

# The Role of Natural Killer Cells and Regulatory T Cells while Aging with HIV

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## Abstract

Combined antiretroviral therapy (cART) has increased the quality of life of people living with HIV (PLHIV). Consequently, the number of PLHIV >50 years is increasing worldwide. Patients on cART are known to remain in a pro-inflammatory state. The latter is linked to the development of non-AIDS related chronic conditions. Although the number of aging PLHIV is increasing, the effect of HIV infection on the process of aging is not fully understood. Understanding the complexity of aging with HIV by investigating the effect of the latter on different components of the innate and adaptive immune systems is important in order to reduce the impact of these comorbid conditions and improve the quality of life of PLHIV. The role of killer immunoglobulin receptors (KIRs), expressed on the surface of NK cells, and their human leukocyte antigen (HLA) ligands in the clearance, susceptibility to or disease progression following HIV infection is well established. However, data on the effect of KIR-HLA interaction in aging HIV-infected population and the development of non-AIDS related comorbid conditions are lacking. Moreover, conflicting data exist on the role of regulatory T (Treg) cells during HIV infection. The purpose of this review is to advance the current knowledge on the role of NK cells and Tregs while aging with HIV infection.

## Introduction

The use of combination antiretroviral therapy (cART) has improved the survival rate of human immunodeficiency virus (HIV)-infected individuals. cART contributed to an increase in the life expectancy of people living with HIV (PLHIV) approaching that of HIV-negative individuals <sup>[1, 2]</sup>. Globally, access to antiretroviral therapy increased from 8 million in 2010 to 21.7 million in 2017 with 59% of all PLHIV in 2017 accessing treatment (2018 Global HIV statistics, fact sheet. <https://www.unaids.org/en/resources/fact-sheet>). In the United States, approximately 50% of PLHIV are aged 50 years and older (CDC, <https://www.cdc.gov/hiv/group/age/olderamericans/index.html>). Despite the fact that new cases within PLHIV aged more than 50 years are declining, the CDC reported 1 in 6 HIV diagnoses in 2016 in this group. The global proportion of PLHIV aged 50 years or older is expected to reach an estimated 21% by 2020 <sup>[3]</sup>. 80% of this population lived in low- and middle-income countries (LMICs) in 2016 and the percentage of PLHIV50<sup>+</sup> is likely to increase in high- and low-income countries. Despite the success of cART, the rate of non-AIDS comorbidities are increasing in treated PLHIV leading to an increased number of deaths exceeding those of AIDS deaths <sup>[4-7]</sup>. These comorbidities include cardiovascular disease <sup>[8-13]</sup>, liver disease <sup>[14, 15]</sup>, renal disease <sup>[9, 11, 16]</sup>, diabetes <sup>[8-10, 12, 16, 17]</sup>, neurocognitive abnormalities<sup>[18, 19]</sup>, as well as non-AIDS defining malignancies including liver, brain, anal and lung cancers <sup>[20, 21]</sup>. Importantly, as PLHIV age, the number of medications to treat comorbid conditions increases leading to an increase in drug-to-drug interaction (DDI), adverse drug effects (ADE), increased hospitalization and lower adherence to antiretroviral therapy <sup>[22]</sup>.

Overlapping with chronic diseases of aging, these non-AIDS comorbidities have been associated with residual and continuous immune activation maintained in ART-treated HIV-infected individuals <sup>[6, 13, 19, 23, 24]</sup>. The persistence of HIV in infected individuals results in increasing levels of proinflammatory mediators (TNF- $\alpha$ , TGF- $\beta$ , IFN- $\alpha$ , IL-1 $\beta$ , IL-12, IL-6, IL-8, sCD14, sCD163, MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, IP-10) <sup>[23, 25]</sup>, C-reactive protein and D-dimers contributing to immune activation (IA) <sup>[24]</sup>. HIV-associated IA is also reported to affect the thymus, despite partial regain of functionality following cART, leading to defective development of regulatory T cells (Tregs). Moreover, microbial translocation and

loss of Th17 were also suggested to contribute to IA [24, 26]. While many of these biomarkers have been proposed as predictors of serious non-AIDS related pathologies (SNAEs), the association with and the ability to predict poor clinical prognosis was not evident [27]. Nevertheless, accelerated IA stimulates viral replication resulting in replicative senescence of T cells and premature immune aging [23]. A number of “immune parallels” have been advanced between HIV infection and aging [23, 24]. The following major factors have been suggested: a) the accumulation of CD28<sup>-</sup>CD8<sup>+</sup> T cells leading to the generation of highly differentiated senescent cells expressing CD57 as well as shortening of telomeres in CD8<sup>+</sup> T cells, another marker of senescence; b) the decline of naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cell counts to levels comparable to 85 years old uninfected individuals. Moreover, the constant inflammation is also maintained among HIV-infected individuals co-infected with Cytomegalovirus (CMV), Hepatitis C virus (HCV), Hepatitis B virus (HBV) and Epstein Barr virus (EBV) and may lead to higher risk of development of non-AIDS comorbidities [24].

As the number of older adults diagnosed with HIV is increasing, the HIV and Aging Consensus Project has published on the specific risk factors affecting older adults with HIV infection [28]. The group recommended screening HIV-infected individuals for diabetes, kidney functions, hypertension (salt intake, weight, exercise) as well as cognitive impairment and depressive disorders. Moreover, assessment of the Framingham Risk Score was also recommended along with cholesterol and blood pressure. Although biological aging in HIV-infected individuals is believed to start earlier compared to uninfected healthy subjects (55 vs 65 years, respectively) [29], the impact of aging with HIV infection and subsequent pathways leading to disease manifestation is not fully understood. With the absence of a chronic care model especially in limited resource countries, these non-AIDS morbidities pose a major risk on treated HIV-infected people progressing into an older population. Understanding the complexity of aging with HIV is important to develop and prioritize interventions leading to reduced immune dysfunction, the development of comorbid conditions and improving the quality of life of PLHIV. The purpose of this review is to advance the current knowledge on natural killer cells (NK) cells, their human leukocyte antigen (HLA) ligands as well as regulatory T (Treg) cells in people living and aging with HIV and their potential role in the development of non-AIDS comorbidities.

## Natural Killer Cells, HIV and Aging

### *Natural Killer Cells and Killer Immunoglobulin Receptors*

NK cells constitute an important component of the innate immune system involved in immune-surveillance and the regulation of the cross-talk between the innate and the adaptive immune responses via secretion of chemokines/cytokines [30, 31]. NK cells, in contrast to B and T cells, lack the expression of cluster of differentiation 3 (CD3), as well as antigen-specific receptors such as B-cell receptors (BCR) or T-cell receptors (TCR). NK cells express CD56 and/or CD16; thus are phenotypically defined as CD3<sup>-</sup>CD56<sup>+</sup>CD16<sup>+</sup> [30, 32]. On the basis of the density of CD56, NK cells are divided into three subsets: terminally differentiated cytotoxic CD56<sup>dim</sup>/CD16<sup>+</sup> cells, less differentiated, cytokine producing CD56<sup>bright</sup>/CD16<sup>-</sup> cells and a minor CD56<sup>-</sup>CD16<sup>+</sup> subset characterized by poor antiviral activity [31-33]. In healthy individuals, CD56<sup>bright</sup>CD16<sup>-</sup> and CD56<sup>dim</sup>CD16<sup>+</sup> subsets constitute 5-10% and up to 90% of the peripheral NK cell population, respectively [34]. CD56<sup>bright</sup> NK cells are immature peripheral cells characterized by longer telomeres compared to the more mature CD56<sup>dim</sup> NK cells [35, 36]. CD57, present on terminally-differentiated CD4 and CD8 T cells, is also expressed on different subsets of NK cells [37]. This marker is expressed on CD56<sup>dim</sup>CD16<sup>+</sup> (terminally differentiated cytotoxic) and CD56<sup>-</sup>CD16<sup>+</sup> (inflammatory) NK cells. The cytokine producing and regulatory CD56<sup>bright</sup>/CD16<sup>-</sup> do not express CD57. Consequently, CD57<sup>+</sup> NK cells are mainly cytotoxic yet poorly responsive to cytokine stimulation [38, 39].

NK cells play a crucial role in early responses to infections; these cells kill target cells (infected or transformed) via: membrane disruption of target cell through secretion of perforin and granzymes; caspase-dependent apoptosis through the engagement of death receptors on target cells with their ligands on NK cells (e.g. Fas-ligand (FasL) or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)); and antibody-dependent cellular cytotoxicity (ADCC) through the binding of Fcγ receptor III (FcγRIII or CD16) on NK cells to the Fc portion of the antibody opsonizing the target cell [30, 31]. Importantly, activation of NK cells contribute to adaptive immunity following exposure to type I interferons (IFN) namely IFN-α and IFN-β secreted by dendritic cells (DCs) as well as pro-

inflammatory cytokines such as interleukin (IL)-12, IL-15 and IL-18<sup>[40, 41]</sup>. Activated NK cells mainly secrete IFN- $\gamma$  which in turn enhances the maturation and effector functions of DCs, macrophages and granulocytes<sup>[40]</sup> and shapes T cell responses in lymph nodes<sup>[41]</sup>. They also produce pro-inflammatory cytokines (tumor necrosis factor (TNF)- $\alpha$ ), IL-10, IL-3, IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF)<sup>[41, 42]</sup>. Chemokines including C-C motif-chemokine ligand (CCL)-2, CCL-3, CCL-4, and CCL-5, chemokine (C motif) ligand XCL-1 (lymphotactin) and chemokine (C-X-C motif) ligand CXCL-8 (IL-8) are also produced by NK cells. These chemokines play an important role in the co-localization of NK cells to inflamed sites along with other innate immune cells (i.e. DCs).

NK cells express distinct and broad range of germline-encoded receptors that are either inhibitory or activating. These receptors include leukocyte immunoglobulin-like receptors (LIR), natural cytotoxicity receptors (NCR) (e.g. NKp30, NKp44, NKp46), C-lectin receptors (e.g. NKG2A, NKG2C, NKG2D), killer cell lectin-like receptor (KLR) (e.g. KLRG1), signaling lymphocyte activation (SLAM) family, Fc $\gamma$  receptor CD16, and most importantly killer cell immunoglobulin-like receptors (KIRs)<sup>[42-44]</sup>. The effector function of NK cells depends on the balance between inhibitory and activating signals. The inhibitory NK cell receptors (KIRs, LIRs and NKG2A) function to inhibit NK cells from killing normal self-cells by recognizing major histocompatibility complex (MHC) class I, expressed on the surface of normal nucleated cells in humans. Tumor cells and pathogen-infected cells often downregulate MHC class-I expression to evade recognition by T cells. The downregulation of MHC-I is detected by these inhibitory receptors resulting in lower inhibitory signals and higher activating signals driving a potent NK cell response. KLRG1, another inhibitory cell receptor<sup>[45]</sup> is expressed on almost 60% of human NK cells specifically the mature CD56<sup>dim</sup>CD16<sup>+</sup> NK cell subset<sup>[46, 47]</sup>. The function of KLRG1 is still not fully understood.

KIRs are highly polymorphic type 1 transmembrane glycoproteins composed of two (2D) or three (3D) extracellular immunoglobulin (Ig) domains as well as short (S) or long (L) cytoplasmic tails<sup>[42, 48-50]</sup> determining the inhibitory or activating functions of KIRs. Genes encoding KIRs are located in the leukocyte receptor complex (LRC) on chromosome 19q13.4<sup>[51]</sup>. Sixteen genes encode KIR of which 7 are activating (KIR2DS1, 2DS2, 2DS3,

2DS4, 2DS5A, 2DS5B, and 3DS1), 8 are inhibitory (KIR2DL1, 2DL2, 2DL3, 2DL5A, 2DL5B, 3DL1, 3DL2, and 3DL3) and one (KIR2DL4) predominantly activating but can transmit inhibitory signals (AFND; <http://www.allelefreqencies.net> and <sup>[52]</sup>). Two KIR haplotypes are defined in humans: haplotype A and haplotype B <sup>[49, 53]</sup>. Haplotype A encodes inhibitory receptors (KIR2DL1, KIR2DL3, KIR3DL1, KIR3DL2, KIR3DL3, KIR2DP1, KIR3DP1, and KIR2DL4) and one activating receptor KIR2DS4. Haplotype B on the other hand carries a variety of gene combinations and encodes more activating receptors as compared to haplotype A; these include: KIR3DL3, KIR2DS2, KIR2DL2, KIR2DL5B (inhibitory) KIR2DS3, KIR2DP1, KIR2DL1, KIR3DP1, KIR2DL4, KIR3DS1, KIR2DL5A (inhibitory), KIR2DS5, KIR2DS1, and KIR3DL2 <sup>[53]</sup>. The balance between the inhibitory and activating signals regulates the function of NK cells. Despite the high allelic polymorphism of KIR genes, almost all individuals possess the following genes referred to as framework loci: KIR3DL3, KIR3DP1, KIR2DL4, and KIR3DL2 <sup>[49, 53]</sup>.

#### *NK cells, KIRs and Human Leukocyte Antigens Ligands during HIV Infection*

KIRs bind to MHC class I molecules; the latter includes the classical human leukocyte antigen: HLA-A, HLA-B and HLA-C. The majority of HLA-I ligands to activating KIRs are still not identified except for KIR2DS4: HLA-A3/11 and HLA-C (C1 or C2). In contrast, the inhibitory KIR2DL1, KIR2DL3, KIR3DL1 and KIR3DL2 bind to HLA-C2, HLA-C1, HLA-Bw4 and HLA-A3/A11, respectively. A number of KIR-HLA pairs have been implicated in the control or the progression of several human viral diseases including human influenza virus, viral hepatitis (HCV and HBV), human cytomegalovirus (HCMV) and HIV <sup>[51, 54]</sup>. Moreover, the frequency of expression of certain KIRs and the interaction with their HLA ligands were implicated in controlling HIV-disease progression in HIV-infected subjects <sup>[31]</sup>. Below we summarize the available literature on the effect of HIV infection on NK cells distribution and KIRs expression.

It is well established that HIV infection results in the accumulation of phenotypically and functionally aberrant NK cells resulting from alterations in the distribution of NK cell subsets (CD56<sup>-</sup>, CD56<sup>dim</sup> and CD56<sup>bright</sup>) <sup>[43]</sup>. The weakly functional CD56<sup>-</sup> NK cells, rarely present in healthy individuals, increase during chronic HIV infection,

chronic HCV infection <sup>[55]</sup> and in response to HCMV infection <sup>[56]</sup>. Earlier studies reported a significant increase in CD56<sup>-</sup> NK cells subset in untreated viremic HIV-infected individuals <sup>[57]</sup>. However, the expression of CD56 on NK cells was similar to that of healthy donors following successful ART for >2 years, as previously reported by the same group <sup>[58]</sup>. CD56<sup>-</sup> NK cells isolated from the same individuals expressed higher levels of inhibitory KIRs (KIR2DL2) and LIR1 suggesting lower cytolytic activity. Recently, the expansion of different NK cell subsets was studied in ART-naïve viremic controllers (VC) (viral load of 40-2,000 copies/ml of plasma), viremic non-controllers (VNC) (viral load of >5,000 copies/ml of plasma) and elite controllers (EC) (viral load of <40 copies/ml of plasma). The results showed the expansion of CD56<sup>-</sup>CD16<sup>+</sup>CD7<sup>+</sup> NK cells in VNC as compared to HIV-negative controls <sup>[59]</sup>. Importantly, CD56<sup>dim</sup>CD16<sup>+</sup> NK cells with increased expression of sialic acid-binding immunoglobulin-like lectin 7 (Siglec-7), were abundant in EC and healthy controls. The frequency of these cells was associated with levels of HIV DNA and thus suggesting the role of these cells in immune control of HIV. The loss of Siglec-7 is suggested to be associated with dysfunctional NK cells occurring during HIV infection <sup>[60]</sup> as well as HCV infection <sup>[61]</sup>. The expression of Siglec-7 was also reported to decrease on NK cells of chronically HIV-infected individuals <sup>[59, 62]</sup>. NK cells from healthy donors expressing Siglec-7 have higher functional activity defined by an increased expression of CD107a and higher IFN- $\gamma$  production <sup>[63]</sup>. Importantly, the expression of Siglec-7 on NK cells of HIV-infected individuals was restored following 18 months of successful ART <sup>[62]</sup>. Collectively these results suggest that high HIV viral load is associated with expansion of phenotypically and functionally aberrant NK cell subset characterized by impaired functional activity.

The CD56<sup>dim</sup> CD16<sup>+</sup> subset, which constitutes the majority of circulating NK cells in healthy subjects, was relatively reduced in treatment-naïve HIV-infected subjects with a parallel increase in the percentage of CD56<sup>-</sup>CD16<sup>+</sup> subset <sup>[64]</sup>. Recent studies also reported the reduction in CD56<sup>dim</sup> NK cells in treatment-naïve (median age 25 years) and treated HIV patients (median age 30 years) compared to uninfected subjects <sup>[65]</sup>. In a prospective randomized clinical trial evaluating the effect of cART on the adaptive and innate immunity following primary HIV infection, a decrease in CD56<sup>dim</sup>CD16<sup>+</sup> subset and a reciprocal increase in CD56<sup>bright</sup> subset were detected <sup>[66]</sup>. Similarly, in non-responders (NRs) (median

age 45 years) on highly active ART (HAART) for a median of 36 months, CD56<sup>bright</sup> NK cells were higher in frequency and absolute number as compared to responders and healthy subjects; moreover, this subset inversely correlated with CD4<sup>+</sup> T cell count [67]. The NRs were defined as HIV-infected patients on HAART with CD4<sup>+</sup> cell count of <350 cells/ $\mu$ l as compared to >500 CD4<sup>+</sup> cells/ $\mu$ l in responders. These results suggest that alterations in the NK cell subsets could contribute to HIV disease progression. [68-71]

The expression of CD57, another predominant marker on NK cells linked to senescence of the latter as well as T cells [72], on CD56<sup>dim</sup> CD16<sup>+</sup> NK cells was also studied in a cohort of ART-naïve or untreated HIV-infected individuals [73]. The results of this study showed an increased expression of CD57 on CD56<sup>dim</sup> NK cells with a relative increase in the frequency of the CD57<sup>bright</sup> CD56<sup>dim</sup> CD16<sup>+</sup> NK cells among HIV-seropositive compared to the CD57<sup>dim</sup> CD56<sup>dim</sup> CD16<sup>+</sup> NK subset. Similar to previous reports [64, 74], this study confirmed the significant loss of CD56<sup>dim</sup> CD16<sup>+</sup> NK cells during HIV infection. Importantly, CD57-expressing CD56<sup>dim</sup> CD16<sup>+</sup> NK cells were characterized by an increasing prevalence of KIR<sup>+</sup> and granzyme B<sup>+</sup>, i.e., a mature phenotype. This differentiated subset is less functional as indicated by the decreased expression of the proliferation marker Ki-67 and the degranulation marker CD107a [73]. Recently, Gondois-Rey et al [75] reported on the alterations of NK cell subsets during primary HIV infection. A mature NK cell profile, i.e. patients with higher frequency of CD57<sup>+</sup> CD56<sup>dim</sup> NK cells compared to patients with higher frequencies of CD56<sup>dim</sup> NKG2A<sup>+</sup> or CD56<sup>dim</sup> NKG2A<sup>-</sup> NKG2C<sup>-</sup> CD57<sup>-</sup> subpopulations, was associated with better response to cART and significant decrease in viral load (<50 copies/mL) 3 months following initiation of cART. Despite the small sample size, the authors advanced the potential significance of assessing the role of mature NK cells during the course of HIV infection and its impact on diseases prognosis.

The majority (90%) of PLHIV and 50% of healthy individuals are seropositive for CMV [76]. Consequently, few studies attempted to study the phenotypes of NK cells among the former. CD57, suggested to expand in response to CMV [77], was reported among older (50-70 years) treated HIV<sup>+</sup> individuals whether CMV<sup>+</sup> or CMV<sup>-</sup>. Despite the success of cART, human CMV (HCMV) infection/reactivation in PLHIV was reported to contribute to HIV disease progression, immune senescence and phenotypical and functional alterations in

NK cells<sup>[56, 78]</sup>. While CD57<sup>+</sup> NK cells were higher among HIV infected individuals compared to healthy controls<sup>[73]</sup> and associated with reduced viral load<sup>[75]</sup>, the status of HCMV infection among study participants was assessed. More studies are needed to elucidate the confounding role exerted by HCMV on NK cell subsets and the potential role of the latter in disease progression and control among HIV-positive individuals.

#### *KIR/HLA-I Interaction during HIV Infection*

It is clear that the interaction between KIRs and their corresponding HLA ligands is important in differential responses to many viral infections such as HCV<sup>[79]</sup>, HBV<sup>[80]</sup> and other disease conditions<sup>[81-84]</sup>. Specifically, KIR2DL2 and or KIR2DL3 along with HLA-C1 were associated with severe influenza infection<sup>[54]</sup> while KIR2DS2 and KIR2DS3 were suggested to be associated with susceptibility to chronic HBV infection<sup>[85]</sup>. Similarly, KIR2DL3/HLA-C1 and KIR2DS3/HLA-C2 ligands were described as protective and risk factor for the development of chronic HCV infection, respectively<sup>[79]</sup>. Moreover, KIR/HLA interaction was also advanced as potentially involved in preventing the generation of protective immune responses against HCMV in patients undergoing kidney transplant or hematopoietic stem cell transplantation (HSCT)<sup>[86]</sup>.<sup>[54][85][87][80][88][89][79][90][86]</sup>

Ample data exist on the interaction between specific KIRs and their HLA-I ligands on target cells and their impact on the clinical outcome during the course of HIV infection<sup>[31]</sup>. HLA-B alleles, with the Bw4 epitope including HLA-B\*57 and HLA-B\*27, are associated with the control of HIV replication and consequently delaying progression to AIDS<sup>[91]</sup>. HLA-Bw4-80I are ligands for KIR3DL1 (inhibitory receptor) and putative ligands for the NK cell activating receptor KIR3DS1; these KIR alleles in combination with the former are associated with protection from severe disease outcome among HIV-infected individuals<sup>[54, 92-95]</sup>. When compared to other alleles, KIR3DL1\*004 allele interacts with HLA-Bw4-80I and significantly slows the progression to AIDS in HIV-infected subjects<sup>[95]</sup>. Recently and while comparing long-term non-progressors (LTNP) to progressors, increased frequencies of KIR3DL1-Bw4T80, KIR3DL1-Bw4/Aw4 and KIR3DL1-Aw4 was reported in the former in a South Indian Cohort<sup>[96]</sup>. Progressors on the other hand possessed KIR3DL1 with homozygous Bw6. Several early studies also reported on the interaction between KIR3DS1

and its putative ligand HLA-Bw4-80I resulting in delay of disease progression and protection from opportunistic infections in chronically HIV-infected individuals <sup>[92, 97-100]</sup>. Alter et al. were the first to show *in-vitro* that NK cells control the replication of HIV in a receptor-ligand specific manner <sup>[92]</sup>. In this study, the level of replication of HIV *in-vitro* using a p24 Gag ELISA was determined in infected CD4<sup>+</sup> (iCD4<sup>+</sup>) T cells co-incubated with autologous NK cells. Both NK and CD4<sup>+</sup> T cells were isolated from HIV-negative individuals (N=36) who possessed KIR3DS1/Bw4-80I genes (N=8), KIR3DS1 gene alone (N=8), HLA-Bw4-80I alone (N=10) or neither KIR3DS1 nor HLA-Bw4-80I genes (N=10). Results showed a significant reduction in HIV replication *in-vitro* when iCD4<sup>+</sup> T cells were co-incubated with NK cells isolated from individuals expressing both KIR3DS1 and its ligand HLA-Bw4-80I. This result was compared to those of cells isolated from other individuals who either lack the expression of these genotypes or express either KIR3DS1 or HLA-Bw4-80I. NK cells expressing KIR3DL1 were also able to reduce HIV replication *in-vitro* but to a lesser extent than NK cells expressing KIR3DS1 <sup>[100]</sup>. Upon co-culture with iCD4<sup>+</sup> T cells, NK cells isolated from individuals possessing KIR3DL1\*h/\*y-HLA-B\*57 or KIR3DS1-HLA-Bw4-80I combinations secreted higher levels of chemokines (CCL3, CCL4 and CCL5) than NK cells from individuals with KIR3DL1\*I/\*x-HLA-B\*57 genotypes or from those who are Bw6 homozygote. Based on the expression on NK cell surface, KIR3DL1 genotype is divided into high (\*h), low/intermediate (\*I), and null (\*004) unexpressed alleles. Homozygous KIR3DL1 can be divided into two groups: \*h/\*y (with \*y being either \*h allele or \*004) with no \*I alleles and \*I/\*x (with \*x being another \*h, \*I, or \*004) with at least one \*I allele <sup>[95, 101]</sup>. Similarly, a higher intracellular expression of these chemokines as well as IFN- $\gamma$  and the activation marker CD107a were detected in these NK cells suggesting a higher functional potential of these cells upon stimulation with autologous iCD4<sup>+</sup> T cells and higher ability to inhibit HIV replication in infected CD4<sup>+</sup> T cells. Recently, the interaction reported between KIR2DL2/S2/ and HLA-C\*1202 and KIR2DL2/S2/HLA-C\*14:03 in treatment naïve HIV-infected individuals exerted a protective effect through suppression of viral replication *in vitro* in a Japanese cohort <sup>[102]</sup>.

Moreover, Alter et al. (2009) were the first to report on the impact of HLA-I ligands on clonal expansion and KIR expression on NK cells during acute HIV infection <sup>[93]</sup>. The

levels of KIR3DS1 on NK cells of individuals with acute HIV infection and expressing HLA-B Bw4-80I was significantly higher than those expressing HLA-B Bw6 and those who are HIV negative. The levels of KIR3DL1 expression showed a similar pattern to KIR3DS1. These results demonstrate the dependent expansion of KIR3DS1 and KIR3DL1 in the presence of their putative ligand.

### NK Cells and KIRs in Aging

Phenotypic and functional alterations in NK cells due to aging have been reported [32, 38, 103-105], whereas scarce data exist on the effect of aging on KIR expression on NK cells. Below we summarize data on the impact of aging on the number, phenotype and function of NK cells.

Overall, a decrease in the immature CD56<sup>bright</sup> NK cell subset accompanied by an increase of the mature CD56<sup>dim</sup> NK cell subset is observed suggesting an expansion in NK cell frequency with age due to the latter [38, 106]. Consequently, NK cell subsets are remodeled in aging with a shift in balance toward the more mature CD56<sup>dim</sup> subset. Despite this change, the production of IFN- $\gamma$  is maintained in the elderly by the CD56<sup>bright</sup> cell subset suggesting a compensatory mechanism for cytokine production and immune regulation in this group [107]. Significantly lower CD56<sup>bright</sup> NK cell number was detected among subjects more than 60 years old compared to those aged 20-40 years [108-110]. Similar results were recently reported in individuals >60 years old compared to younger subjects [109, 110]. Consequently, an expansion and accumulation of the mature NK cell subsets is clearly detected in agreement with the findings of Le Garff-Tavernier et al. (2010) whereby the frequency and the absolute number of CD56<sup>bright</sup> subset gradually decreased with age along with increased proportion of CD56<sup>dim</sup>/CD56<sup>bright</sup> and thus higher frequency of mature NK subset in elderly individuals (>60 years) [103]. Importantly, Le Garff-Tavernier et al. [103] extensively analyzed the phenotypic and functional characteristics of NK cells from healthy subjects of different ages: newborn (cord blood), adult (18-60 years), old (60-80 years) and very old (80-100 years). IFN- $\gamma$  was produced following treatment of NK cells from different age groups with IL-2 and IL-18. Significantly higher levels of IFN- $\gamma$  were detected in cord blood as compared to other age groups. NK cells isolated from very old

subjects exerted low cytolytic activity *ex vivo* that was reversed following IL-2 activation suggesting the potential recovery of age related changes along with an increase in the frequency of CD56<sup>dim</sup> NK cells subset expressing the terminally differentiation marker CD57. The latter is associated with a mature NK cell phenotype with increased cytotoxic capacity and decreased response to cytokines [38, 103, 105]. Importantly, CD57<sup>+</sup> NK cells are characterized by a strong ADCC [111]. CMV infection was associated with the expansion of terminally-differentiated effector memory CD8 T cells while aging is associated with the decline of naïve CD8 T cells proportions [112]; moreover, it was linked to induce the expression of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells [113], the latter reported to play a role in the control of infection [113-116]. Recently, the effect of HCMV seropositivity and aging on NK cells was evaluated in young (18-35 years) and old (>70 years) healthy individuals [112]. CD57<sup>+</sup> NK cells, CD56<sup>dim</sup>CD16<sup>+</sup>CD57<sup>+</sup> and CD56<sup>-</sup>CD16<sup>+</sup>CD57<sup>+</sup> NK cells from young CMV-seropositives as well as CD56<sup>-</sup>CD16<sup>+</sup>CD57<sup>+</sup> NK cells from elderly expressed higher levels of CD94/NKG2C compared to CMV-seronegatives. Importantly, the expression of CD57 on CD56<sup>-</sup>CD16<sup>+</sup> NK cells subpopulation was higher among the older CMV-seropositive compared to younger CMV-seropositive individuals. Moreover, the immature CD56<sup>bright</sup> NK cells decreased among elderly and expressed higher levels of granzyme A, i.e. differentiated, compared to the younger group. This study highlighted the role of CMV in shaping NK cell subsets in different age groups and suggests the significance of determining the status of CMV infection especially in people aging with HIV.

Variable results have been reported regarding the expression of KIRs as well as other NK receptors. The expression of KLRG1 appears to be reduced in elderly [117, 118]. Interestingly, a lower frequency of KLRG1<sup>+</sup>CD57<sup>-</sup>NK cells was reported among CMV<sup>+</sup> young individuals compared to higher frequency of KLRG1<sup>-</sup>CD57<sup>+</sup>NK cells leading authors to advance the role of CMV rather than aging in shaping NK cell phenotypes [119]. NKG2A expression was reported to either decrease or remain the same in elderly (reviewed in [38, 106]). Interestingly, the age-related changes on NK cells have been proposed to contribute to the increased frequency of senescent cells detected in aged tissues [120] as well as low immune responses in older subjects [38]. In this study, the surface expression of the inhibitory receptor CD94/NKG2A was reported to be significantly lower in all age groups

compared to newborns similar to previous report <sup>[121]</sup>. Moreover, NKp30 and NKp46 (activating NK receptors) are significantly lower in adults, old and very old individuals as compared to newborns. The frequency of NK cells expressing these receptors was previously reported to decrease in elderly subjects (>60 years) compared to younger adults (18-59 years) <sup>[109]</sup>. Le Garff-Tavernier et al. reported the increased expression of the following in adult controls compared to cord blood with similar levels detected in other age groups: KIR2DL1/DS1 (bind group 2 HLA-Cw), KIR2DL2/DL3/DS2/ (bind HLA-C3 group 1), KIR3DL1/DS1 (HLA-Bw4 ligand). Moreover, an increase in the expression of the inhibitory receptor LIR-1/ILT-2 was detected in very old subjects.

As previously discussed, during the course of untreated and uncontrolled HIV infection, the frequency of CD56<sup>dim</sup> NK cells decline whereas CD56<sup>bright</sup> NK cells reciprocally increase which is not in parallel with the impact of aging on these subsets. Large studies are needed to investigate the dynamics affecting NK cell subsets, KIR expression and the interaction with their HLA-I ligands among PLHIV above 50 years during the course of HIV infection. Elucidating these mechanisms would have implications in developing therapeutic approaches that could enhance NK cells activity through exploitation of KIRs and KIR-HLA ligands combination aiming at reducing the incidence of comorbidities and deaths due to them. **Regulatory T cells in HIV and Aging**

### *Tregs*

Regulatory T (Treg) cells, a subset of CD4<sup>+</sup> T cells, play a major role in maintaining immune homeostasis. These cells are characterized by their ability to maintain immune tolerance to both self-antigens in autoimmune disorders and non-self-antigens in various infectious diseases <sup>[122-125]</sup>. Based on their site of differentiation, Tregs are classified into thymic (tTregs) and peripheral Tregs (pTregs) <sup>[126-132]</sup>. The development and maturation of tTregs are mediated by the interaction between B7 molecules expressed on antigen-presenting cells (APCs), CD28 expressed on thymocytes and IL-2, respectively <sup>[127, 131-133]</sup>. pTregs are derived from naïve CD4<sup>+</sup> T helper cells in the peripheral lymphoid organs. Peripherally-induced Tregs are generated following weak TCR and CD28 stimulation/or exposure to subimmunogenic peptides for a long period of time resulting in expression of

forkhead box P3 transcription factor (FoxP3)<sup>[126, 131-133]</sup>. Tregs are phenotypically defined by the expression of FoxP3<sup>[127]</sup>. FoxP3 binds to nuclear factor of activated T cells (NFAT) to form a complex modulating the suppressive function of Tregs. The latter is mediated via increasing the expression of T cell activation markers (CTLA-4 and CD25) on CD4<sup>+</sup> T cells and inhibiting the transcription of IL-2<sup>[134-138]</sup>. Moreover, TGF- $\beta$  plays an important role in the generation of pTregs *in vitro* and *in vivo*<sup>[126, 131-133]</sup>.

CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells constitute the most investigated immunophenotype. This phenotype plays a vital role in attenuating cell-mediated immunity and suppressing activated CD4<sup>+</sup> T cell function<sup>[122, 139-143]</sup>. Tregs suppress the function and proliferation of Th1, Th2 and Th17 CD4<sup>+</sup> and CD8<sup>+</sup> T cells<sup>[122-124, 144]</sup>. Tregs exert their immunosuppressive function mainly via: 1) release of anti-inflammatory mediators including IL-10, IL-35, and TGF- $\beta$ <sup>[145, 146]</sup>; 2) CTLA-4 expressed on Tregs blocking the CD28-costimulation<sup>[147, 148]</sup>; and 3) binding to IL-2 and thus competing with non-Treg cells and inhibiting activation<sup>[125, 126, 130, 137, 142, 149-153]</sup>.

It is well established that increased Tregs frequency is detected in aged mice and humans and thus contributing to age-associated immune suppression (reviewed in<sup>[133, 141, 154]</sup>). Briefly, animal studies suggested the accumulation of Tregs in lymphoid organs during aging leading to increased susceptibility to infection and reduced vaccine responses. For example, Influenza infection induced antigen-specific FoxP3<sup>+</sup> Treg cells as well as increased levels of memory Tregs following secondary infection<sup>[155, 156]</sup>. Recently, vaccine-induced Tregs suppressed anti-influenza immune responses *in vitro* and *in vivo* in the mice model<sup>[157]</sup>. When addressing the impact of aging on vaccine responses, Van Der Geest et al. (2014) reported a decline in naïve Tregs and increased ratios of Treg and T effector (T<sub>eff</sub>) cells in the CD4<sup>+</sup> T cell memory compartment of healthy aged volunteers. These data suggest a bias toward Tregs in the elderly which was also shown to inversely correlate with immune responses following influenza vaccine. Similarly, a significant increase in FoxP3<sup>+</sup>CD4<sup>+</sup> T cells was detected in the skin of older individuals infected with Varicella Zoster virus (VZV) above 60 years old<sup>[158]</sup>. CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs were also higher among individuals with lung cancer compared to healthy controls<sup>[159]</sup>. Importantly, these cells as well as FoxP3 mRNA also increased with aging in healthy people<sup>[160]</sup>. Along the same lines,

aging has been recognized to program the generation of a suppressive immune milieu affecting DCs and T cells in mice <sup>[161]</sup> and consequently loss of their function in tumor-bearing mice <sup>[162]</sup>. Overall, the process of aging is characterized by a decline of the thymus function and its involution. Despite the decline in tTreg and pTreg differentiation with age, older individuals accumulate mature Tregs in blood and secondary lymphoid organs. This phenomenon would lead to suppression of immune responses and thus enhancement of immunosenescence.

### *Tregs, HIV infection and aging*

The role of Tregs in HIV and SIV infections has been recently reviewed and is gaining attention in an attempt to manipulate these cells for the development of HIV therapeutic strategies <sup>[127, 163, 164]</sup>. However, the debate about the beneficial or detrimental role of Tregs during HIV infection is still ongoing. Importantly, naïve Tregs are potential targets for HIV infection due to the increased expression of HIV co-receptors, CCR5 and CXCR4 <sup>[127, 130, 137, 153, 163, 165, 166]</sup>. The infection of Tregs *in vitro* with HIV (X4) was reported to result in downregulation of FoxP3 expression, decreased production of TGF- $\beta$  and increased production of IL-4; suggesting loss of the suppressive function of HIV-infected Tregs <sup>[167]</sup>.

The interaction between IL-2 and IL-2R $\alpha$  receptor (CD25) expressed on the surface of Tregs has been linked to the homeostatic role exerted by these cells <sup>[168, 169]</sup>. IL-2 facilitates the TGF- $\beta$ -mediated transformation of naïve T cells to FoxP3<sup>+</sup>-expressing Tregs. Consequently, high levels of IL-2 produced by activated T cells are suggested to result in the expansion of Tregs. This homeostatic balance, previously described in autoimmune diseases <sup>[169]</sup>, is disturbed in HIV-infected individuals due to viral mediated alterations of Tregs phenotype. The latter is defined by the loss of IL-2R- $\alpha$  on Tregs thus, decreasing their suppressive capacity <sup>[170]</sup>. Previous reports confirmed the loss of Treg suppressive function following loss of CD25 expression on the surface of Tregs isolated from healthy individuals <sup>[171]</sup>. Further studies are needed to investigate the role of these balances in HIV and therefore, therapeutic agents targeting Tregs and IL-2.

Importantly, Tregs suppress HIV-specific CD8<sup>+</sup> cytotoxic T cell responses resulting in an increased viral load<sup>[172]</sup> and contributing to viral persistence and disease progression [130, 131, 137, 173, 174]. The latter was confirmed *ex vivo* among controllers expressing protective HLA-B\*27 and HLA-B\*57 alleles. The HLA-B\*27 or HLA-B\*57-restricted CD8<sup>+</sup> T cells were able to escape the suppressive mechanism of Tregs and retain their proliferative capacity [129, 175]. These restricted CD8<sup>+</sup> T cells were able to up-regulate granzyme B production upon antigenic stimulation and consequently becoming less susceptible to suppression by Tregs. These results, in addition to the low frequency of Tregs, are suggested to contribute to robust HIV-specific CD8<sup>+</sup> T cell responses in controllers.

Similarly, the role of Tregs was further studied among HIV exposed seronegative (HESN) individuals<sup>[176, 177]</sup>. HESN individuals constitute an important model to study resistance and/or control of HIV infection. Briefly, PBMCs from HESN adults from HIV serodiscordant cohort were used to assess the function of Tregs and protection from HIV<sup>[177]</sup>. Treg-depleted PBMCs were stimulated *in vitro* with Gag, Pol, Env and Vpu peptide pools. Two subsets of Tregs were detected: a subset suppressing HIV-specific CD4<sup>+</sup> T cell proliferation and another one unable to suppress the latter. When compared to cells able to suppress HIV-specific CD4<sup>+</sup> T cell proliferation, Tregs unable to suppress the latter produced MIP-1  $\beta$  and showed increased T cell activation and Treg frequencies. The role of Tregs in immunological reconstitution was also studied among treated HIV-infected individuals<sup>[178]</sup>. The percentages of peripheral and lymphoid FoxP3<sup>+</sup> Tregs were assessed in immunological non-responders (INR) (CD4 T cells <200 cells/  $\mu$ L), intermediate responders (IR) (CD4 T cells 200-500 cells/  $\mu$ L) and immunological responders (RES) (CD4 T cells >500 cells/  $\mu$ L) following cART. INR had significantly higher percentages of Tregs than other groups including controls with higher percentages producing IL-10. Moreover, this group showed a higher percentage of activated Tregs. HIV controllers showed lower Treg frequencies and counts, compared to uninfected subjects, thus potentially leading to stronger HIV-specific T cells responses as well as T cell activation<sup>[179]</sup>. Similarly, many studies reported higher frequencies of Tregs among untreated HIV-viremic individuals compared to elite controllers, long-term non-progressors as well as HIV-infected

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individuals on treatment <sup>[174, 179-182]</sup>. The lower frequencies of Tregs reported in these groups were similar to those reported in healthy controls.

In an attempt to analyze immunological markers potentially contributing to comorbidities, ART-treated HIV-infected individuals older than 45 years of the AGE<sub>HIV</sub> Cohort Study with undetectable levels of viremia, showed high percentages of Tregs associated with increased immune activation <sup>[183]</sup>, the hallmark of HIV infection <sup>[184]</sup>. Increased levels of immune activation were confirmed in this group by detection of increased percentages of CD38<sup>+</sup> HLA-DR<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells, regulatory T cells and PD-1-expressing CD4<sup>+</sup> T cells. These results confirmed previous reports <sup>[185, 186]</sup>. The role of Tregs was further evaluated in non-progressing HIV infection. The percentages of activated Tregs in LTNPs (with ongoing viral replication) and EC (with low-to-undetectable levels of viral replication) were found to be higher than those detected among progressors while percentages of resting Tregs were lower <sup>[178, 187]</sup> suggesting the beneficial role of these cells in immune reconstitution and non-progression. Recently, lower levels of HIV DNA and viral mRNA were detected in FoxP3 expressing-CD4<sup>+</sup> T cells <sup>[188]</sup>. The latter were either infected *in vitro* and are in agreement with previously reported data <sup>[189]</sup> or isolated from HIV infected individuals and tested *ex vivo* <sup>[188]</sup>. Tregs were also suggested to play a role in inhibiting HIV expression in neighboring CD4<sup>+</sup> T cells. Briefly, Tregs from HIV infected individuals were co-cultured with conventional and memory CD4<sup>+</sup> T cells. Antibody-mediated blocking of Tregs' inhibitory surface markers (CTLA-4, PD-1, and GARP) increased HIV DNA integration and viral mRNA expression in neighboring CD4<sup>+</sup> T cells; suggesting contact with cell surface molecules as the mechanism behind potentially protecting neighboring CD4<sup>+</sup> T cells from HIV infection and inhibiting viral replication <sup>[188]</sup>.

An emerging body of evidence suggests further the detrimental role of Tregs in inhibiting HIV-specific immune responses. Following treatment interruption, as expected, HIV RNA in plasma and HIV-DNA in PBMCs increased, and CD4<sup>+</sup> T cell counts decreased. The proportion of HLADR<sup>+</sup> CD38<sup>+</sup> CD8<sup>+</sup> T cells, i.e. assessing immune activation, was associated with the expansion of Tregs in HIV-infected individuals undergoing treatment interruption <sup>[190]</sup>. Similar results were observed in the gastrointestinal tract whereby the frequency of mucosal Tregs increased during chronic HIV infection among non-controllers

compared to controllers and uninfected controls and was associated with viral load and immune activation <sup>[174]</sup>. These results suggested yet again the role of Tregs in promoting viral persistence and HIV-disease progression. It is clear that conflicting data exist regarding the impact of Tregs on either enhancing or reducing HIV disease progression <sup>[191-196]</sup> as well as controlling immune activation. A significant complication exerted by Tregs lies in being recognized as a major reservoir for SIV/HIV (Reviewed by <sup>[125, 127]</sup>). pTregs were described as latently infected with HIV without addressing tissues Tregs in humans. Importantly, increased numbers of SIV-infected Tregs were detected in gut-associated lymphoid tissue (GALT) of untreated macaques <sup>[197]</sup>. Further investigations are needed to understand the persistence of HIV in tissue Tregs compared to pTregs and consequently understand their role in immunosenescence.

In summary, the “split personality of Tregs” <sup>[136, 163]</sup>, i.e. detrimental role contributing to disease progression via suppression of HIV-specific T cell responses and a beneficial one resulting in eliminating viral spread, reestablishing immune constitution at low CD4<sup>+</sup> T cell counts, and preventing immune hyper-activation among infected subjects, requires further investigation for clearer conclusions on disease outcome in HIV-infected individuals.

<sup>[133, 141, 154][155, 156][157][158][159][160][161][162]</sup>Limited data exist on the impact of aging on Tregs during HIV infection. Tenorio et al <sup>[138]</sup> studied the impact of aging on Tregs in treatment-naïve young (18-30 years) and older ( $\geq 45$  years) HIV<sup>+</sup> individuals. The results of this study indicated significantly higher total percentages of Tregs among older HIV-positives; the latter were compared to younger seropositives as well as healthy age-matched controls. Moreover, a positive correlation was shown between age of HIV-infected subjects and Treg percentages while a negative one was reported among HIV-negative subjects. These results were reported in a small sample size and consequently should be cautiously interpreted. Tregs have been suggested to exert a detrimental role contributing to disease progression via suppression of HIV-specific T cell responses; consequently, the increase in their percentages during HIV infection as well as their expansion in aging healthy individuals might contribute to an accelerated disease progression in aging HIV-infected subjects <sup>[138]</sup>. In an attempt to understand the changes

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on NK cells phenotypes and frequency (summarized above) among immunological non-responders (NR), Giuliani et al. [67] reported an overall decrease in Treg numbers. Currently, the consensus on the fate of Tregs during HIV infection is still controversial especially among NRs whereby similar [198], lower [199] and higher [186, 187, 200, 201] frequencies were reported when compared to responders. This study also reported an inverse correlation between reduced Treg counts and CD56<sup>bright</sup> NK cells among NRs. The expansion of the latter was suggested to shift the homeostatic control of Trgs leading to reduced immune reconstitution in this group. It is worth mentioning that *in vitro* studies in humans as well as studies in mice model suggest the ability of Tregs to suppress the cytolytic activity and proliferation of NK cells [68-71].

## Conclusion

HIV-infected individuals receiving ART are known to have a shorter life-expectancy and higher rate of age-associated diseases compared to age-matched uninfected individuals [9-11]. While comorbidities are associated with the natural aging process, the use of ART, polypharmacy, immune activation, and chronic inflammation due to HIV are linked to an increased risk of comorbidities in older HIV patients [9, 11, 202]. Importantly, HIV infection and aging exert similar impact on NK cell subsets and Tregs. Consequently, new treatment challenges are faced to control the concomitant effect of aging with HIV on immune reconstitution. . Further studies are needed to understand the role of NK cells, the KIR-HLA interaction and Tregs in older HIV-positive individuals with comorbidities and potentially targeting these for the development of innovative and effective therapeutic interventions.

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## Author Disclosure

The authors declare that they have no conflict of interest.

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The Role of Natural Killer Cells and Regulatory T Cells while Aging with HIV (DOI: 10.1089/AID.2019.0134)

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