;22(12):1487-91.
64. Jennes W, Verheyden S, Demanet C, Adje-Toure CA, Vuylsteke B, Nkengasong JN, et al. Cutting edge: resistance to HIV-1 infection among African female sex workers is associated with inhibitory KIR in the absence of their HLA ligands. J Immunol. 2006;177(10):6588-92.
65. Altfeld M, Addo MM, Rosenberg ES, Hecht FM, Lee PK, Vogel M, et al. Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. AIDS. 2003;17(18):2581-91.
66. Brockman MA, Schneidewind A, Lahaie M, Schmidt A, Miura T, Desouza I, et al. Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A. J Virol. 2007;81(22):12608-18.
67. den Uyl D, van der Horst-Bruinsma IE, van Agtmael M. Progression of HIV to AIDS: a protective role for HLA-B27? AIDS Rev. 2004;6(2):89-96.
68. Leslie A, Matthews PC, Listgarten J, Carlson JM, Kadie C, Ndung'u T, et al. Additive contribution of HLA class I alleles in the immune control of HIV-1 infection. J Virol. 2010;84(19):9879-88.
69. Scott-Algara D, Truong LX, Versmisse P, David A, Luong TT, Nguyen NV, et al. Cutting edge: increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravascular drug users. Journal of immunology (Baltimore, Md : 1950). 2003;171(11):5663-7.
70. Lohman-Payne B, Slyker JA, Moore S, Maleche-Obimbo E, Wamalwa DC, Richardson BA, et al. Breast milk cellular HIV-specific interferon γ responses are associated

with protection from peripartum HIV transmission. AIDS (London, England). 2012;26(16):2007-16.
71. Montoya CJ, Velilla PA, Chougnet C, Landay AL, Rugeles MT. Increased IFN-gamma production by NK and CD3+/CD56+ cells in sexually HIV-1-exposed but uninfected individuals. Clinical immunology (Orlando, Fla). 2006;120(2):138-46.
72. Quillay H, Costa HE, Durie M, Marlin R, Cannou C, Madec Y, et al. NK cells control HIV-1 infection of macrophages through soluble factors and cellular contacts in the human decidua. Retrovirology. 2016;13(39).
73. Vega JA, Villegas-Ospina S, Aguilar-Jiménez W, Rugeles MT, Bedoya G, Zapata W, et al. Haplotypes in CCR5-CCR2, CCL3 and CCL5 are associated with natural resistance to HIV-1 infection in a Colombian cohort. Biomédica. 2017;37(2).
74. Chung AW, Kumar MP, Arnold KB, Yu WH, Schoen MK, Dunphy LJ, et al. Dissecting Polyclonal Vaccine-Induced Humoral Immunity against HIV Using Systems Serology. Cell. 2015;163(4):988-98.
75. Ackerman ME, Mikhailova A, Brown EP, Dowell KG, Walker BD, Bailey-Kellogg C, et al. Polyfunctional HIV-Specific Antibody Responses Are Associated with Spontaneous HIV Control. PLOS Pathogens. 2016;12(1):e1005315-e.
76. O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. T cell– and B cell–independent adaptive immunity mediated by natural killer cells. Nature Immunology. 2006;7(5):507-16.
77. Reeves RK, Li H, Jost S, Blass E, Li H, Schafer JL, et al. Antigen-specific NK cell memory in rhesus macaques. Nature immunology. 2015;16(9):927-32.
78. Lima JF, Oliveira LMS, Pereira NZ, Mitsunari GE, Duarte AJS, Sato MN. Distinct Natural Killer Cells in HIV-Exposed Seronegative Subjects With Effector Cytotoxic CD56dim and CD56bright Cells and Memory-Like CD57+NKG2C+CD56dim Cells. JAIDS Journal of Acquired Immune Deficiency Syndromes. 2014;67(5):463-71.
79. Gondois-Rey F, Chéret A, Granjeaud S, Mallet F, Bidaut G, Lécuroux C, et al. NKG2C+memory-like NK cells contribute to the control of HIV viremia during primary infection: Optiprim-ANRS 147. Clinical & translational immunology. 2017;6(7):e150-e.
80. CDC. Monitoring selected national HIV prevention and care objectives by using HIV surveillance data—United States and 6 U.S. dependent areas—2011. HIV Surveillance Supplemental Report. 2013;13(8).
81. Barbosa Junior A, Szwarcwald CL, Pascom AR, Souza Junior PB. [Trends in the AIDS epidemic in groups at highest risk in Brazil, 1980-2004]. Cad Saude Publica. 2009;25(4):727-37.
82. Baral S, Sifakis F, Cleghorn F, Beyrer C. Elevated risk for HIV infection among men who have sex with men in low- and middle-income countries 2000-2006: a systematic review. PLoS Med. 2007;4(12):e339.
83. Beyrer C, Baral SD, van Griensven F, Goodreau SM, Chariyalertsak S, Wirtz AL, et al. Global epidemiology of HIV infection in men who have sex with men. Lancet (London, England). 2012;380(9839):367-77.
84. CDC. HIV in the United States and Dependent Areas. HIV surveillance report. 2017(Volume 28).
85. McDermott DH, Beecroft MJ, Kleeberger CA, Al-Sharif FM, Ollier WE, Zimmerman PA, et al. Chemokine RANTES promoter polymorphism affects risk of both HIV infection and disease progression in the Multicenter AIDS Cohort Study. AIDS. 2000;14(17):2671-8.
86. Mowat AM, Viney JL. The anatomical basis of intestinal immunity. Immunol Rev. 1997;156:145-66.
87. Pope M, Haase AT. Transmission, acute HIV-1 infection and the quest for strategies to prevent infection. Nat Med. 2003;9(7):847-52.

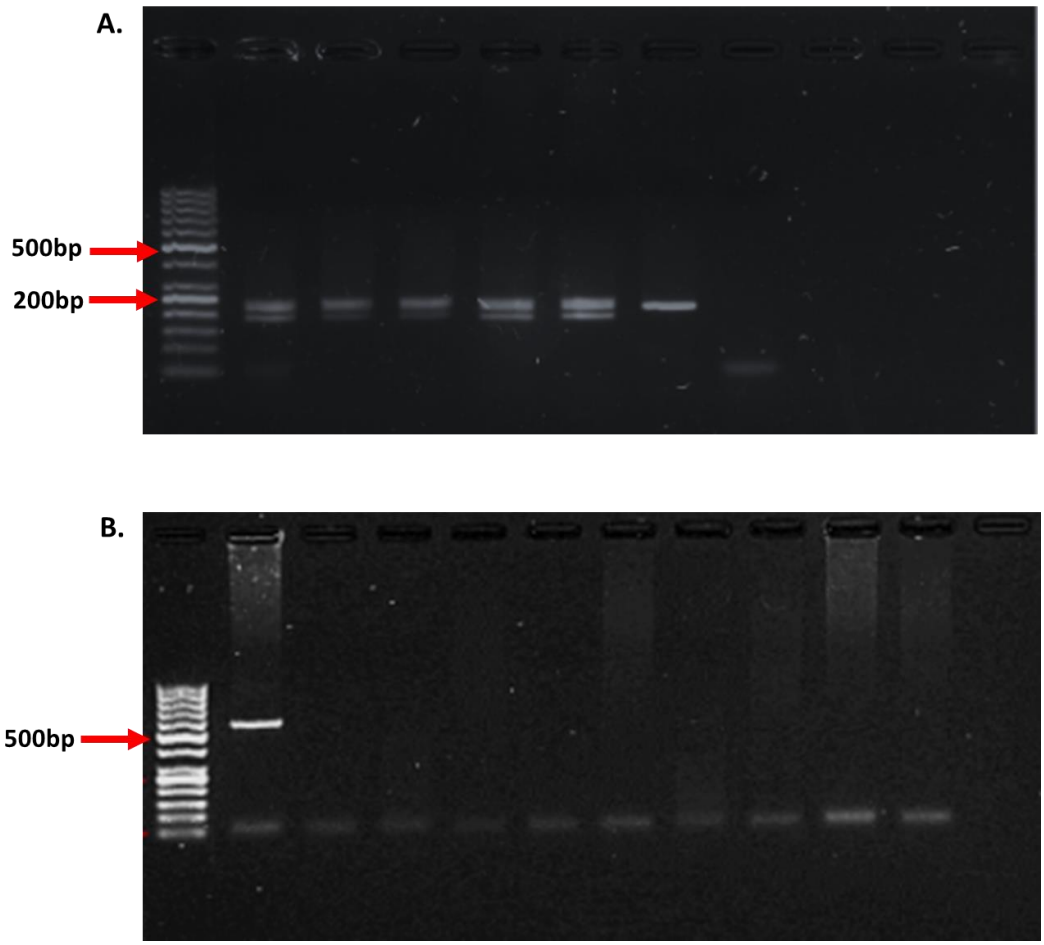
88. Ribeiro Dos Santos P, Rancez M, Pretet JL, Michel-Salzat A, Messent V, Bogdanova A, et al. Rapid dissemination of SIV follows multisite entry after rectal inoculation. PLoS One. 2011;6(5):e19493.
89. Lauby JL, Millett GA, LaPollo AB, Bond L, Murrill CS, Marks G. Sexual risk behaviors of HIV-positive, HIV-negative, and serostatus-unknown Black men who have sex with men and women. Arch Sex Behav. 2008;37(5):708-19.
90. Flowers P, Knussen C, Li J, McDaid L. Has testing been normalized? An analysis of changes in barriers to HIV testing among men who have sex with men between 2000 and 2010 in Scotland, UK. HIV Med. 2013;14(2):92-8.
91. Blas MM, Alva IE, Cabello R, Carcamo C, Kurth AE. Risk behaviors and reasons for not getting tested for HIV among men who have sex with men: an online survey in Peru. PLoS One. 2011;6(11):e27334.
92. Wagner GJ, Aunon FM, Kaplan RL, Rana Y, Khouri D, Tohme J, et al. A qualitative exploration of sexual risk and HIV testing behaviors among men who have sex with men in Beirut, Lebanon. PLoS One. 2012;7(9):e45566.
93. Brito AM, Kendall C, Kerr L, Mota RM, Guimaraes MD, Dourado I, et al. Factors Associated with Low Levels of HIV Testing among Men Who Have Sex with Men (MSM) in Brazil. PLoS One. 2015;10(6):e0130445.
94. MUNDIAL FYF. “COMPORTAMIENTO SEXUAL Y PREVALENCIA DE VIH EN HOMBRES QUE TIENEN RELACIONES SEXUALES CON HOMBRES EN TRES CIUDADES DE COLOMBIA: BOGOTÁ, MEDELLÍN Y SANTIAGO DE CALI. 2018.
95. Pines HA, Karris MY, Little SJ. Sexual Partner Concurrency Among Partners Reported by MSM with Recent HIV Infection. AIDS Behav. 2017;21(10):3026-34.
96. Tieu HV, Nandi V, Frye V, Stewart K, Oquendo H, Bush B, et al. Concurrent partnerships and HIV risk among men who have sex with men in New York City. Sex Transm Dis. 2014;41(3):200-8.
97. Marmor M, Sheppard HW, Donnell D, Bozeman S, Celum C, Buchbinder S, et al. Homozygous and heterozygous CCR5-Delta32 genotypes are associated with resistance to HIV infection. J Acquir Immune Defic Syndr. 2001;27(5):472-81.
98. Trecarichi EM, Tumbarello M, de Gaetano Donati K, Tamburrini E, Cauda R, Brahe C, et al. Partial protective effect of CCR5-Delta 32 heterozygosity in a cohort of heterosexual Italian HIV-1 exposed uninfected individuals. AIDS Res Ther. 2006;3:22.
99. Gupta A, Padh H. The global distribution of CCR5 delta 32 polymorphism: role in HIV-1 protection. BMC Infectious Diseases. 2012;12:O16-O.
100. Liu S, Kong C, Wu J, Ying H, Zhu H. Effect of CCR5-Delta32 heterozygosity on HIV-1 susceptibility: a meta-analysis. PLoS One. 2012;7(4):e35020.
101. Paxton WA, Liu R, Kang S, Wu L, Gingeras TR, Landau NR, et al. Reduced HIV-1 Infectability of CD4+Lymphocytes from Exposed-Uninfected Individuals: Association with Low Expression of CCR5 and High Production of β-Chemokines. Virology. 1998;244(1):66-73.
102. Thomas SM, Tse DB, Ketner DS, Rochford G, Meyer DA, Zade DD, et al. CCR5 expression and duration of high risk sexual activity among HIV-seronegative men who have sex with men. AIDS. 2006;20(14):1879-83.
103. de Silva E, Stumpf MP. HIV and the CCR5-Delta32 resistance allele. FEMS Microbiol Lett. 2004;241(1):1-12.
104. Björkström NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. Blood. 2010;116(19):3853-64.
105. Le Garff-Tavernier M, Beziat V, Decocq J, Siguret V, Gandjbakhch F, Pautas E, et al. Human NK cells display major phenotypic and functional changes over the life span. Aging Cell. 2010;9(4):527-35.

106. Goodier MR, White MJ, Darboe A, Nielsen CM, Goncalves A, Bottomley C, et al. Rapid NK cell differentiation in a population with near-universal human cytomegalovirus infection is attenuated by NKG2C deletions. Blood. 2014;124(14):2213-22.
107. Foley B, Cooley S, Verneris MR, Pitt M, Curtsinger J, Luo X, et al. Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. Blood. 2012;119(11):2665-74.
108. Bjorkstrom NK, Lindgren T, Stoltz M, Fauriat C, Braun M, Evander M, et al. Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. J Exp Med. 2011;208(1):13-21.
109. Beziat V, Dalgard O, Asselah T, Halfon P, Bedossa P, Boudifa A, et al. CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. Eur J Immunol. 2012;42(2):447-57.
110. Petitdemange C, Becquart P, Wauquier N, Beziat V, Debre P, Leroy EM, et al. Unconventional repertoire profile is imprinted during acute chikungunya infection for natural killer cells polarization toward cytotoxicity. PLoS Pathog. 2011;7(9):e1002268.
111. Lopez-Vergès S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. Blood. 2010;116(19).
112. Nielsen CM, White MJ, Goodier MR, Riley EM. Functional Significance of CD57 Expression on Human NK Cells and Relevance to Disease. Front Immunol. 2013;4:422.
113. Penack O, Gentilini C, Fischer L, Asemissen AM, Scheibenbogen C, Thiel E, et al. CD56dimCD16neg cells are responsible for natural cytotoxicity against tumor targets. Leukemia. 2005;19(5):835-40.
114. Jiang Y, Zhou F, Tian Y, Zhang Z, Kuang R, Liu J, et al. Higher NK cell IFN-gamma production is associated with delayed HIV disease progression in LTNPs. J Clin Immunol. 2013;33(8):1376-85.
115. Chung AW, Navis M, Isitman G, Wren L, Silvers J, Amin J, et al. Activation of NK cells by ADCC antibodies and HIV disease progression. J Acquir Immune Defic Syndr. 2011;58(2):127-31.
116. Luetke-Eversloh M, Hammer Q, Durek P, Nordstr??m K, Gasparoni G, Pink M, et al. Human cytomegalovirus drives epigenetic imprinting of the IFNG locus in NKG2Chi natural killer cells. PLoS pathogens. 2014;10(10).
117. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol. 2004;75(2):163-89.
118. Samuel CE. Antiviral actions of interferons. Clin Microbiol Rev. 2001;14(4):778-809, table of contents.
119. Chaix J, Tessmer MS, Hoebe K, Fuseri N, Ryffel B, Dalod M, et al. Cutting edge: Priming of NK cells by IL-18. J Immunol. 2008;181(3):1627-31.
120. Fehniger TA, Cai SF, Cao X, Bredemeyer AJ, Presti RM, French AR, et al. Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs. Immunity. 2007;26(6):798-811.
121. Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. Immunity. 2007;26(4):503-17.
122. Bukowski JF, Biron CA, Welsh RM. Elevated natural killer cell-mediated cytotoxicity, plasma interferon, and tumor cell rejection in mice persistently infected with lymphocytic choriomeningitis virus. J Immunol. 1983;131(2):991-6.
123. Barton ES, White DW, Cathelyn JS, Brett-McClellan KA, Engle M, Diamond MS, et al. Herpesvirus latency confers symbiotic protection from bacterial infection. Nature. 2007;447(7142):326-9.

124. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Jacobs C, Xavier RJ, et al. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. Clin Immunol. 2014;155(2):213-9.
125. Marquardt N, Ivarsson MA, Blom K, Gonzalez VD, Braun M, Falconer K, et al. The Human NK Cell Response to Yellow Fever Virus 17D Is Primarily Governed by NK Cell Differentiation Independently of NK Cell Education. J Immunol. 2015;195(7):3262-72.
126. Goodier MR, Rodriguez-Galan A, Lusa C, Nielsen CM, Darboe A, Moldoveanu AL, et al. Influenza Vaccination Generates Cytokine-Induced Memory-like NK Cells: Impact of Human Cytomegalovirus Infection. J Immunol. 2016;197(1):313-25.
127. Gumá M, Cabrera C, Erkizia I, Bofill M, Clotet B, Ruiz L, et al. Human Cytomegalovirus Infection Is Associated with Increased Proportions of NK Cells That Express the CD94/NKG2C Receptor in Aviremic HIV-1–Positive Patients. The Journal of Infectious Diseases. 2006;194(1):38-41.
128. Drews E, Adam A, Htoo P, Townsley E, Mathew A. Upregulation of HLA-E by dengue and not Zika viruses. Clin Transl Immunology. 2018;7(9):e1039.
129. Martini F, Agrati C, D'Offizi G, Poccia F. HLA-E up-regulation induced by HIV infection may directly contribute to CD94-mediated impairment of NK cells. Int J Immunopathol Pharmacol. 2005;18(2):269-76.
130. Liu LL, Landskron J, Ask EH, Enqvist M, Sohlberg E, Traherne JA, et al. Critical Role of CD2 Co-stimulation in Adaptive Natural Killer Cell Responses Revealed in NKG2C-Deficient Humans. Cell Rep. 2016;15(5):1088-99.
131. Bryceson YT, Ljunggren HG, Long EO. Minimal requirement for induction of natural cytotoxicity and intersection of activation signals by inhibitory receptors. Blood. 2009;114(13):2657-66.
132. Cichocki F, Cooley S, Davis Z, DeFor TE, Schlums H, Zhang B, et al. CD56dimCD57+NKG2C+ NK cell expansion is associated with reduced leukemia relapse after reduced intensity HCT. Leukemia. 2016;30(2):456-63.
133. Elmaagacli AH, Steckel NK, Koldehoff M, Hegerfeldt Y, Trenschel R, Ditschkowski M, et al. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. Blood. 2011;118(5):1402-12.
134. Nguyen S, Beziat V, Dhedin N, Kuentz M, Vernant JP, Debre P, et al. HLA-E upregulation on IFN-gamma-activated AML blasts impairs CD94/NKG2A-dependent NK cytolysis after haplo-mismatched hematopoietic SCT. Bone Marrow Transplant. 2009;43(9):693-9.
135. Farquhar C, Rowland-Jones S, Mbori-Ngacha D, Redman M, Lohman B, Slyker J, et al. Human leukocyte antigen (HLA) B*18 and protection against mother-to-child HIV type 1 transmission. AIDS Res Hum Retroviruses. 2004;20(7):692-7.
136. Chaudhari DV, Chavan VR, Ahir SP, Kerkar SC, Mehta PR, Mania-Pramanik J. Human leukocyte antigen B distribution in HIV discordant cohort from India. Immunol Lett. 2013;156(1-2):1-6.
137. Valenzuela-Ponce H, Alva-Hernandez S, Garrido-Rodriguez D, Soto-Nava M, Garcia-Tellez T, Escamilla-Gomez T, et al. Novel HLA class I associations with HIV-1 control in a unique genetically admixed population. Sci Rep. 2018;8(1):6111.
138. Payne R, Muenchhoff M, Mann J, Roberts HE, Matthews P, Adland E, et al. Impact of HLA-driven HIV adaptation on virulence in populations of high HIV seroprevalence. Proc Natl Acad Sci U S A. 2014;111(50):E5393-400.
139. Raulet DH. Missing self recognition and self tolerance of natural killer (NK) cells. Semin Immunol. 2006;18(3):145-50.

140. Alter G, Rihn S, Walter K, Nolting A, Martin M, Rosenberg ES, et al. HLA class I subtype-dependent expansion of KIR3DS1+ and KIR3DL1+ NK cells during acute human immunodeficiency virus type 1 infection. J Virol. 2009;83(13):6798-805.
141. Boudreau JE, Mulrooney TJ, Le Luduec JB, Barker E, Hsu KC. KIR3DL1 and HLA-B Density and Binding Calibrate NK Education and Response to HIV. J Immunol. 2016;196(8):3398-410.
142. Boulet S, Song R, Kamya P, Bruneau J, Shoukry NH, Tsoukas CM, et al. HIV protective KIR3DL1 and HLA-B genotypes influence NK cell function following stimulation with HLA-devoid cells. J Immunol. 2010;184(4):2057-64.
143. Song R, Lisovsky I, Lebouche B, Routy JP, Bruneau J, Bernard NF. HIV protective KIR3DL1/S1-HLA-B genotypes influence NK cell-mediated inhibition of HIV replication in autologous CD4 targets. PLoS Pathog. 2014;10(1):e1003867.
144. Le Luduec J-B, Hsu KC. KIR3DL1 and HLA-Bw4 Allotypes Predict The Extent Of NK Cell Licensing. Blood. 2013;122(21):1043-.
145. O'Connor GM, Vivian JP, Gostick E, Pymm P, Lafont BA, Price DA, et al. Peptide-Dependent Recognition of HLA-B*57:01 by KIR3DS1. J Virol. 2015;89(10):5213-21.

7. SUPPLEMENTARY INFORMATION



Supplementary figure 1. Δ32 and proviral DNA PCR products. **A.** Representative gel of Δ32 PCR products. From left to right, positive control for the heterozygous phenotype (to bands 225bp and 193bp) and four heterozygous individuals. The last line shown a 225bp for homozygous wild type phenotype. **B.** Representative gel of proviral DNA products. From left to right, positive control (562bp product from integrated viral DNA), 8 individuals included in the study and the negative control.

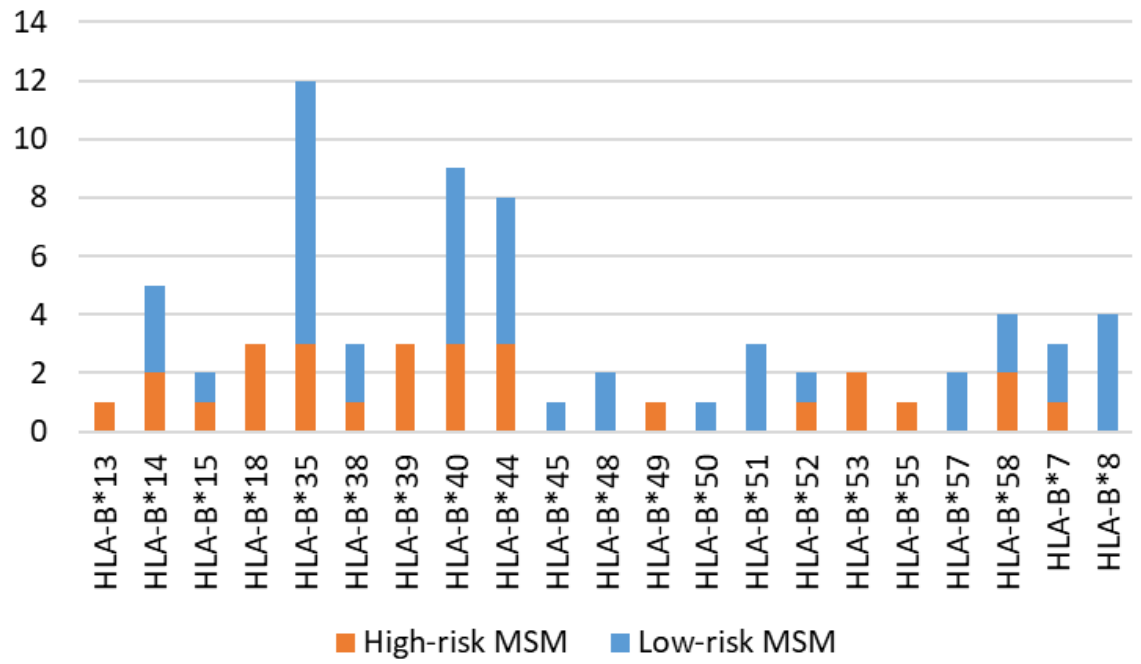
***Supplementary Table 1*.** Primer sequences for amplification of HIV proviral DNA

	Primer sequence	Location (Env)
First round	**ED3:** Fw 5´-TTAGGCATCTCCTATGGCAGGAAGAAGCGG-3´	5956-5985
	ED14: Fw 5´-CCTCAGCCATTACACAGGCCTGTCCAAAG-3´	7960-7931
Second round	**ED31:** Fw 5´-CCTCAGCCATTACACAGGCCTGTCCAAAG-3´	6816-6844
	ED33: Fw 5´-TTACAGTAGAAAAATTCCCCTC-3´	7359-7380

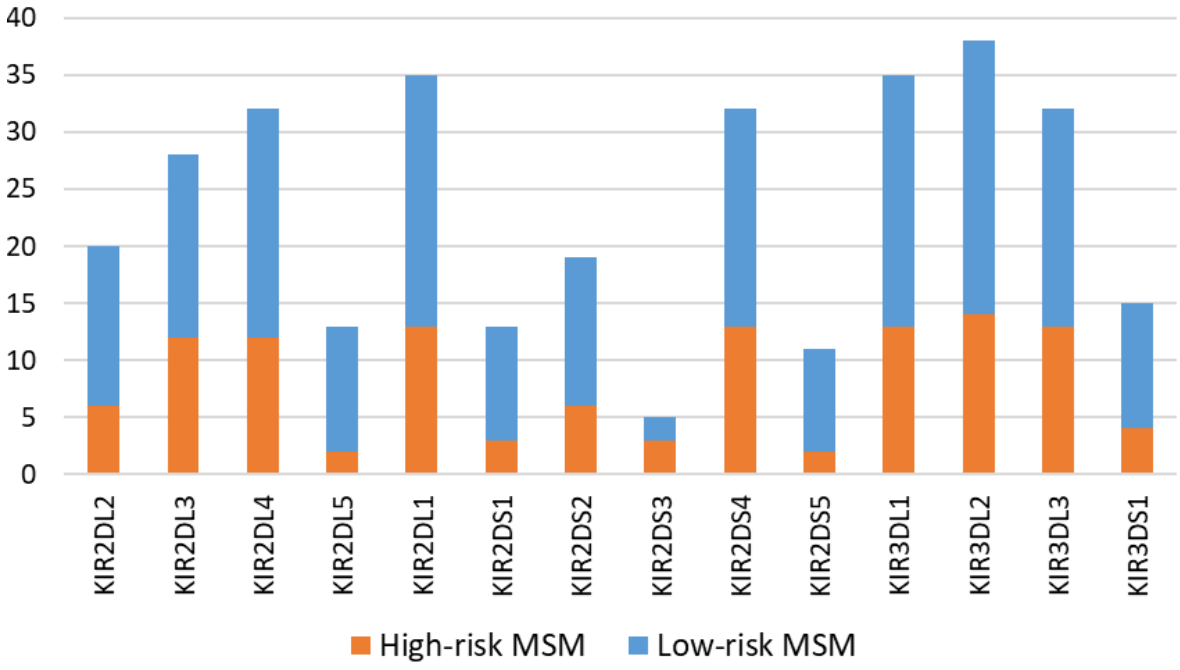
Supplementary Table 2. Primer sequences and PCR conditions for gene expression evaluation.

Gene	Primer Sequence	T melting
PGK	Fw 5`-GTTGACCGAATCACCGACC-3` Rv 5`-TCGACTCTCATAACGACCCGC-3`	60°C
RANTES	Fw 5`-CCATGAAGGTCTCCGCGGCA-3` Rv 5`-GTGGGCGGGCAATGTAGGCAA-3`	64°C
MIP -1β	Fw 5`-CTGCCTTCTGCTCTCCAGCG-3` Rv 5`-GGAGCAGAGGCTGCTGGTCT-3`	60°C
MIP-1α	Fw 5`- TGCATCACTTGCTGCTGACACG-3` Rv 5`- CAACCAGTCCATAGAAGAGG-3`	61°C
Perforin	Fw 5`- CCGCTTCTACAGTTTCCATGT-3` Rv 5`-GTGCCGTAGTTGGAGATAAGC-3`	52°C
Granzyme	Fw 5`- CACTGTTGGGGAAGCTCCAT-3` Rv 5`-TGGGGGATGGGTCTTTTCAC-3`	54°C
IFN-γ	Fw 5`-TCGTTTTGGGTTCTCTTGGC -3` Rv 5`-TCTGTCACTCTCCTCTTTCCAA-3`	59°C
IL-22	Fw 5`-CCCTATATCACCAACCGCAC-3` Rv 5`-CACTCATACTGACTCCGTGG-3`	58°C
TNF-α	Fw5`- CCCATGTTGTAGCAAACCCTC-3` Rv 5`- TATCTCTCAGCTCCACGCCA-3`	60°C

All amplifications were done with the same protocol. Initial enzyme activation step of 94°C for 10 min. Then, denaturation at 94°C for 10 sec; annealing at T° melting for 30 sec; extension at 72°C for 30 sec, 40 times, and a final extension step at 72°C for 2 min.



Supplementary figure 2. Allelic frequency of HLA-B



Supplementary figure 3. Allelic frequency of KIR

Supplementary Table 3. HLA-B alleles distribution among groups

***Supplementary Table 4*.** KIR alleles distribution among groups

	High-risk MSM % (n)	***Low-risk MSM % (n)***	***p***
KIR2DL2	46.1 (6)	56 (14)	ns
KIR2DL3	92.3 (12)	64 (16)	ns
KIR2DL4	92.3 (12)	80 (20)	ns
KIR2DL5	15.3 (2)	44 (11)	ns
KIR2DL1	100 (13)	88 (22)	ns
KIR2DS1	23 (3)	40 (10)	ns
KIR2DS2	46.1 (6)	52 (13)	ns
KIR2DS3	23 (3)	8 (2)	ns
KIR2DS4	100 (13)	76 (19)	ns
KIR2DS5	15.3 (2)	36 (9)	ns
KIR3DL1	100 (13)	88 (22)	ns
KIR3DL2	100 (13)	96 (24)	ns
KIR3DL3	100 (13)	76 (19)	ns
KIR3DS1	30.8 (4)	44 (11)	ns