Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen Departement Biomedische Wetenschappen

In vitro assessment of allogeneic NK cell responses against HIV-1 infected T cells

In vitro evaluatie van de allogene NK cel responsen tegenover HIV-1 geïnfecteerde T cellen

Hens Jef

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Biomedische Wetenschappen aan de Universiteit Antwerpen

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Acknowledgements

Before continuing to the actual doctoral thesis, I want to thank some people who made it possible for me to even start and ultimately finish my PhD. For having my back and supporting me during my endeavors, I thank you.

First of all, I want to thank **Dr. Wim Jennes** for introducing me the field of the topic. Without him, I would not have been so determined to start and finish my PhD. Especially our hour-long talks and brain storm sessions were fueling my excitement and commitment for the experiments conducted by the lab. Even after his days at the ITM, me and Wim met to discuss my new results and he was still able to enlighten me as a true expert.

Also the contribution of **Prof. Dr. Luc Kestens** to my PhD was of significant value, for one by entrusting me with this 4-year journey. As my promotor, Luc guided me through the process and pointed out obstacles ahead, but for most entrusted me with the choices I made and the direction my PhD was heading. Luc was always available (almost 24/7) for my questions, which I appreciated a lot. I also would like to acknowledge **Dr. Odin Goovaerts** for supervising me during my PhD. Odin arrived 1,5 year into my PhD, but started immediately by catching up with my progress and was from thereon out closely involved with my experiments. As a master in the English language and a determined mentor, Odin was a great help in getting my papers to publication-ready quality. During my experiments, my lab work was facilitated by the helping hand of **Ann Ceulemans**. Besides helping me get data, Ann was a formidable colleague who was eager to help and contribute to the scientific progress. To all the others colleagues during my four years at the lab of immunology, thank you for making it an unforgettable experience.

Besides the lab of immunology, I found a lot of help in the lab of Virology. Besides the mental support during the coffee breaks, the lab of Virology also supported some of my experiments. I would like to thank **Prof. Dr. Guido Vanham** and **Prof. Dr. Kevin Ariën** for their consult in defining new experiments and allowing these experiments to be conducted in the L3 lab. In the L3 lab, I was

assisted by many lab technicians (Johan Michiels, Leo Heyndrickx and Coloma Costas), but I must thank **Elisabeth Willems** specifically for training me to become acquainted for working in the L3-lab.

This PhD really was a big test for me in different aspects of my life, with many ups but also downs. For believing in me and constant mental support, I want to thank my parents and my friends, but most of all my girlfriend **Eline Beerten**.

I want to end this section with some words for my uncle **Peter Mariën**, constantly striving forward, taking only yes for an answer. Up until this day, he is a constant inspiration for my way of life, professionally as well as personally, and I am grateful for my memories of our time.

Table of content

List of Abbreviations

7-AAD	7-Aminoactinomycin D
ADCC	Antibody-dependent cell cytotoxcity
AIDS	Acquired immune deficiency system
aKIR	Activating Killer-cell immunoglobulin-like receptor
APC	Allophycocyanin
ART	Anti-retroviral therapy
BAT-3	HLA-B associated transcript 3
BM	Bone Marrow
CCL	CC chemokine
CCR	CC chemokine receptor
cDC	Conventional dendritic cells
CIP4	Cdc42-interacting protein 4
CLP	Common lymphocyte progenitor
CMV	Cytomegalovirus
CNS	Central nervous system
CTL	Cytotoxic T lymphocytes
CXCR	CXC chemokine receptor
dC	Discordant couples
DC	Dendritic cells
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	Deoxyribonucleic acid
EIAV	Equine Infectious Anemia Virus
ELISA	Enzyme-linked immunosorbent assay
ESN	Exposed seronegatives
FasR	Fas receptor
FGT	Female genital tract
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
GALT	Gut-associated lymphoid tissue
GATA-3	GATA binding protein 3

GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp120/41	Glycoprotein 120/41
GTPase	Guanosine triphosphatase
GVHD	Graft versus host disease
GVL	Graft versus leukemia
HA	Hemagglutinin
HAART	Highly-active antiretroviral therapy
Haplo-HSCT	Haploidentical hematopoietic stem cell transplantation
HIV-1	Human immune-deficiency 1
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
ICAM	Intercellular adhesion Molecule
iDC	Immature dendritic cell
IEL	Intra-epithelial lymphocyte
IFN	Interferon
iKIR	Inhibitory KIR
IL	Interleukine
ILC	Innate lymphoid cells
IL-R	Interleukine receptor
IN	Intergrase
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
IQR	Inter-quartile range
KARAP	Killer cell activating receptor-associated protein
KIR	Killer-cell immunoglobulin-like receptor
LFA-1	Lymphocyte function-associated antigen 1
Lin	Lineage
LN	Lymph nodes
LP	Lamina propria
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MALT	Mucosal-associated lymphoid tissue

mDC	Myeloid-derived dendritic cells
MdFI	Median fluorescence intensity
MGT	Male genital tract
MIC-A/B	MHC class I polypeptide-related sequence A & B
MIP3-α	Macrophage inflammatory protein-3 alpha
MOI	Mode of infection
mRNA	Messenger RNA
MTOC	Microtubule organizing center
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKP	Natural killer precursor
NKp44L	NKp44 ligand
NTB-A	NK-T-B-antigen
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
p/cSMAC	Peripheral/central supramolecular activation cluster
pDC	Plasmacytoid dendritic cells
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein complex
PHA	Phytohaemagglutinin
PIC	Pre-integration complex
PLT	Peripheral lymphoid tissue
PR	Protease
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RORγt	RAR-related orphan receptor gamma t
RPMI	Roswell park memorial institute
RRE	Rev response element
RT	Reverse transcriptase
SEM-1/2	Semenogelins
SEVI	Semen-derived enhancer of viral infection
SIV	Simian immune-deficiency virus

SLT	Secondary lymphoid tissue
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
STD	Sexual transmitted diseases
TAR	Tat-dependent RNA regulatory element
TCR	T cell receptor
T/F	Transmitter/founder
Th	T helper cell
TNF-α	Tumor necrosis family alpha
ULBP	UL16 binding protein
UNAIDS	The Joint United Nations Programme on HIV and AIDS
WASp	Wiskott–Aldrich Syndrome protein
WIP	WASP-interacting protein
ZAP70	Zeta-chain-associated protein kinase 70

Abstract

The immune system protects against all incoming threats. To this end, it composes different immune cells that can recognize each kind of pathogen. Natural killer (NK) cells are innate immune cells and identify virally-infected and tumoral cells. NK cells travel throughout the periphery, surveilling target cells for the presence of ligands for its receptors. Upon recognition, NK cells directly kill the threat or remain tolerant for the surveilled cell. NK cell receptors recognize inhibitory or activating ligands, transducing an inhibitory or activating signal. The balance of all these signals decides if activation of the NK cell is allowed or not. In healthy conditions, human leukocyte antigen (HLA) class I molecules transduce an inhibitory signal when binding the corresponding killer-cell immunoglobin-like receptors (KIR) on NK cells. These KIR-HLA interactions are also of vital importance during NK cell maturation. Binding the self-HLA ligand grants full functionality to the NK cell expressing the KIR, whereas the absence of this bond results in hyporesponsive NK cells. After this maturation, the absence of the self-HLA will result in the activation of mature NK cells as the inhibitory signal is lacking, known as the missing-self response.

Recent findings associated protection against the acquisition of the human immune-deficiency virus 1 (HIV-1) with NK cell alloreactivity induced by this missing-self principle, which was significantly more prevalent in discordant couples (only one partner HIV-positive) as compared to concordant couples (both partners HIV-positive). The data suggest that KIR-HLA incompatibility between both sexual partners was the driving force behind alloreactive NK cell responses and subsequent protection from HIV transmission. To investigate the mechanisms behind this epidemiological observation, we conducted *in vitro* experiments to verify the cytotoxic potential of KIR-HLA incompatible driven NK cell against CD4+ T cells derived from HIV-1 patients (Chapter 4). We observed missing-self responses specifically by single KIR expressing NK cells, providing evidence for alloreactive NK cell responses in KIR-HLA incompatible conditions. Between the various types of KIR-HLA mismatches, we measured differences in NK cell activation potential. Interestingly, the KIR2DL1-induced missing-self NK cell response was the strongest, in accordance with the previously observed resistance to HIV-1 in

discordant couples. Moreover, the size of the missing-self KIR+ NK cell population was only correlated with NK cell cytotoxicity when NK cell donors with an activating KIR repertoire were selected, indicating a vital role for activating signals in these missing-self responses.

The anti-viral capacities of alloreactive missing-self responses were further explored by composing co-cultures with *in vitro* HIV-1 infected T cells and staining them for activating ligands related to HIV-1 infection (Chapter 5). Exposure to HIV-1 increased the NK cell cytotoxicity in the missing-self context. However, the increased cytotoxicity was not directed towards the productively infected T cells, but rather the HIV-1 exposed T cells. Moreover, missing-self responses against productively infected T cells were only measured in the presence of the activating ligand MHC class I polypeptide-related sequence A & B (MIC-A/B).

In conclusion, we provide evidence for the NK cell-activating potential of an allogeneic missing-self response and noticed KIR2DL1+ NK cells to be the strongest responder. After exposure of target CD4+ T cells to HIV-1, the missing-self NK cell response increased, suggesting an activating role for HIV-1. However, the missing-self effect against productively infected cells was low and only measured in the presence of activating ligands, such as MIC-A/B but not FasR. In this respect, we suggest that the anti-HIV-1 effect of alloreactive missing-self responses is limited and is therefore suggested to have a restricted impact on the transmission of HIV-1.

Samenvatting

Het immuun systeem staat in voor de bescherming van het lichaam tegen alle inkomende pathogenen. Het bestaat uit verschillende soorten cellen die elk verantwoordelijk zijn voor het elimineren van een bepaald pathogeen. NK cellen maken deel uit van het aangeboren immuun systeem en zijn in staat om viraal-geïnfecteerde en tumorale cellen te herkennen en te elimineren. NK cellen inspecteren de betreffende cel door het opsporen van liganden op de doelwitcel, die kunnen binden aan receptoren op de NK cellen. Bij herkenning kunnen NK cellen de gevaarlijke cellen direct doden of niet. NK cellen kunnen zowel activerende als inhiberende liganden herkennen, die op hun beurt een activerend of inhiberend signaal doorsturen. De balans van al deze signalen bepaalt of de NK cel geactiveerd wordt of niet. In gezonde toestand zorgt een binding van de KIR receptor met een HLA class I molecule voor een inhiberend signaal. Deze KIR-HLA verbinding speelt ook een belangrijke rol tijdens de maturatie van NK cellen. Als een KIR+ NK cel tijdens de maturatie bind met zijn HLA ligand zal deze NK cel zijn functies later ten volle kunnen uitvoeren. In afwezigheid van zijn HLA ligand zal de KIR+ NK cel zijn functies maar minimaal kunnen uitvoeren. Na maturatie zal de afwezigheid van een self-HLA ligand voor activatie van de NK cel zorgen omdat het inhiberend signaal wegvalt, wat ook wel de missing-self response wordt genoemd.

Recent onderzoek in koppels die ofwel beiden besmet waren met HIV (concordant) ofwel met één besmette en één niet-besmette partner (discordant), vond een link tussen bescherming tegen HIV-1 transmissie en deze missing-self responsen tegenover niet-eigen of allogene cellen. Hierdoor werd beweerd dat de KIR-HLA incompatibiliteit tussen de seksuele partners de drijvende kracht was achter de alloreactieve NK cel responsen. Om deze hypothese verder te onderzoeken hebben we *in vitro* experimenten opgezet om het effect van deze KIR-HLA incompatibiliteit op NK cel alloreactiviteit te bepalen (Chapter 4). NK cellen met één KIR waren voornamelijk gevoelig aan de activatie door KIR-HLA incompatibiliteit, wat aantoont dat missing-self responsen wel degelijk plaatsvinden in deze allogene context. Alsook zagen we een verschil in het potentieel om de NK cel te activateren tussen de verschillende KIR-HLA mismatches, met als sterkste de KIR2DL1 missing-self response. Interessant genoeg was het net deze KIR/HLA mismatch die gerelateerd was met de HIV-1 resistentie in de

voorafgaande epidemiologische studie. Daarbij zagen we ook nog dat de grootte van de KIR+ NK cel populatie onder invloed van de KIR-HLA mismatch enkel correleerde met de NK cel cytotoxiciteit als de NK cel donor een activerend KIR repertoire bevatte. Dit toont aan dat activerende signalen in deze missing-self response ook een belangrijke rol hadden.

Om de antivirale capaciteiten van de allogene missing-self NK cel response meer direct te onderzoeken, zetten we co-culturen op met *in vitro* HIV-1 geïnfecteerde T cellen waarbij we de activerende liganden gelinkt aan HIV-1 infectie registreerden. Om de activerende signalen beter te kunnen bestuderen, gebruikte we antilichamen om de activerende receptoren en liganden te meten (Chapter 5). *In vitro* blootstelling aan HIV-1 zorgde voor een sterkere missing-self NK cel response tegenover allogene CD4+ T cellen, wat suggereerde dat HIV-1 een activerend effect heeft. Merkwaardig genoeg was deze sterkere missing-self response was niet gericht naar productief geïnfecteerde cellen, maar eerder naar HIV-1 blootgestelde cellen. Meer zelfs, missing-self NK cel responsen tegenover productief geïnfecteerde cellen waren zwak en enkel aanwezig als deze cellen de activerende liganden, maar niet FasR, tot expressie brachten.

In conclusie kunnen we stellen dat allogene NK cel responsen sterker waren in de afwezigheid van de HLA ligand, met de KIR2DL1+ NK cel als sterkste KIR-HLA mismatch. Blootstelling aan HIV-1 deed de missing-self NK cel cytotoxiciteit tegenover allogene CD4+ T cellen stijgen, terwijl cytotoxiciteit tegenover productief geïnfecteerde T cellen beperkt bleef. Desondanks waren er missing-self responsen te meten tegenover productief geïnfecteerde cellen in de aanwezigheid van MIC-A/B stimulatie.

In deze context suggereren de resultaten dat allogene missing-self responsen maar een beperkt antiviraal effect hebben dat mogelijks niet voldoet om de transmissie van HIV-1 in alle gevallen tegen te houden.

Chapter 1 - Introduction

A short introduction in the field of interest

Since the discovery of the human immunodeficiency virus type 1 (HIV-1) in the late 1970s, between 59.9 and a 100 million people have been infected, making HIV-1 one of the most widespread viruses in the world[1]. In 2017, an estimated 37.5 million people were globally living with HIV-1, with less than half of them having access to antiretroviral treatment (ART)[1]. As immunization by a potent HIV-1 vaccine is still unavailable[2,3], the search for other preventive or curative mechanisms continues[4-6].

Clearly, in most cases, the human immune response is unable to prevent infection or control viral replication after infection. Since no curative treatment is available as yet, clinicians use a combination of antiretroviral drugs to suppress the active replication without entirely eliminating the virus. Nevertheless, ART has been shown to prevent infection, besides the use of condoms and oral pre-exposure prophylaxis with antivirals. However, in some cases, resistance to HIV-1 acquisition is seen in individuals with a high exposure rate, exposed sero-negatives (ESN): sex workers, intravenous drug users, and mother-child transmission. By investigating these cases, multiple anti-HIV-1 immune mechanisms have been suggested to be partly or fully protective[7].

Although the HIV-1 virus expanded swiftly across the globe, the efficacy rate of vaginal sexual transmission itself is rather low compared to other viral infections (receptive: 0.01% and insertive: 0.005%)[8,9]. Herein, the genital immunity plays a vital role as it can induce a swift anti-viral immune response (reviewed in[5]). During vaginal sexual transmission, the survivability of HIV-1 is low and, in most cases (80%), a single viral clone is responsible for transmission[10]. Therefore an anti-viral immune response is much more likely to be HIV-1 protective during this timeframe compared to later stages of infection. Able to produce such a swift anti-viral immune response, NK cells are put forward as promising mediators of HIV-1 protection. Investigating NK cell receptors and ligands in ESN and slowly progressing patients revealed associations with beneficial HIV-1 outcomes. Recent research even suggests that NK cells can prevent against HIV-1 transmission before the actual acquisition[11].

1. The human immunodeficiency virus (HIV)

1.1. Introduction

HIV is a lentivirus, a member of the retroviridae family. The lentiviral subfamily contains five strongly related viruses, each infecting a specific species: HIV-1 and -2 (humans), SIV (primates), FIV (cats), EIAV (horses), and Visna (sheep). HIV-1 originated in West-central Africa, where a mutated SIV virion was able to infect and replicate in humans, initiating the first HIV-1 infection. HIV transmission can occur by vertical (mother-to-child) and horizontal (blood contamination and sexual intercourse) pathways. During sexual transmission, free HIV particles or cell-associated virus infect and replicate in mucosal/peripheral CD4+ T-cells, dendritic cells (DCs) or macrophages to subsequently spread out over the body[5]. Direct blood-to-blood infection (vertical transmission or contamination) is more effective as it circumvents the vulnerable mucosal stage and immediately spreads out to the periphery. During the clinical course of the infection, the CD4+ T-cell count steadily decreases and the progressive weakening of the host immune system eventually results in the development of the 'acquired immune deficiency syndrome' (AIDS)[12,13]. The diagnosis of AIDS is applied when the patient's CD4+ T cell count drops below 200 cells/ mm^3 blood or when one of the AIDS-defining opportunistic infections or cancers occurs.

1.2. The origin of HIV-1

In the first three decades of the 20th century, HIV-1 originated from West-Central Africa after several cross-species transmissions (SIVcpz) from chimpanzee to human[14]. During the manipulation of chimpanzee meat, the hunter or hunter's wife became infected through blood-to-blood contamination. In total, four different HIV-1 strains (M, N, O, and P) arose from four separate SIV contaminations, starting their chain of infections[15]. The HIV-1 M strain (M for main) was transmitted by the chimpanzee subfamily *Pan troglodytes troglodytes* and is responsible for 90% of all human infections worldwide[16,17] (Fig.1.1). The infectious advantage of HIV-1 M can be brought back to a genetically determined higher

"fitness" as compared with the "founder" viruses of groups O, P, and N[18]. Group M can be further subdivided into subtypes (A-K), but also recombinant forms between the subtypes exist[19].

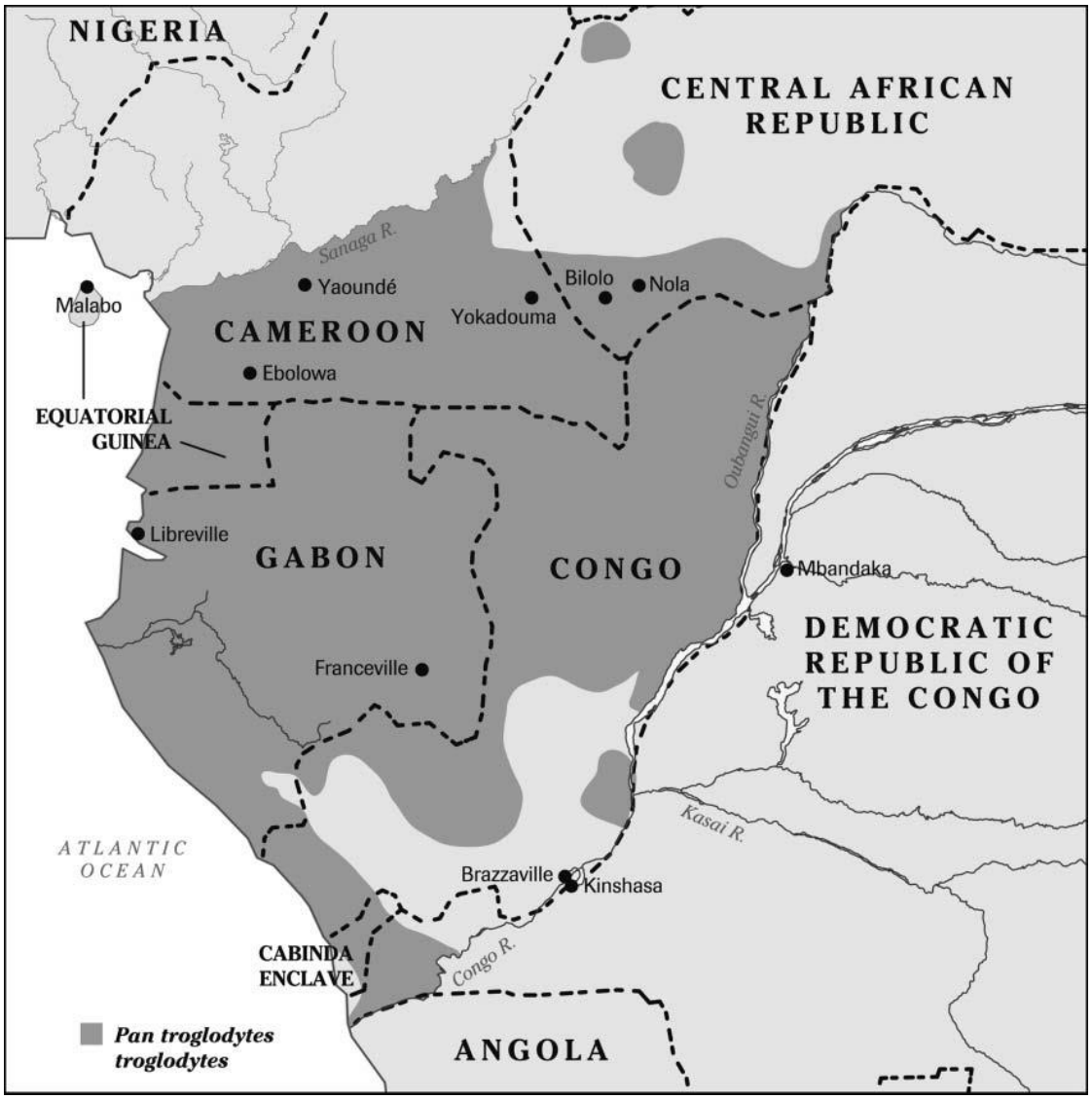

Figure.1.1: Map of central Africa with the habitat of the Pan troglodytes troglodytes, source of HIV-1: *Within this area, the first SIV cross contaminations resulting in HIV-1 should've occurred. Later on, following urbanization, HIV-1 spread to larger cities such as Leopoldville and Brazzaville (shown on the map) and eventually spread throughout Africa and reached the other side of the Atlantic ocean, Haiti (source[15]).*

Also other factors were crucial for HIV-1 to develop into a worldwide epidemic, as it is implausible that no SIVcpz cross-species transmission occurred before the 20th century. Therefore, two features of 19th-century African colonization were seen as fundamental catalyzers for the rapid spread of the HIV-1 virus: urbanization and healthcare[15]. Colonization led to urbanization, attracting in particular

unmarried men to these towns. The change in demographics led to concomitant sexual partnerships (*femmes Libres* in Leopoldville and Brazzaville), resulting in viral persistence and the spreading of the virus. Secondly, healthcare mainly focused on tropical diseases[20] (syphilis, yaws, leprosy and sleeping sickness) whereby treatment consisted of a long regimen of intravenously administered drugs. Unaware of viral contamination, used needles were shared between patients, spreading the virus over a larger population[21,22]. Since 1960, mass migration towards Leopoldville-Brazzaville, following the abrupt decolonization, further catalyzed the growth of the HIV-1 infected population, both via heterosexual (high-risk prostitution[23]) and intravenous transmission (shared needles in clinics[20]). Between 1960 and 1970, the HIV-1 virus expanded over the African continent. HIV-1 also reached Haiti, presumably by one of the many Haitian technical assistants recruited to Congo during this turbulent time[15]. Once arrived at the other side of the Atlantic, HIV-1 spread to the US, likely due to sexual tourism. From the US, HIV-1 spread to all parts of the world with strong linkage to Western Europe and Latin America. From here on out, HIV-1 diversity flourished [24-28], and a pandemic arose.

1.3. HIV-1 Epidemiology

In 2017, the world health organization and UNAIDS released an updated epidemiologic report about HIV infections (Fig.1.2). More than two out of 3 individuals with HIV-1 worldwide reside within Africa (25.7 million). In Africa, ART is available for 15.3 million (59.53%), of which children (0-14 years) have the lowest access to treatment. South-Africa has the largest epidemic in the world, with 7.1 million people infected (19% of the global burden). In Asia and the Pacific, 5.2 million people are infected with HIV, and 2.7 million (52%) of them have access to ART. In contrast to the areas in Africa, Asia focuses on ART access for infected children (71%), compared to pregnant women (56%) and (young) adults (53%). In Western and Central Europe and North America, 2.2 million people are living with HIV, of which 1.7 million have access to treatment (77%). Also Latin America, with 1.8 million HIV infected people, has a relatively high access rate (61% or 1.1 million)[1]. The figure (Fig.1.2) shows the presence of HIV in every part of the world, with high endemic areas in Africa and Asia. Most HIV infections occur in (South) Africa, accompanied and driven by low access to ART regimens.

In 2016, the UNAIDS set treatment goals for the year 2020, also known as "90-90-90" in an attempt to end the AIDS epidemic. Worldwide, 90% of the HIV-1 infected individuals should know their HIV status. Of these diagnosed patients, 90% should have access to retroviral therapy, and of the patients receiving treatment 90% will have achieved viral suppression. In 2019, at least 61 cities worldwide, mostly from high-income countries, had already attained the three goals or at least one of them[29]. To complete these goals, not only the supply of ART is necessary, but also increasing the awareness and eliminating discrimination towards HIV. Besides, social determinants such as employment seem to play a role in achieving the 90-90-90 goals[30]. By collaborating with local and federal political regimens, UNAIDS continues to raise awareness and attempts to break the social stigma surrounding HIV and ART.

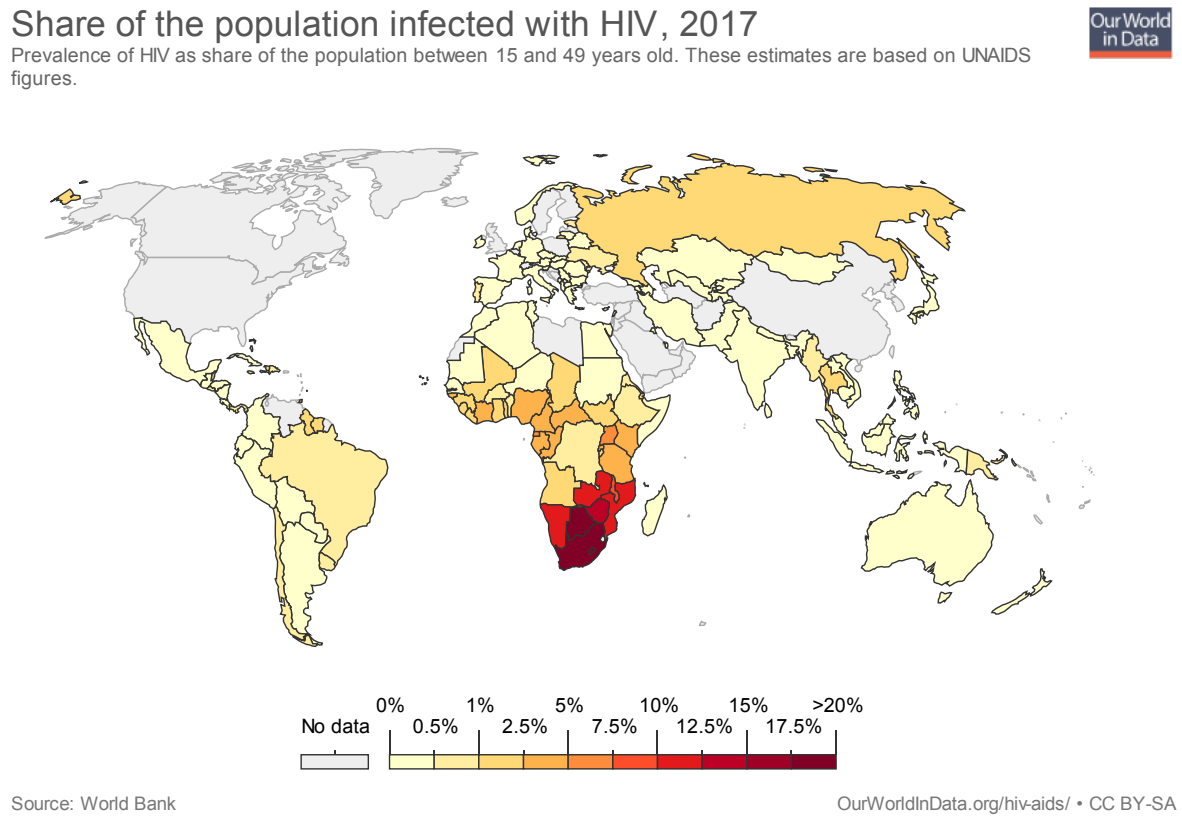

Figure.1.2: Global heat map of HIV-1 epidemiology in countries according to UNAIDS data 2017[31]*: South and Eastern Africa have the highest endemic HIV-1 infected population. HIV-1 endemic populations are also found in Western and Central Africa, Latin America, and Central Asia (source[1]).*

1.4. Virion structure and HIV-1 genome/proteome

As a member of the retrovirus family, HIV-1 virions contain a cone-shaped capsid core and possess a genome with nine genes (Fig.1.3). The morphology of a mature HIV-1 particle (Fig.1.3A) is a spherical lipid bilayer with a diameter of approximately 120 nm. The HIV envelope is composed of the host's membrane acquired during budding, containing molecules such as HLA and CD3[32]. The viral surface glycoproteins gp120 and gp41, necessary for viral entry, are anchored to the envelope. Underneath lay a matrix of p17 proteins surrounding the conical capsid, consisting of p24 capsid proteins. Inside the capsid, two copies of unspliced positive single-stranded RNA are tightly bound to nucleoproteins and enzymes essential to the development of the virion (reverse transcriptase, integrase,…)[33].

The genome of HIV-1 contains nine genes: three structural (*gag, pol* and *env*), two regulatory (*tat* and *rev*), and four accessory regulatory genes (*nef, vpr, vif,* and *vpu*) (Fig.1.3B). Transcription and translation of the three structural genes produces large viral polypeptides. Subsequently, these polypeptides are proteolyzed into proteins responsible for the structure of the virion[34]. The *gag* gene generates four viral proteins: p17/MA (matrix), p24/CA (capsid), nucleocapsid (inside core), and p6 (essential for Vpr assembly functioning). The *env* gene encodes the envelope proteins, gp120 and gp41. The three *pol* derived proteins protease (PR), reverse transcriptase (RT), and integrase (IN) are encapsulated within the core and perform vital enzymatic functions. During viral assembly and exocytosis of the virions, PR cleaves Gag and Gag-Pol polyproteins into the viral structural components, p17/MA, p24/CA, p7/NC, and p6; and also PR, RT, and IN from the Pol polyprotein[36,37]. Both RT and IN are necessary for the integration of viral RNA in the newly infected cell. Before integration, the single-stranded RNA is reverse transcribed into double-stranded DNA by RT[38,39]. Following reverse transcription, the viral DNA is integrated by IN into the host chromosome[40,41]. When the infected cell is stimulated, transcription of the host genome is accompanied by the transcription of the viral genome, inducing the production of virions. The early transcribed Tat protein enhances the transcription of the viral genome[42-44]. The Rev protein stimulates the export of unspliced and single-spliced HIV-1 transcripts to the cytoplasm by binding[45,46] and inhibiting the cleavage of viral messenger RNA (mRNA)[47].

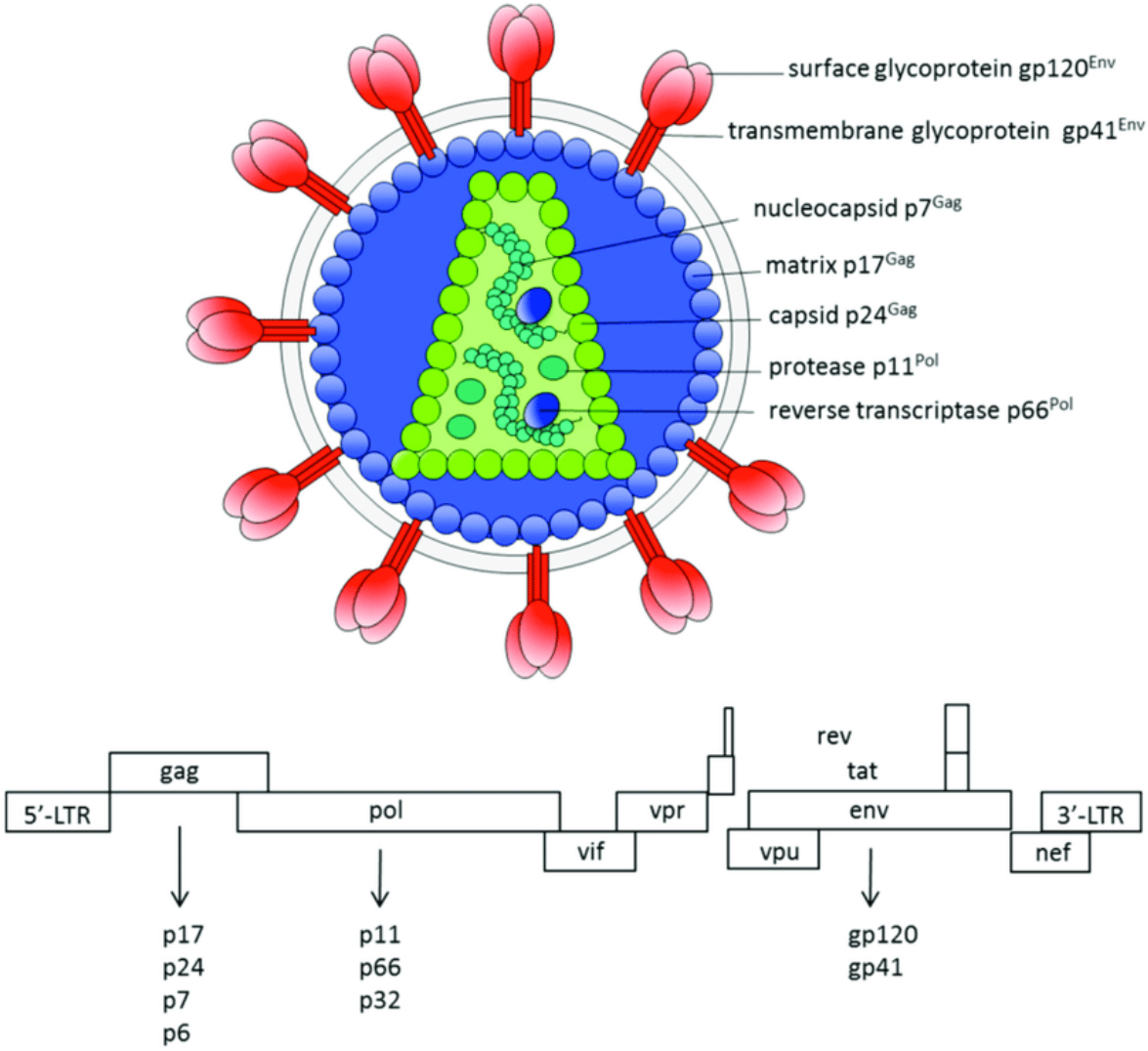

Figure.1.3: HIV-1 genome and virion structure: *Figure A) displays a fully matured budded HIV-1 virion. Figure B) represents the HIV-1 genome with the collocations of the 9 HIV-1 genes and the derivative 15 HIV-1 proteins. (source[35]).*

The accessory proteins Nef and Vpu both downregulate the expression of CD4, although in different ways. Nef facilitates the receptor degradation of membrane-bound CD4 by directing them to lysosomes[48], whereas Vpu targets newly synthesized intracellular CD4 complexes[49,50]. CD4 downregulation prevents precarious Env-CD4 interactions impairing proper budding of Env incorporated virions. Upon entry, the virion is uncoated and the RNA genome is reverse transcribed into dsDNA, which is transported to the nucleus, mediated by the accessory protein Vpr[41]. The HIV-1 protein Vif is essential for the generation of replication-competent HIV-1 virions by inhibiting the host's restriction factor APOBEC3G from entering the virion during budding[51].

1.5. HIV-1 replication cycle

1.5.1. Early phase

HIV-1 virions infect cells that express the CD4 molecule at the cell membrane, including CD4+ T cells, macrophages, and DCs. Infection occurs through a direct fusion of the external gp120 protein of the HIV virion with the amino-terminal Ig domain of the CD4 molecule (Fig.1.4). Yet, the gp120-CD4 interaction does not suffice for entering the target cell. The binding with CD4 evokes two conformational changes of gp120, first allowing it to bind with its co-receptor CXC chemokine receptor (CXCR) 4 or CC chemokine receptor (CCR) 5, followed by the revealing of the gp41 to the membrane. Gp41 merges with the cell membrane, completing the fusion with the target cells[33]. Upon entry, the intact capsid is released in the cytoplasm and transported to the nucleus for chromosomal integration[41]. Before nuclear transport, the capsid is uncoated to enter the nucleus via a nucleus pore, only allowing the passage of DNA size molecules[52]. Subsequently, a Vif and RT-driven reverse transcription of the viral genome results in the synthesis of the pre-integration complex (PIC) containing viral DNA, IN, RT, NC, and Vpr. The PIC is transported in the nucleus by Vpr, linking the PIC to the cellular nuclear import machinery. Once actively transported in the nucleus, the viral DNA is integrated into the host's genome by IN[33].

1.5.2. Late phase

When infected cells become mitotically active, transcription of the host and viral genome results in the formation of viral mRNA. The early transcribed Tat and Rev proteins, respectively, enhance viral transcription and facilitate the export of structural unspliced (Gag and Gag-Pol) or single spliced (Env, Vif, Vpr, and Vpu) mRNA. At the endoplasmic reticulum (ER), the *env* mRNA is translated into the gp160 polyprotein (gp120-gp41 precursor) and oligomerizes to a trimeric structure[53,54]. Post-translational modification in the endoplasmatic reticulum and Golgi-apparatus cleave the precursor into the associated gp120-gp41 trimeric glycoprotein. To assure undisturbed transport of gp120-gp41 to the host's cell membrane for proper viral assembly, Nef and Vpu downmodulate the intracellular and transmembrane expressed CD4 to inhibit gp120-CD4 interactions[48-50]. Due to a translational frameshift,

the associated Gag and Gag-Pol polyproteins are translated in the ribosomes. The N-terminal p17 domain of the Gag polyprotein binds with the cell membrane[55-57] and interacts with the cytoplasmic tail of gp41[58]. About 1200-2000 copies of the Gag polyprotein encapsulate two copies of the unspliced viral genome to form an immature virion. Upon budding, the Gag and Gag-Pol polyproteins are cleaved by HIV-1 PR, generating the structural proteins p17, p24, p7, as well as the enzymes PR, RT, and IN; resulting in the maturation of the virion[33,59].

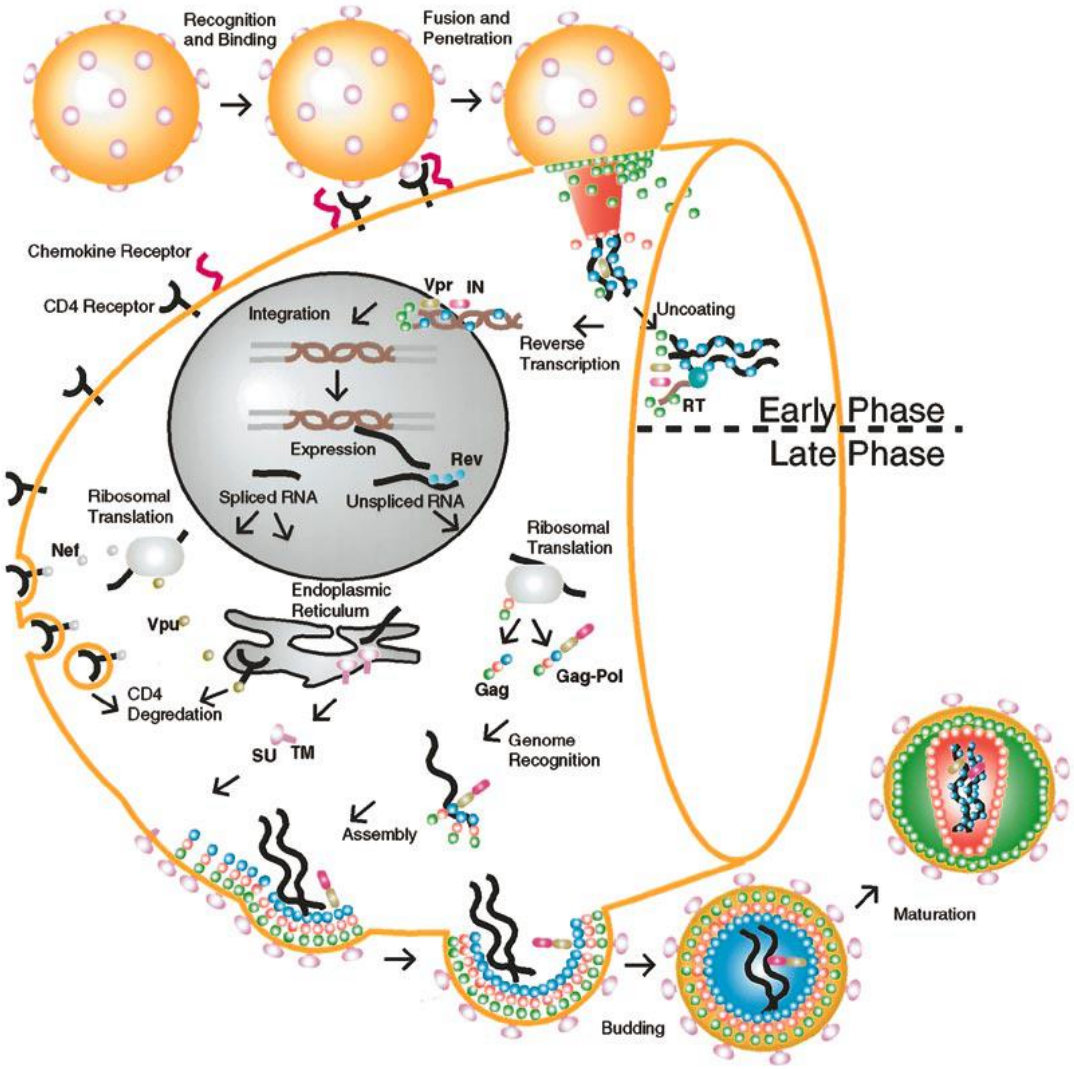

Figure.1.4: Intracellular HIV-1 cell cycle with the distinction of early and late phase*: The figure displays the main steps in the viral cycle: fusion with the target cell, integration of the viral genome, viral replication, and budding. Also, the viral proteins with enzymatic functions in viral replication are presented: Vpr-induced transportation of the viral genome in the nucleus, IN integrating the viral genome, Rev facilitating the export of unspliced proteins and, Nef and Vpu downregulating CD4. (SU:gp120, TM:gp41) (source [33]).*

1.6. HIV-1 Transmission

1.6.1. HIV-1 Transmission: A vulnerable timeframe

In most cases, HIV-1 is transmitted during sexual intercourse by semen or vaginal secretions containing free HIV virions as well as cell-associated virus (CD4+ T cells and macrophages)[60,61]. However, male-to-female vaginal sexual transmission efficacy of HIV-1 during these events is rather low (1-8 in 1000 sexual exposures)[62]. In 80% of the cases, sexual HIV-1 transmission derives from a single transmitted/founder HIV-1 clone[10]. Subsequent to sexual transmission, 97% of these viruses are R5 trophic (CCR5 as co-receptor, macrophage-tropic), which is less cytopathic compared to the X4 trophic variant (CXCR4 as co-receptor, CD4 T cell-trophic)[63,64]. An excellent location for HIV-1 transmission is the single-layer epithelium of the endocervix and transformation zone (Fig.1.5.), containing a high concentration of target cells (DCs, CD4+ T cells, and macrophages)[65,66]. Three to four days post vaginal exposure, the sparsely transmitted virions are only capable of infecting 40-50 cells, as seen in primate HIV-1 transmission models[13,67]. Subsequent propagation of the infection results in the dissemination of the virus to regional lymph nodes (LN) and later on to the gastro-intestinal associated lymphoid tissue (GALT). Systemic infection with HIV-1 preludes the substantial impairment of the immune system. In contrast, transmission can be seen as a vulnerable timeframe for HIV-1 to encounter a protective immune response, if it is sufficiently potent and quick.

1.6.2. The superior migration capacities of virally-infected cells

During unprotected sexual intercourse, HIV-1 is transmitted by the exposure of the uninfected anal, vaginal or penile tract to the infected semen or anal/vaginal fluid. In male-to-female transmission, both viral particles, as well as virally infected cells can transmit the virus[69]. In the semen, macrophages and CD4+ T cells constitute the main source of infected cells, with an average infection rate of 0,2% in untreated HIV-1 individuals, as well as SIV infected macaques[70]. The viral particles in the semen derive from the bloodstream or tissue are produced by CD4+ cells in the semen[71].

In both *in vivo* murine[72-75] and simian models[76-78], cell-associated HIV-1 virus was able to migrate over the epithelial layer and infect the host after vaginal inoculation. In human *ex vivo* and *in vitro* cervicovaginal tissue explant models, multiple studies confirmed the migration of HIV-1 infected cells over the epithelial monolayers and cervical tissue, infecting the sub-epithelial cells[79-86]. Also, the HIV-1 genotype of acutely infected women correlated with the genotype of the cell-associated rather than the cell-free virus found in the semen of the donor[69]. *In vitro,* cell-to-cell infection of naive lymphocytes by infected cells (CD4+ T cells and macrophages) was 100 to 1000 fold more efficient compared to cell-free infection of cells. This efficiency was ascribed to the generation of a viral synapse, bringing the virion in the proximity of the target cell's CD4 receptor[87-89]. Also, membrane protrusions such as filopodia and tunneling nanotubes are gateways for cell-to-cell infection[90].

Besides, a static short-lived HIV-1 virion would become trapped in the genital mucus, whereas an "encapsulated" cell-associated virus can withstand the antiviral properties of the genital mucus[91,92]. In line with these results, infected T cells present in the semen are called "Trojan horses", for their capacity to safely navigate the virus across the barrier and release it once transmitted. Upon encountering the anal/vaginal epithelium, the cell-associated virus can transmigrate the cervicovaginal epithelium and infect the submucosal target cells[74,75,81-86]. This transmigration is modulated by the lymphocyte function-associated antigen 1 (LFA-1) -ICAM interaction between the infected cell and vaginal epithelial cells[93,94]. On the other hand, a cell-free virus can interact with host epithelial cells by binding heparan sulfate proteoglycans and glycosphingolipids[95,96] as well as DC-SIGN/Langerin[97-99]. Although different pathways are used, the cell-associated virus is considered to be at least as effective as viral particles in penetrating the cervical epithelial layer.

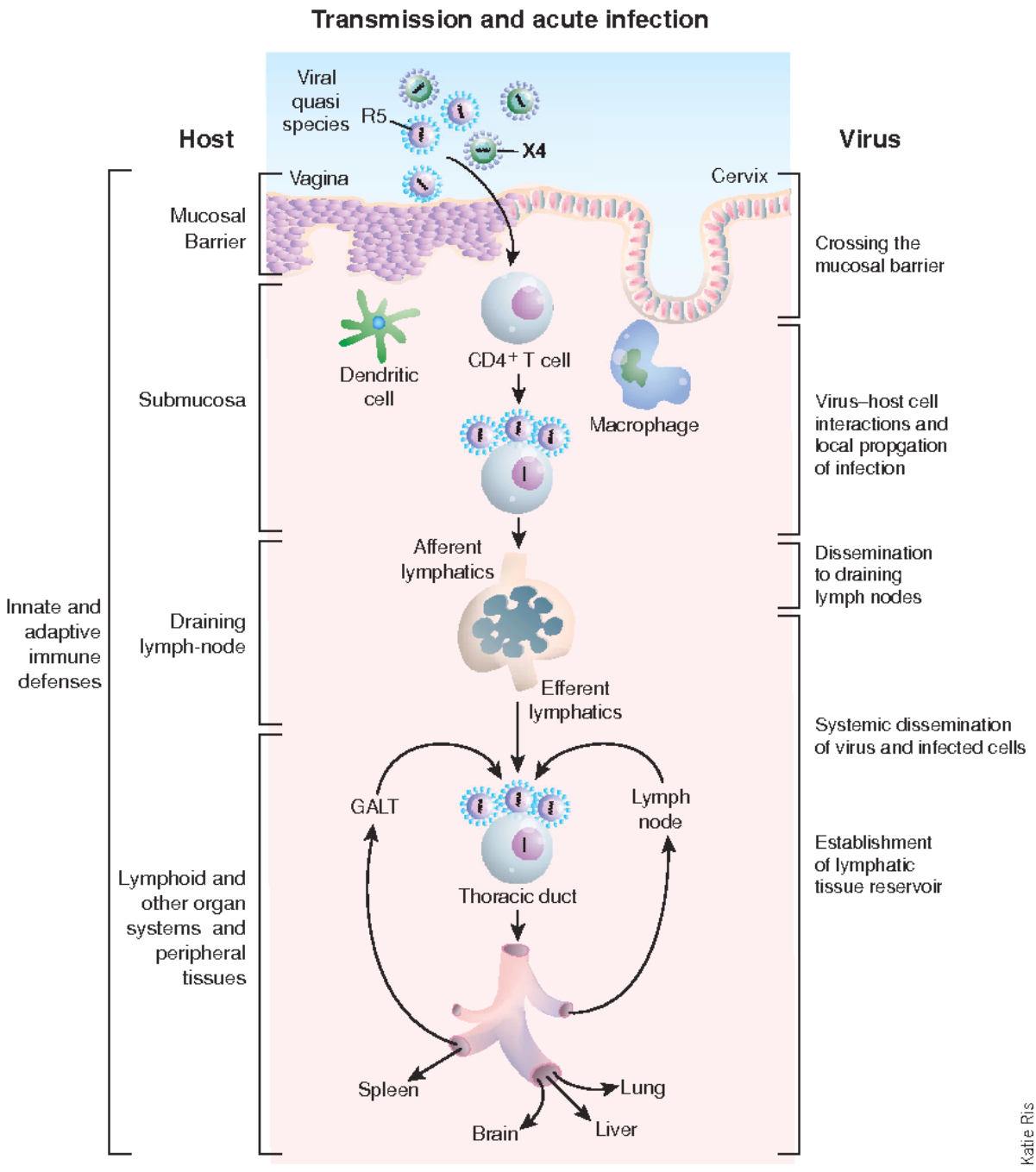

Figure 1.5: Sexual HIV-1 transmission and subsequent initial propagation: *During male to female sexual transmission, the virus can cross the multiple layer vaginal epithelium (1) or the single columnar epithelium of the transformation zone (2) and cervix (3). Predominantly CCR5 tropic viruses can infect T cells, macrophages or DCs at the portal of entry. Subsequent propagation of infection results in small founder foci. The increasing viral replication leads to systemic dissemination by migrating to draining lymph nodes (source[68]).*

1.6.3. Factors influencing HIV-1 transmission

Besides intrinsic viral "fitness", the infection efficiency of HIV-1 is also subjected to factors affecting the infectiousness of the HIV-1 donor and the susceptibility of the naïve recipient[100]. First of all, a high viral load in the HIV-1 donor benefits transmission by an increased concentration of virus in the blood or genital tract/secretions[101,102]. In accordance, susceptibility to HIV-1 depends on the frequency of target cells at the site of transmission[102,103]. Secondly, the integrity of the genital mucosa/physical barriers is necessary for a proper anti-viral defense. A healthy genital tract is a robust barrier containing mucus, epithelial layers, antibodies, and defensive proteins (α-defensins, immunoglobulins, and complement)[104]. Infection with sexually transmitted diseases (STD) interrupts the genital barrier by ulcerations and inflammation[100]. The disrupted or inflamed epithelium facilitates the transport of virions and infected cells[105], increasing both donor infectivity as well as recipient susceptibility. STDs also induce urethral inflammation, attracting both target cells in naïve recipients as well as HIV-1 virions and infected cells in HIV-1 donors[106-108]. As these co-factors highly influence HIV-1 transmission, effective treatment, and prevention of STD's lead to improved HIV-1 prevention.

In male-to-female sexual HIV-1 transmission, semen functions as a vehicle for viral particles and virally infected cells (CD4+ T cells and macrophages[70]). When introduced into the female genital tract (FGT), semen modulates the anti-viral barriers, irrespective of HIV-1 exposure (Fig.1.6.). Semen neutralizes the acidic environment of the cervical mucus, increasing HIV-1 particle diffusion, which would otherwise trap the virus[91]. Semen also contains a prostatic alkaline phosphatase (SEVI)[109] and semenogelins (SEM1 & SEM2)[110], two amyloid fibrils promoting the attachment of the virus to potential target cells. Also the complement inhibiting factor CD59 is observed in semen, preventing the opsonization of the virions and infected cells[111]. Indirectly, seminal plasma triggers the production of CC chemokine (CCL) 2 by ectocervical epithelial cells, facilitating viral transmission by attracting target cells such as CD4+ T cells to the vaginal mucosa[112]. In the semen of HIV-1 patients, cytokines (IL-7 and G-SCF) are upregulated, which supports viral survival and replication during transmission[113,114]. The presence of these cytokines in the semen is related to inflammation of the male

genital tract induced by STDs and is also associated with a high viral load and virally infected cell concentrations in the seminal plasma[100,115-118].

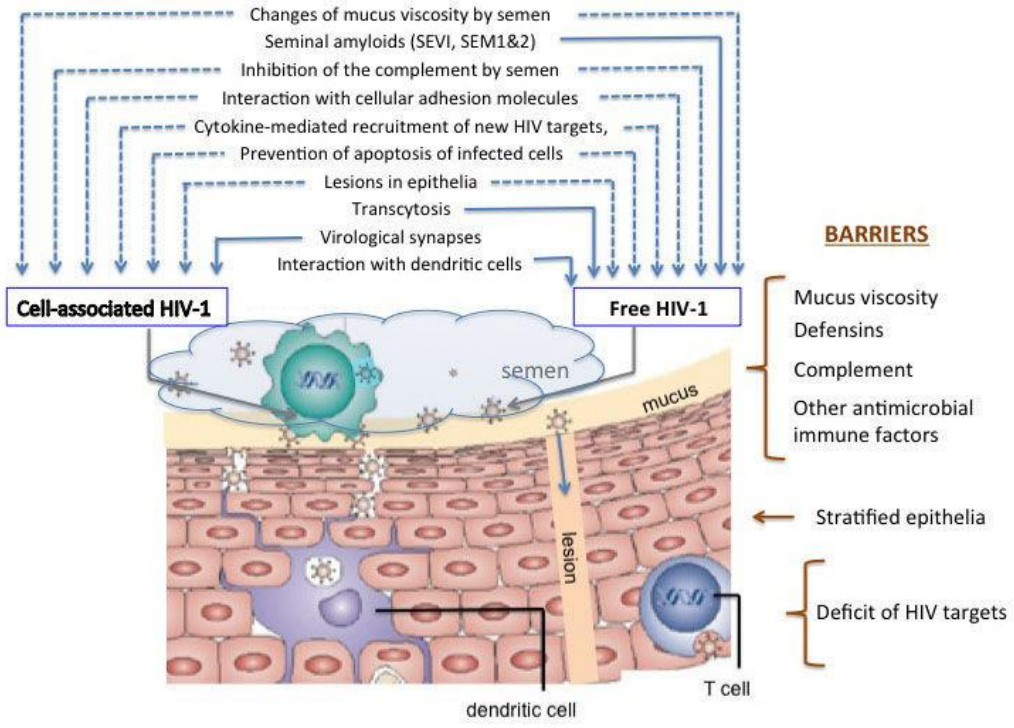

Figure 1.6: Factors influencing the efficiency of HIV-1 vaginal transmission: *Barriers that have to be overcome (brown) are opposed to factors facilitating transmission (blue). In-depth, certain factors contribute equally to the transmission of cell-associated as well as cell-free virus (dashed blue lines), whereas others mainly promote the transmission of the cell-associated or cell-free virus (solid blue lines) (source[119]).*

1.7. HIV-1 Pathogenesis

1.7.1. HIV-1 acute infection

The first transmission events are followed by an eclipse phase of one to three weeks, with local reproduction and formation of small foci consisting of 40 to 50 infected target cells[13,67]. The preferred initially infected target cells are CCR5+ CD4+ T cells (active memory T cells), whereas also plasmacytoid DC's (pDCs) or macrophages are known to take up HIV-1 at the site of infection and cluster with CD4+ T cells. After 3-4 days, these clusters start to expand locally by infecting new target cells such as pDCs, attracted from the mucosal epithelium by the viral-upregulated MIP3-α/CCL20. In turn, infected pDCs attract CD4+ T cells and macrophages, accelerating the generation of the small

founder populations[5]. From there, the virus spreads to the local LN and GALT[12]. In the GALT, a large proportion of leukocytes are Th17 cells that sustain the enteric homeostasis by producing IL-17 and IL-22, as well as the anti-microbials for the mucosal barrier[120,121]. HIV-1 infection of the GALT leads to the loss of gut integrity by depleting the (Th17) CD4+ T cell subset and the upregulation of inflammatory cytokines[122,123]. The loss of gut membrane integrity results in the influx of bacterial products (Lipopolysaccharide), that forces the systemic immune system into a constant state of inflammation/activation. Eventually, this will wear out the immune response, as HIV-1 keeps infecting these activated target cells[124,125]. Between 7 and 21 days post-infection, the virus becomes detectable in the blood plasma[126], which heralds the end of the eclipse phase[127]. Upon reaching the systemic LN, the infection starts to reproduce exponentially in the presence of high concentrations of CCR5+ CD4 T cells[128]. These events can be diagnosed by a peak in the viral load in the blood and depletion of circulating CD4+ T cells, sometimes accompanied by a "flu-like" syndrome.

1.7.2. HIV-1 chronic infection

After 2-3 weeks, the first wave of adaptive immune responses, mainly cytotoxic CD8+ T cells (CTL) and neutralizing antibodies counter the spreading viremia and halt the depletion of CD4+ T cells. The plasma viral load drops to a labile equilibrium value, known as the "viral set point" (Fig.1.7)[125]. Although acute or unchecked viral replication is stopped, adaptive immune cells are incompetent to eliminate all HIV-1 virions or infected cells. Mutations in the HIV-1 genome, resulting in a highly diverse repertoire of HIV-1 antigens, keep the virions and infected cells one step ahead of the immune system. Unable to cope with the mutation speed of HIV-1, the immune system becomes more and more "exhausted", and slowly but surely, the CD4+ T cell concentration decrease[125]. In this chronic stage, the virus spreads to various tissues to establish the HIV-1 reservoir (e.g., the central nervous system, the germinal centers of lymph follicles…), out of reach for the immune system. At a concentration lower than 200 cells/mm³, a chronic HIV-1 patient is diagnosed with AIDS[129]. At this stage, the level of impairment makes the immune system incapable of controlling opportunistic infections and cancers[130].

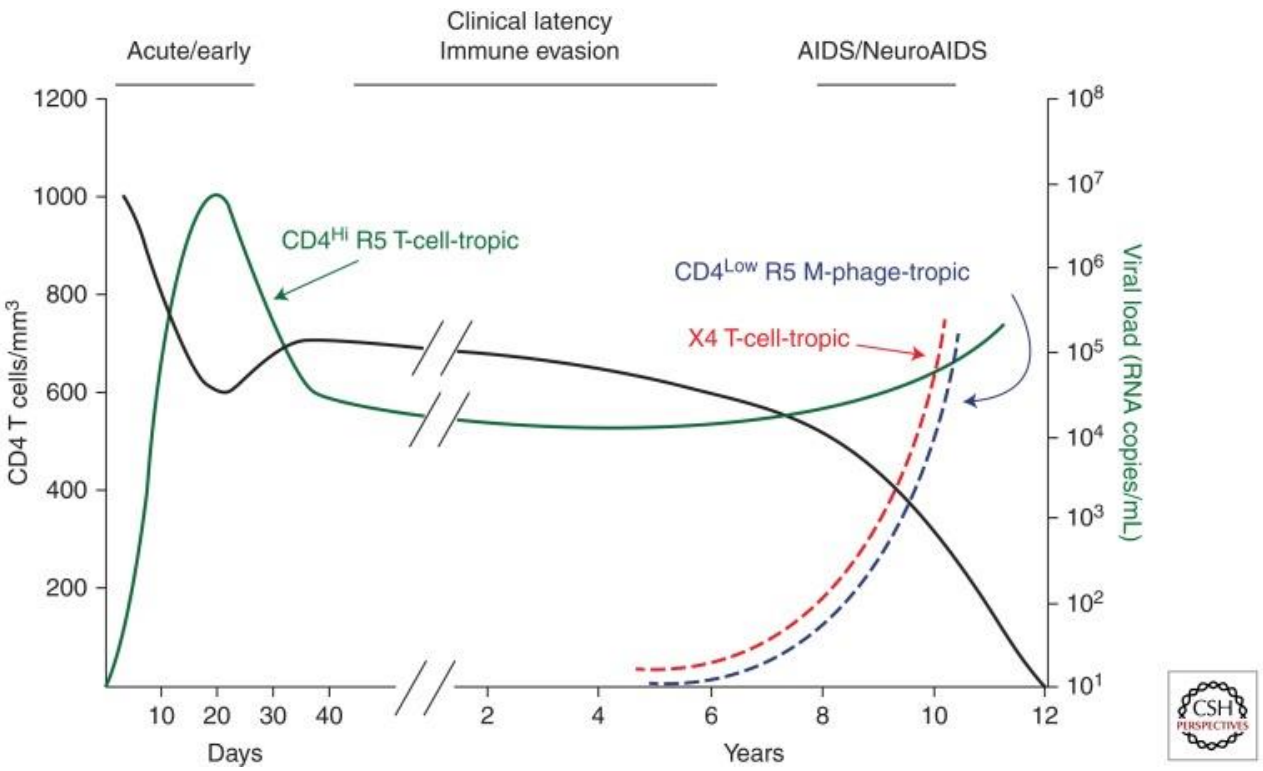

Figure.1.7: HIV-1 viral load and CD4+ T cell concentrations in the different stages of HIV-1 disease progression: *The viral load (mRNA copies/ml) and CD4+ T cell concentrations (# cells/mm³) are two diagnostic markers used in the follow-up of HIV-1 patients. These parameters can predict the severity of the disease progression and the efficiency of the current ART treatment. On the graph, the drop in CD4+ T cell count and the peak of viral load during the acute phase is known as the "viral zenith" and is also a predictive marker for disease progression. An early viral set point will result in a limited loss of CD4+ T cells and viral load growth, high viral set point and is associated with a slow progress towards AIDS. Whereas a late adaptive immune intervention or exponential growth of the virus, high viral set point, is related to a rapid progression towards AIDS. Towards the end of the chronic phase, the highly mutated HIV-1 population also contains HIV-1 virions capable of infecting naïve CD4+ T cells (red, X4) and macrophages (blue, R5), ultimately impairing the immune system and generating an additional reservoir, respectively (source[124]).*

1.7.3. HIV-1 reservoirs

Most studies use blood as a primary source of HIV-1 isolation for genetic sequencing. However, to what extend the HIV-1 sequences in the blood resemble the sequences found in tissue is unclear. As seen by Anderson et al.[71] (Fig.1.8), phylogenetic trees of HIV-1 sequences found in tissue (semen) and blood exposed three potential compartment interplays. In most cases, viral sequences are comparable between blood and tissue as a result of consistent migration and exchange between tissue and blood. In contrast, HIV-1 replication without inter-compartment exchange can occur and results in the generation

of tissue-specific viral sequences[71]. Lymphoid tissue is the primary source of the diversity of HIV-1 sequences found in the blood. In the LN, homogenous foci of infected cells reside at the outside of the germinal centers within the paracortex[131]. Each focus independently produces virions that transduce from the tissue to the blood. Conversely, blood-derived sequences can take over the LN sequences, as is seen with CTL escape isolates or drug-resistant recombinants[3,132].

HIV-1 virions are also found in the cerebrospinal fluid and seem associated with tissue-related pathologies of the central nervous system (CNS), suggesting the occurrence of viral replication[133]. The viral sequences found in the CNS are compartmentalized[134] and diverted into macrophage-R5 trophic viruses[135]. The compartmentalization suggests a limited exchange of HIV-1 virions or infected cells over the blood-brain barrier[124]. Another location where the HIV-1 resides is the male (MGT) and FGT. In the MGT, multiple anatomical sites (prostate, testes)[136,137] accommodate HIV-1 target cells. Although a transparent mixed interplay was suggested[138,139]; apparent differences in viral sequences between semen-/testis-derived and blood-derived target cells suggest stable local replication and compartmentalization[71,140-143]. In the FGT, local foci of HIV-1 infected cells could not be pinpointed to any distinct anatomical sites. Nevertheless, HIV-1 virions and infected cells are found in FGT mucus and (sub)epithelial border. Although tissue-specific virus strains were detected at the FGT, the differences were ascribed to clonal amplification, a mechanism related to compartmentalization in short time studies, but not related to the long term compartmentalization[144-146]. Other tissues known to house the HIV-1 reservoir are the kidneys, lungs, and breasts[124].

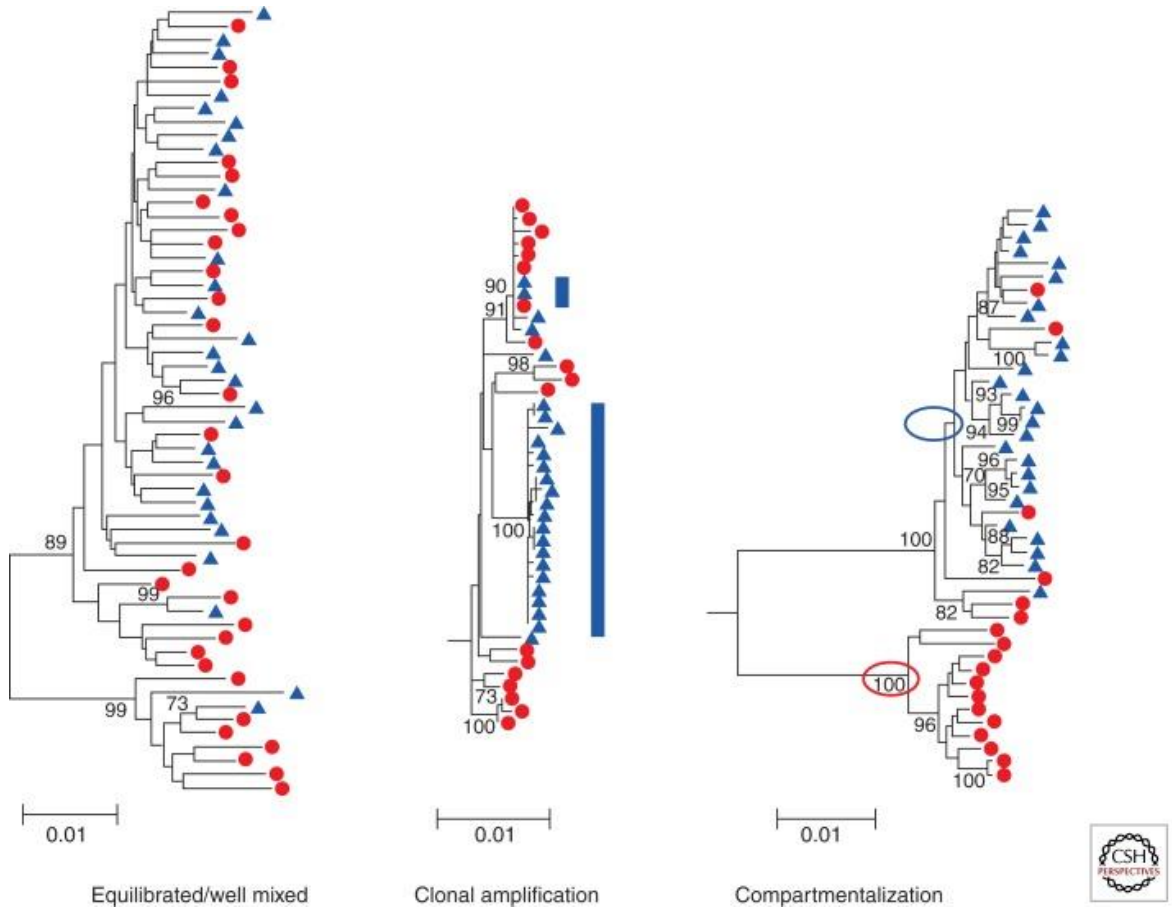

Figure.1.8: Phylogenetic trees of HIV-1 sequences in blood and semen: *These phylogenetic trees point out the variations in genomic diversity between blood (Red) and semen (blue). In the first tree, there is an equal exchange between viral sequences of the blood and tissue, called a mixed model. In the second tree, a dense concentration of tissue target cells with nearly identical amplified clones diverted from the blood sequences. However, there is still a slight exchange between tissue and blood. In the third tree, a constant distance in the genetic lineage between blood and tissue is generated by local replication and diversification foci in the tissue, distinct from the sequences found in the blood, but not separated*[71].

1.8. Clinical complications of HIV-1

1.8.1. Diagnosis of HIV-1

During the eclipse phase, infected individuals display nonspecific viremic symptoms. Complaints about fever, sore throat, lymphadenopathy, and rash are, in most cases, mistaken for a generic flu-like infection. Without any indications, the subclinical HIV-1 infection can disseminate without being noticed or treated[147]. Relevant early biomarkers used for diagnosis of HIV-1 and follow-up, are viral RNA and HIV-1 antibodies detected in the blood. The first detectable marker is viral RNA (viral load)

and is highly present in the blood after the first few weeks of HIV-1 infection. Anti-HIV-1 antibodies are present during these few weeks, but not at a detectable concentration. Hence detection is only possible 1 to 2 months post infection[125]. Fiebig and colleagues[148] differentiated six stages based on the stepwise presence of various viral and immunological markers. The different stages correspond to the presence of HIV-1 viral RNA (Fiebig I), the viral p24 antigen (Fiebig II) and the detection of HIV-1 antibodies using ELISA (Fiebig III) and Western Blot (indeterminant banding pattern (Fiebig IV), diagnostic banding pattern without p31 reactivity (Fiebig V) and diagnostic banding pattern with p31 reactivity (Fiebig VI)).

At the end of the acute phase, the appearance of anti-HIV-1 adaptive immune responses reflects a stagnation of the viral load (viral zenith) before a drop. After the introduction of adaptive immunity in the balance, the viral load stabilizes towards equilibrium, the viral set point. In most cases, the viral set point is used as a predictive value for the rate of AIDS progression. A low viral set point is related to viral control, whereas a viral set point exceeding 100,000 copies/ml is related to rapid progression towards AIDS[125]. As the viral load is a somewhat incomplete marker to predict disease outcome[149], markers of systemic immune activation seem to be a more useful tool for predicting the progression risk[64,150,151]. In Fig.1.9.,

1.8.2. AIDS-related complications

The more severe clinical complications of HIV-1 infection appear in the final stage of the infection, AIDS. At this stage, HIV has impaired the immune system to such a degree that commensal and other opportunistic pathogens are free to spread and propagate. Pathogens such as *Pneumocystis jirovecii*, mycobacteria, cytomegalovirus, Toxoplasma gondii, Cryptococcus as well as malignancies such as non-Hodgkins lymphoma and Kaposi's sarcoma commonly take advantage of the impaired T cell compartment of HIV-1 infected individuals. Also, the humoral defense is impaired and infected individuals become prone to *Streptococcus pneumoniae* infection[152,153]. All of the complications and opportunistic pathogens related to HIV-1 infection are combined in Table.1.1[154].

Complications in Patients with HIV/AIDS

System	*Direct effect of HIV infection*	*Common complications*	*Associated pathogens*	*Antiretroviral treatment-related adverse effects*
Neuropsychiatric	HIV-associated neurocognitive disorders, neuropathy, radiculopathy, myelopathy	Primary central nervous system lymphoma Chronic psychiatric disorders	*Cryptococcus neoformans*, CMV, JC virus, *Toxoplasma gondii*	Efavirenz (Sustiva): vivid dreams, sedation NRTIs: peripheral neuropathy
Head and neck	HIV-associated retinopathy	Gingivitis, dental and salivary gland disease	Retinitis: CMV, *T. gondii* Acute retinal necrosis and progressive outer retinal necrosis: HSV, varicella zoster virus Otitis, sinusitis: invasive fungi	—
Cardiovascular	HIV-associated cardiomyopathy Atherosclerosis	Cardiovascular disease, endocarditis	Myocarditis, pericarditis: CMV, invasive fungi, *Mycobacterium* species, *T. gondii*	Abacavir (Ziagen): cardiotoxicity* Protease inhibitors: dyslipidemia
Pulmonary	HIV-associated pulmonary hypertension Emphysema*	Chronic obstructive pulmonary disease, lung cancer (including Kaposi sarcoma and lymphoma)	Pneumonia, pneumonitis: CMV, invasive fungi, *Pneumocystis jiroveci* (formerly *Pneumocystis carinii*), *T. gondii* Pulmonary tuberculosis: *Mycobacterium tuberculosis*	—
Gastrointestinal	HIV-induced enteropathy Nonalcoholic fatty liver disease*	Viral hepatitis, lymphoma, Kaposi sarcoma, HPV-related malignancies	*Candida* species, CMV, HSV, protozoa	NRTIs: pancreatitis Protease inhibitors: diarrhea, fatty liver*
Renal/ genitourinary	HIV-associated nephropathy	Chronic kidney disease not caused by HIV-associated nephropathy,	Sexually transmitted infections (e.g., *Chlamydia trachomatis*)	Protease inhibitors: nephrolithiasis Tenofovir (Viread): nephrotoxicity
Endocrine	Impaired lipid and glucose metabolism HIV-associated wasting Lipodystrophy Hypogonadism,* premature ovarian failure	—	Adrenal gland infiltration: CMV, invasive fungi, *Mycobacterium* species	Protease inhibitors: lipid or glucose disorders, lipodystrophy
Musculoskeletal	Myopathy, myositis	Osteopenia, osteoporosis, osteonecrosis	—	NRTI or NNRTIs: osteomalacia* Protease inhibitors with statins: myopathy
Hematologic or oncologic	Anemia of chronic disease Coagulation disorders*	Lymphoma, multiple myeloma	Bone marrow infiltration (leading to pancytopenia): CMV, invasive fungi, *Mycobacterium* species	Zidovudine (Retrovir) and trimethoprim/ sulfamethoxazole (Bactrim, Septra): anemia
Dermatologic	Eosinophilic folliculitis*	Papulosquamous disorders (e.g., eczema, seborrheic dermatitis, psoriasis); molluscum contagiosum; Kaposi sarcoma	Fungal dermatoses, varicella zoster virus	—

CMV = cytomegalovirus; HIV = human immunodeficiency virus; HPV = human papillomavirus; HSV = herpes simplex virus; NNRTI = nonnucleoside reverse transcriptase inhibitor; NRTI = nucleoside reverse transcriptase inhibitor.

**—Research suggests an association, but evidence is not definitive.*

Table 1.1. A comprehensive overview of all the complications related to HIV-1 infection: *This table combines a few determinants to describe the various HIV-1 complications. Determinants as System, Direct effect of HIV-1 infection, common complications, associated pathogens, antiretroviral treatment-related adverse effect are enlisted above (source[154]).*

1.8.3. Anti-retroviral therapy

In the absence of an effective HIV-1 vaccine to prevent new infections, anti-retroviral treatment (ART) plays a significant role in preventing the intra- and interindividual spread of the virus. Before 1988, the treatment regimen of HIV-1 consisted of the treatment of AIDS-related clinical complications and opportunistic infections. Since the discovery of anti-retroviral agents such as reverse transcriptase and protease inhibitors, HIV-1 morbidity and mortality dramatically decreased[156-159]. When taken correctly, the combi-therapy HAART (Highly Active ART) will repress the virus to undetectable levels in the blood and semen, limiting the risk of HIV-1 transmission. Hence the adage "undetectable equals untransmittable" or "U=U". As the virus gets repressed, the immune system gets the chance to reconstitute[160-162]. If the adherence to treatment is not sufficient, the pressure generated by ART on the highly mutating HIV-1 may result in resistance against various anti-retroviral drugs[163].

ART is a treatment regimen containing one or more pharmacological agents intervening with the HIV-1 replication cycle, such as nucleoside-analog reverse transcriptase inhibitors, non–nucleoside reverse transcriptase inhibitors, integrase inhibitors, protease inhibitors, fusion inhibitors, and coreceptor antagonists (Fig.1.9). Based on their intervention in the viral cycle, at least three drugs out of at least two different classes are prescribed in the regimen to avoid drug resistance[158,164-166]. Some studies even showed that some agents would pressure the virus in a mutational "corner", where it would be more vulnerable to agents from other classes. Despite these actions, resistance against drugs of all classes is documented.

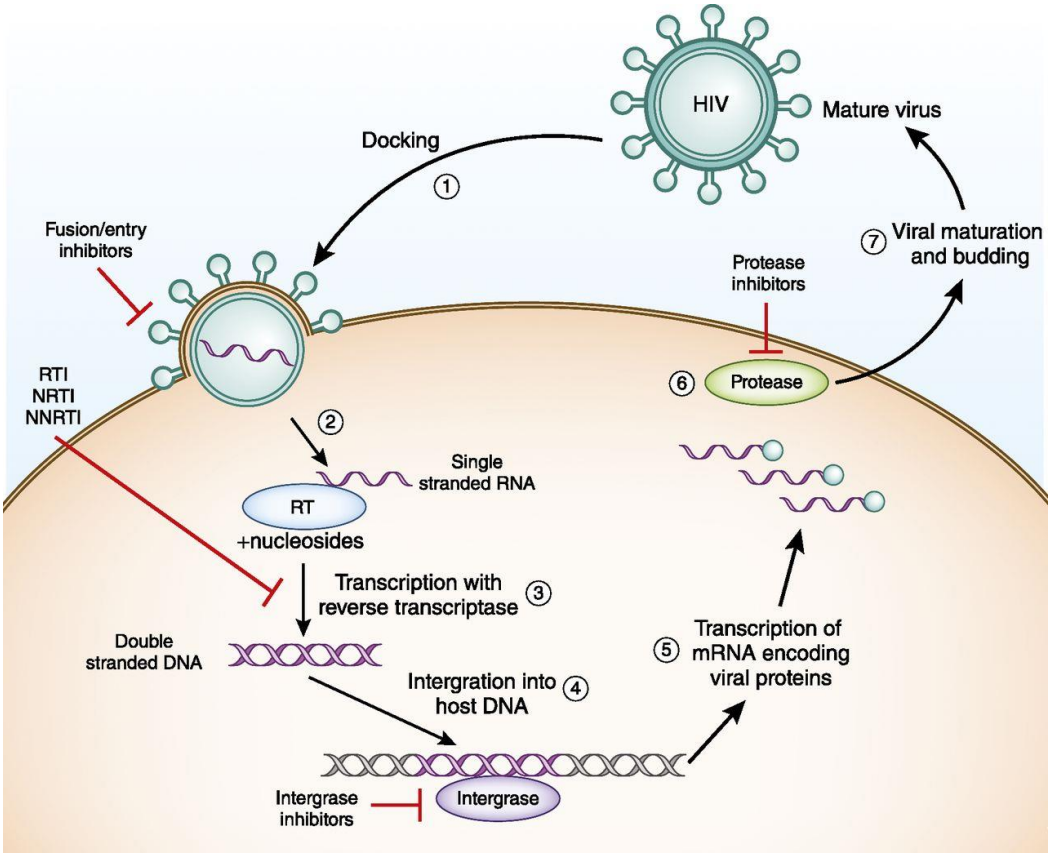

Figure.1.9: Classes of ART drugs and their role in intervening with the HIV-1 replication cycle: *The different steps of the HIV-1 replication cycle are numbered on the figure from one to seven. The different classes of antiretroviral drugs are shown as red lines near the numbered step in the cycle it inhibits. NNRTI, non-nucleoside reverse transcription inhibitor; NRTI, nucleoside reverse transcription inhibitor; RT, reverse transcription; RTI, reverse transcription inhibitor (source[155]).*

2. Natural Killer cells

2.1. The immune system: introduction

A healthy immune system protects against pathogens and malignancies. The immune system consists of secreted molecules (antibodies, defensins, complement factors, cyto- and chemokines) and immune cells, each with their specific role in protecting the host. Within the immune system, two communicating classes of immune cells are distinguished: the innate and the adaptive immune system (Fig.1.10). Innate immune cells originate in the bone marrow and start a constant patrolling from here on out, throughout the periphery of the body. Therefore, innate immune cells are the first ones to encounter pathogens and produce the first line of defense and conduct the antigen presentation to the adaptive immune system. The adaptive immune system becomes activated by this antigen presentation in combination with co-stimulation. Once activated (1-2 weeks post-exposure), the adaptive immune system generates an antigen-specific immune response consisting of cytotoxic cell-to-cell contacts as well as the production of antibodies and cytokines. Cells of the adaptive immune system are also capable of generating a memory of encountered pathogens, resulting in a rapid response upon re-exposure.

The innate immune system contains not only cells but also comprises anti-viral and anti-microbial molecules such as complement, defensins, and chemokines, as well as anatomical barriers (skin, gut, vaginal tract, lungs). The innate immune system is responsible for a variety of functions: recognition and elimination of foreign substances by innate immune cells (macrophages, NK cells, neutrophils), antigen presentation and stimulation of the adaptive immune system (macrophages and DC's), recruitment of immune cells to the site of infection by the production of chemo-attractant factors, and serving as the first line of defense for incoming pathogens by generating a physical (mucus, skin) and a biological (anti-microbial, -viral products) barrier. Innate immune cells recognize pathogens by receptors widely expressed on the membrane or intracellularly in various cell types i.e., pattern recognition receptors (PRR's) recognizing pathogen-associated molecular patterns (PAMP's) and antibody (Fc) receptors binding antibody-restrained pathogens. Other receptors can also be cell-specific such as the KIRs on NK cells and DC-SIGN on DC's.

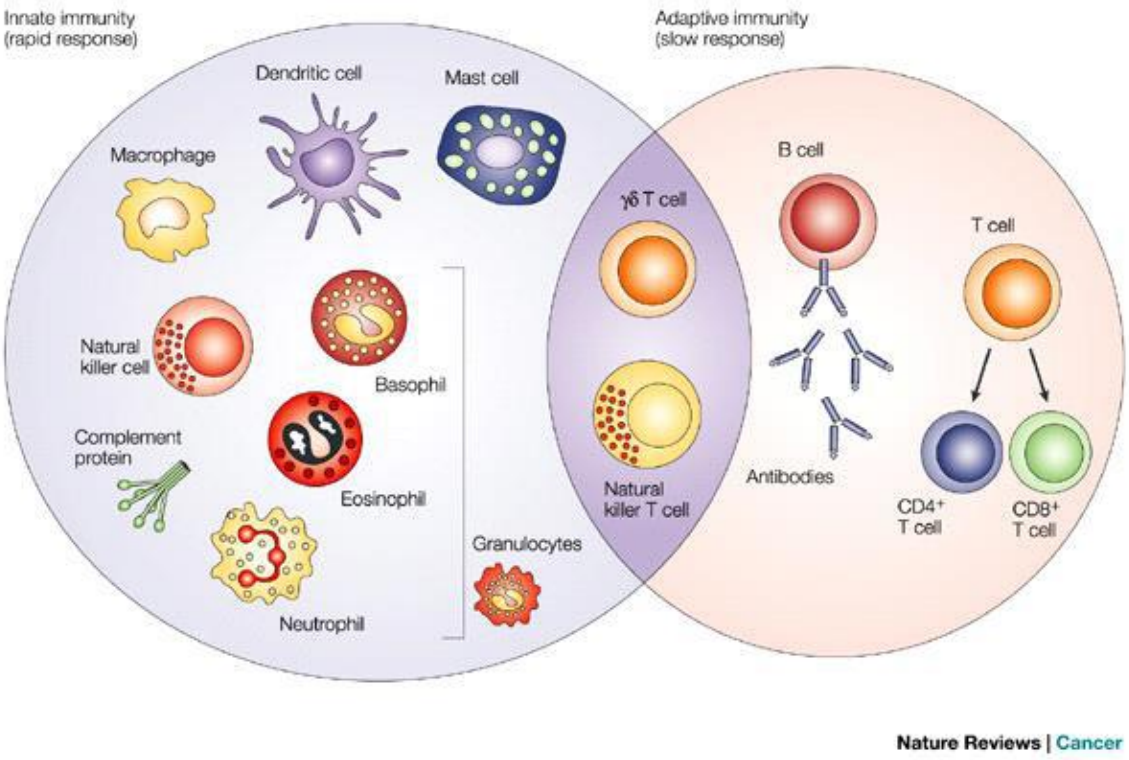

Figure.1.10: Different types of innate and adaptive immune cells: *The immune system is divided into the innate and adaptive immune systems. Cells of the innate immune system can rapidly respond to a threat, whereas adaptive immune cells need prior sensitization before becoming activated. The innate immune system consists of antigen-presenting cells (DC's and macrophages), granulocytes (basophils, eosinophils, and neutrophils), cytotoxic cells (NK cells) and the allergy and parasite related mast cells. The adaptive immune system comprises of two specific cell populations. On the one hand, CD4+ "helper" or CD8+ "cytotoxic" T cells, and on the other hand, B cells responsible for the production of antibodies. Based on receptor profiles, both γδ T and NKT cells are seen as members of both innate and adaptive immunity (source[167]).*

2.2. Natural Killer cells: introduction

2.2.1. Introduction

NK cells are a part of the innate immune system and react to virally-infected and transformed cells. In healthy conditions, NK cells constitute 5–20% of lymphocytes in the blood. NK cells can also reside in peripheral tissues such as the bone marrow (BM), liver, uterus, spleen, and lung, as well as in the secondary lymphoid tissues (SLT), the mucosal-associated lymphoid tissues (MALT) and the thymus[168]. NK cells can directly kill the infected or transformed cells by exocytosis of granules filled

with cytotoxic mediators, perforin and granzyme B. NK cells also exert immunoregulatory functions by producing cytokines (IFN- α/γ, tumor necrosis family (TNF) α, IL-6, -7, -10, -12, -13 and granulocyte-macrophage colony-stimulating factor (GM-CSF)) and chemo-attractant factors (CCL2-5, 8-10 and -26)[169]. Two functionally mature NK cell populations can be identified phenotypically, based on the expression of CD56 and CD16 on the membrane[170-172]: cytotoxic $CD56^{dim}$ CD16+ ($CD56^{Dim}$ NK, produce perforin and granzyme B) and the immunoregulatory $CD56^{bright}$ CD16- ($CD56^{Br}$ NK, secreting cyto- and chemokines). According to the functional diversity, the two NK cell subpopulations express a variety in the receptors.. Most $CD56^{Dim}$ NK cells express inhibitory as well as activating KIR's, whereas $CD56^{Br}$ NK cells primarily express the heterodimeric CD94-NKG2A/C receptors. Whether an NK cell remains quiescent or becomes active is decided by the balance of inhibitory and activating impulses generated by receptor-ligand interactions during encounters with other cells[173-180].

2.2.2. Innate Lymphoid Cells (ILC)

In tissue, cells with both NK and T cell-like characteristics can be distinguished, ILCs. In analogy with T cell functionality, NK cells, ILC-1, -2 and -3 resemble CD8+ T cells, T_{h1}-, T_{h2}- and T_{h17} cells, respectively[181]. In analogy to T cell subsets, specific threats tend to expand and activate different ILC groups: intracellular microbes, viruses, and tumors mainly stimulate the ILC-1s; ILC-2 by large parasites, adipose tissue, tissue injury and allergens; and ILC-3 cells by extracellular microbes, bacteria and fungi[182] (Fig.1.11). Both NK cells and ILC-1's produce IFN-γ[183], whereas maturation, as well as cytolytic response, seem to differ between both cell types. ILC-1 maturation is T-bet related and uses the cytotoxic TNF-related apoptosis-inducing ligand (TRAIL) pathway[184], whereas NK cells secrete cytotoxic granules and mature in the presence Eomes[185-187]. Furthermore, no phenotypical markers can discriminate between NK cells from ILC-1's as most of these innate markers are under influence of the inflammatory state and are tissue-dependent[188-192].

ILCs can differentiate between the various subtypes by skewing the transcription factors (ILC-1, T-bet/Eomes; ILC-2, GATA binding protein 3 (GATA-3); and ILC-3, RAR-related orphan receptor gamma t (RORγt))[193,194]. The cytokine environment dictates this trans-differentiation between ILC

subtypes. IL-7 preserves the ILC-3 characteristics by the induced production of IL-17, and IL-22[195]. When stimulated with IL-1β, -2, -12, -15 or -18, ILC-3 cells acquire ILC-1 characteristics (downregulation of RORγt, upregulation of T-bet, NKp46, and production of IFN-γ)[193,196,197]. In the presence of IL-33 and Thymic stromal lymphopoietin, ILC-2 cells differentiate into ILC-1 cells with the upregulation of the receptors for IL-12 and IL-18. Especially IL-12 seems to be a key factor in the trans-differentiation as it downregulates GATA-3, upregulates T-Bet and enables the production of IFN-γ[198-201]. In general, pro-inflammatory conditions (IL-2/-12/-15/-18) induce the trans-differentiation of ILC-2 and -3 cells into ILC-1. The plasticity of ILC-2 or -3 cells into ILC-1 is reversible. Differentiation of ILC-1 into ILC-3 cells is regulated by CD14(-) DC's producing retinoic acid and IL-23, whereas CD14+ DC's are able to revert ILC-3 cells into ILC-1's[195,202]. On the other hand, IL-4 producing eosinophils are responsible for the trans-differentiation of ILC-1 cells into ILC-2's[245]. Chronic inflammatory diseases, such as chronic obstructive pulmonary disease and Chron's disease, are related to a higher frequency of pro-inflammatory ILC-1 cells and could therefore be responsible for the disturbed homeostasis, lack of ILC-2 and -3, and the subsequent immunopathology[193,203].

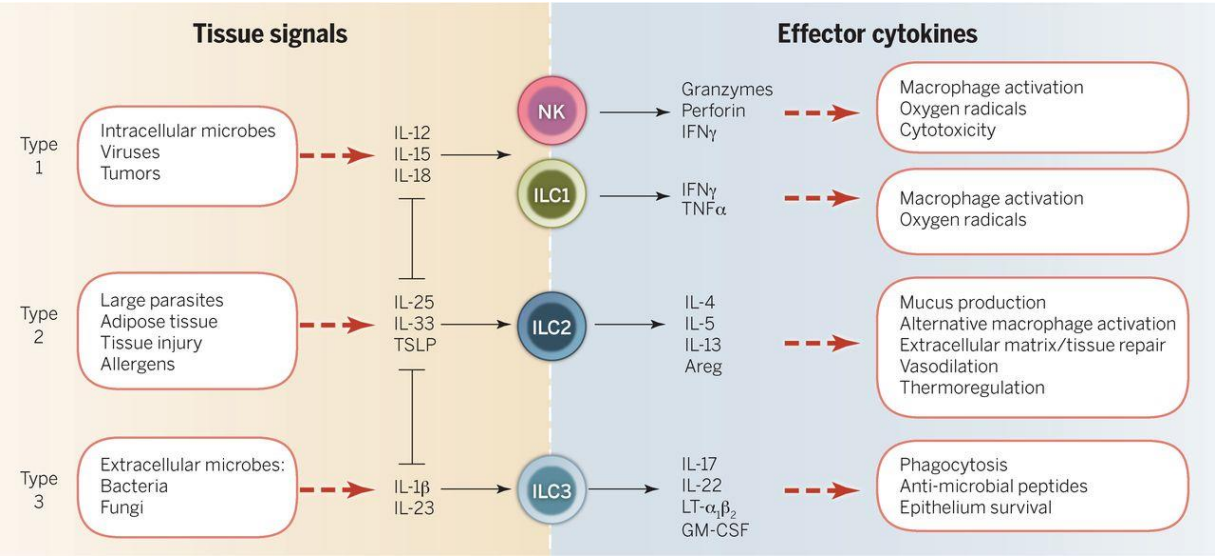

Figure.1.11: Overview of the functionality of the different ILC's: *Both in activation and effector function, the NK, ILC-1, -2, and -3 cells resemble the CD8+ T, T_{h1}-, T_{h2}-, and T_{h17} cells, respectively. ILC-1 and NK cells expand in the presence of intracellular microbes, viruses, and tumors. ILC-2 cells are generated under the pressure of large parasites, adipose tissue, tissue injury, and allergens, whereas ILC-3 differentiation is driven by extracellular microbes, bacteria, and fungi. Areg, Amphiregulin; TSLP, Thymic stromal lymphopoietin; LT-$\alpha_1\beta_2$, Lymphotoxin alpha one beta two (source[182]).*

2.3. NK cell receptors

Unlike adaptive immune cells, innate immune cells are able to respond without prior antigen sensitization. Innate immune cells directly recognize pathogens through PRRs binding PAMPs. NK cells express some PRRs such as all Toll-like receptors, RIG1, NOD2, NRLP3, and MDA5, capable of recognizing viral, tumoral, and fungal antigens[204,205]. Besides, NK cells also express receptors for ligands expressed on all healthy nucleated cells, such as HLA class I and HLA-E molecules. Co-receptors can amplify the response by shifting the balance further to activation and by the formation of the synapse. The expression repertoire of these NK cell receptors determines its functional competence. However, the expression of these receptors is not a steady-state affair, but rather evolves stochastically during the development of NK cells, and further tuned by viral or tumoral stimulation. In the following part, we will discuss the inhibitory and activating receptors modulating the activation of NK cells (Fig.1.12).

2.3.1. Killer immunoglobulin-like receptors (KIR)

The KIR family is the most studied NK cell receptor family, KIRs are involved in NK cell education during development and are associated with multiple disease outcomes. The KIR family contains 17 different KIR genes, of which four framework genes (KIR3DL2, KIR3DL3, KIR2DL4, and KIR3DP1)[206] and one pseudo-gene (KIR2DP1). The KIR genes are located on chromosome 19q13.4 in the leukocyte receptor complex[207] and are highly polymorphic. With the high amount of KIR alleles comes a high diversity in affinity and avidity for the HLA ligands by the different KIR alleles. KIRs transmit an inhibitory or activating signal upon binding their ligand. Classification of the different KIRs is based on the number of external immunoglobulin-like (Ig) domains (2D,3D). The length of the cytoplasmatic tails (‘L’ long, ‘S’ short) on the domains is also a surrogate marker for the signal transduced upon interaction. The generation of an activating signal is related to a short (‘S’) cytoplasmatic tail, whereas a long (‘L’) cytoplasmatic tail transmits an inhibiting signal. Based on the presence, or absence, of activating KIR genes, two major KIR haplotypes can be distinguished: an inhibitory KIR A haplotype or an activating KIR B haplotype, resulting in AA, AB or BB KIR

genotypes. Although a wide variety of KIR haplotypes is found throughout the population, haplotype A chiefly contains three inhibiting KIR genes (KIR2DL1,-2DL3,-3DL1) and one activating KIR gene (KIR2DS4), whereas haplotype B contains three inhibiting KIR genes (KIR2DL5A, -2DL5B and -2DL2) and five activating KIR genes (KIR2DS1,-2DS2,-2DS3,-2DS5 and -3DS1)[208].

2.3.1.1. Inhibitory KIR receptors

During the licensing of immature NK cells (section 2.4.1), the expression of inhibitory KIRs (iKIRs) binding self HLA ligands grants functionality to the NK cell. During these events, the strength of the binding presets the inhibitory threshold that needs to be overcome to activate the NK cell during immune surveillance. In this context, iKIRs are seen as the gatekeepers of auto-immune responses. Inhibitory KIRs can be divided into receptor classes containing 2 or 3 extracellular domains. After binding its HLA-ligand, the iKIR induces phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM) on the cytoplasmatic tails[209,210].

KIR2DL- receptors

The most studied KIR2DL receptors are KIR2DL1 and KIR2DL2 or 3. KIR2DL2 and KIR2DL3 are different alleles of the same gene, whereby KIR2DL2 is related to an activating B haplotype and KIR2DL3 to haplotype A. KIR2DL2 and KIR2DL3 have the same ligand, an HLA-C molecule with an asparagine at position 80, also known as HLA-C1. KIR2DL2 has a stronger affinity for HLA-C1, compared to KIR2DL3[211,212]. If the amino acid is a lysine instead of an asparagine, the HLA-C molecule, HLA-C2, is a ligand for KIR2DL1[211,213]. In contrast, KIR2DL4 is expressed by every mature NK cell and can induce both activating or inhibitory signals upon interaction with its ligand, the non-classic HLA-G molecule[214,215]. KIR2DL5 is 90% identical with KIR2DL4, but contains 2 ITIMs and is therefore explicitly inhibitory[216], although the ligand remains unknown.

KIR3DL- receptors

KIR3DL receptors bind HLA-A and HLA-B molecules containing the Bw4 pattern. The strongest binding can be seen with isoleucine at amino acid 80 on the Bw4 epitope (HLA-Bw4-Ile80)[217,218], compared to a threonine (HLA-Bw4-Thr80). The KIR3DL1 gene locus is the most variable of all KIR

genes[219,220] and resulted in 52 known alleles, of which 14 KIR3DS1 resulting from a fusion between KIR3DL1 and a stimulatory KIR locus[221,222]. The different KIR3DL1 alleles vary in the level of surface expression and affinity for HLA-Bw4[223].

2.3.1.2. Activating KIRs

The bond between an iKIR and its HLA ligand occurs in the presence of autologous healthy peptides, whereas small structural differences of the aKIRs (Activating KIRs) attain preferential binding in the presence of viral or transformed peptides at the HLA binding groove[224]. Binding of an activating KIR (KIR3DS,-2DS) with its HLA-ligand will activate kinases Syk and ZAP70, causing NK cell activation. The activation of these kinases is based on the adaptor protein, DAP12. DAP12 contains an immunoreceptor tyrosine-based activation motif (ITAM)[225,226].

The belief that KIR3DS1 would also bind HLA-Bw4 is mostly supported by indirect evidence for a KIR3DS1 and HLA-Bw4-80I interaction[227]. Nevertheless, a bond between KIR3DS1 and HLA-Bw4-80I remained presumptive by the lack of direct proof[224,228,229]. The only direct evidence of such an interaction resulting in a functional response was demonstrated between KIR3DS1 and the HLA-Bw4 allele B*57:01 and was peptide-dependent[230]. The presence of unconventional peptides in the repertoire, e.g., HIV-1 derived, with an aromatic ring at position eight generated a weak but reproducible bond. As a KIR3DL1 allele, KIR3DS1 is presumed to be derived from KIR3DL1 under the pressure of viruses altering binding affinity for viral peptides. Recently, multiple studies demonstrated another HLA molecule with a strong affinity for KIR3DS1, HLA-F. HLA-F is capable of presenting peptides to KIR3DS1 and was linked to HIV-1 specific NK cell responses as the presentation of viral peptides by HLA-F generated a KIR3DS1 bond, inducing anti-viral responses[231,232].

2.3.2. C-type lectin receptors

2.3.2.1. CD94/NKG2 receptors

CD94/NKG2A,-C, -E, and -F are members of the C-type lectin family. The receptor is a disulfide heterodimer with CD94 and NKG2A/C[233]. The NKG2 receptors interact with the non-classic HLA-E

molecule, which presents peptides from the same peptide pool as the classical HLA-A, B, C molecules[234]. The binding affinity of the peptide-HLA-E complex with the CD94/NKG2A or CD94/NKG2C heterodimer is peptide dependent. The presentation of healthy peptides by HLA-E will favor binding by NKG2A, whereas viral peptides favor NKG2C over NKG2A. The induction of target cell lysis by KIRs or CD94/NKG2A-C seems to be complementary, based on the peptide sequence that controls the binding affinity[234,235]. An ITIM is present in the NKG2A molecule, explaining the inhibitory character[176]. NKG2C is an activating receptor, containing an ITAM on the DAP12 protein[175].

2.3.2.2. NKG2D receptors

Unlike the CD94/NKG2 receptors, the NKG2D receptor is a homodimer expressed on all NK cells. Also virtually all T cell receptor (TCR) γδ+ and CD8+ TCRα/β+ cells express NKG2D. Studies concerning NKG2D expression on NK cells is mostly related to their capacity to lyse malignant cells. The NKG2D receptor plays an essential role in NK cell activation, inducing the cytotoxic degranulation response and the production of IFN-γ[236,237]. The NKG2D receptor binds the stress-induced proteins (MHC class I chain-related gene (MIC) -A/B and UL16 binding protein (ULBP) -1/-2/-3)[180]. MIC-A/B is expressed on epithelial tumors such as breast, ovary, colon, kidney, and lung carcinomas[238,239], but also exposure to HIV-1 induces expression of MIC-A/B[240]. Instead of DAP12 adaptor proteins, the NKG2D homodimer contains DAP10 adaptor proteins. The DAP10 adaptor protein contains Tyr-x-x-Met motifs in the cytoplasmic tail that, upon tyrosine phosphorylation, binds the PI 3-kinase; as a resemblance to the ITAM's on DAP12 adaptor proteins[241].

2.3.3. Natural cytotoxic receptors (NCRs)

In general, NCRs are known to induce NK cell cytotoxicity after binding their ligands, viral-induced or host molecules. The magnitude of the cytotoxic response correlates with the density of NCRs on the cell membrane[242]. NCR-induced NK cell responses mostly play a role in defense against tumors and RNA viruses[243-245]. NCR expression is limited to NK cells and are constitutively expressed (NKp46[178] and NKp30[177]), but is also upregulated upon viral infection (NKp44[179]):

2.3.3.1. NKp46 receptor

NKp46 is expressed on NK cells and induces cytotoxicity, Ca^{2+} mobilization and cytokine production upon stimulation[178]. NKp46 is molecularly characterized as a type I transmembrane glycoprotein belonging to the Ig superfamily with CD3ζ and FcεRIγ adaptor proteins, containing ITAMs[246-248]. In analogy, the NKp46 gene is located close to the KIR locus at 19q13.4. A wide variety of pathogen-derived antigens is known to induce NKp46-mediated cytotoxicity: Hemagglutinin (HA) protein (influenza and vaccinia viruses)[245], Duffy binding-like 1α domain (malaria)[249], vimentin (Mycobacterium tuberculosis)[250], heparan sulfate proteoglycan (tumor cells) [251] and proteins derived from Sendai and Newcastle viruses[252].

2.3.3.2. NKp30 receptor

NKp30 resembles NKp46 in many features: Omni-expressed on NK cells, capable of inducing cytotoxicity, Ca^{2+} mobilization and cytokine production, and activation associated with CD3ζ and FcεRIγ adaptor protein-related ITAMs. The NKp30 gene is located on chromosome 6 in the TNF cluster of the HLA gene complex[177,253]. Three splice variants of the NKp30 can be detected, NKp30a and NKp30b resulting in NK cell activation, whereas NKp30c is immunosuppressive[254]. Paradoxically, the NKp46 stimulating HA, as well as human cytomegalovirus (HCMV) component pp65, inhibits NKp30 activation[255]. One ligand for NKp30 is the host molecule HLA-B-associated transcript-3 (BAT-3). BAT-3 was related to efficient tumor clearing and the "editing" of autologous immature DCs (iDCs)[256]. B7-H6 is expressed explicitly on tumor cells and binds NKp30, inducing tumor lysis. Interestingly, a B7-H6 bond with the inhibitory splice variant of NKp30, NKp30c, will result in the production of the inhibitory IL-10[254].

2.3.3.3. NKp44 receptor

Similar to NKp30, the gene encoding NKp44 is located on chromosome 6. Unlike the other NCRs, the expression of NKp44 is related to the activation of NK cells[179]. Instead of an ITAM, NKp44 has a transmembrane region containing the activating adaptor complex KARAP/DAP12[257,258]. Both virally infected and tumor cells express various ligands for the NKp44 receptor, which in most cases results in

NK cell activation. However, an inhibiting signal is perceived when NKp44 interacts with the proliferating cell nuclear antigen (PCNA) on malignancies. Also, soluble ligands for the NKp44 receptor have been discovered, such as growth hormones or glycoproteins derived from the extracellular matrix. Interestingly, depending on the type of NKp44 ligand, NKp44-expressing cells, tissues, or environmental conditions, an interaction can trigger cytotoxicity, cytokine production, or immune regulation. During HIV-1 infection, exposure of the HIV-1's gp41 triggers the expression of an NKp44 ligand, resulting in NK cell-mediated cytotoxicity. As NKp44 ligand expression was related to the viral load of the patients, the NKp44-NKp44-ligand interaction was suggested to enhance the depletion of CD4+ T cells (reviewed in[259]).

2.3.3.4. NCR expression on other immune cells

Although NCRs were believed to be exclusively expressed on NK cells, NK cell-like immune cells such as ILC's and T cells also express NCRs. *In vitro*, NCR expression on TCRγδ T cells and CD8+ T cells was related to activation by TCR or cytokine stimulation[179,260-264]. *In vivo*, the expression of NKp46 and NKp44 was also observed on TCRαβ intraepithelial lymphocytes (IEL) in patients with intestinal inflammatory celiac disease, driven by a pathological overexpression of IL-15[265]. The two NCRs induced IFN-γ production and perforin degranulation, independent of TCR proliferation[266]. In general, NCR expression on cells other than NK cells is hypothesized to be related to a chronic TCR engagement. The persistent impulse results in the acquisition of a molecular NK cell program, upregulating NCRs. As a result of this, they enable responsiveness without prior sensitization.

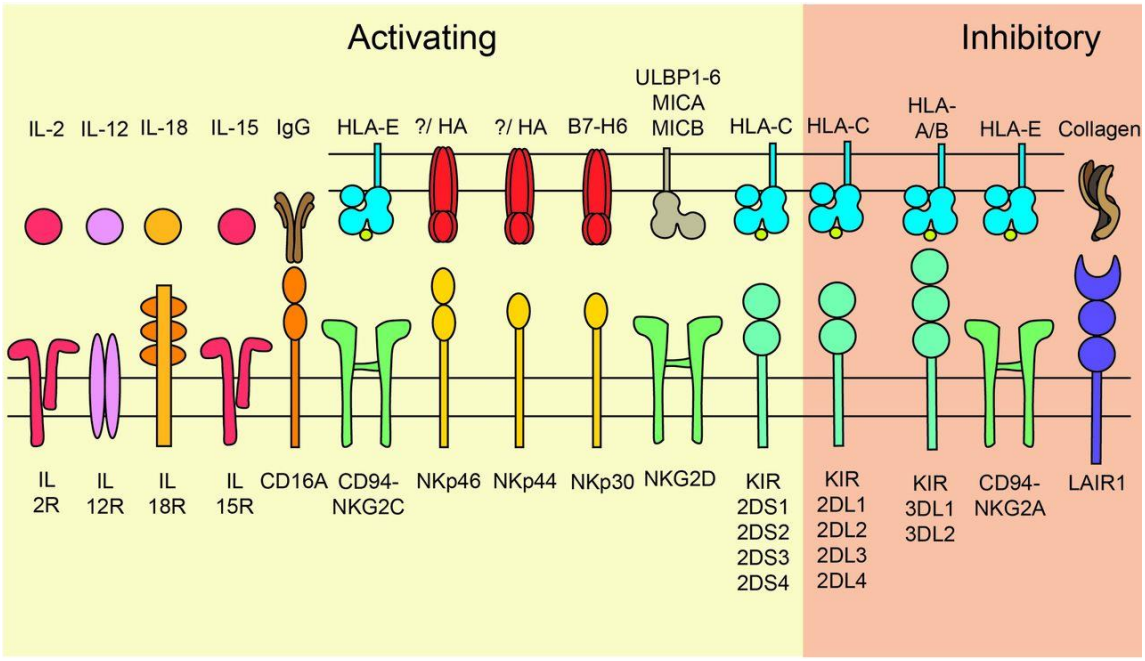

Figure.1.12: The various categories of NK cell receptors: *NK cell receptors can be divided into activating and inhibitory receptors, based on the type of signals transduced upon binding. Besides activation by interleukins, NK cell can be activated by the binding of NKG2C (and -E) with HLA-E, CD16 with Ab, NCRs NKp46, NKp44 and NKp30 with various ligands, NKG2D with stressed ligands (MIC-A/B or ULBP-1-6) and KIRs binding their HLA class I counterpart. Inhibitory receptors are mainly KIRs binding HLA-B/C ligands or NKG2A binding HLA-E. LAIR1, Leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1 is a collagen-receptor that inhibits immune cell function upon collagen-binding)(source[267]).*

2.4. NK cell development

2.4.1. Early stages of NK cell development

NK cells have the same lymphoid precursors as the T-cell lineage, explaining the CD8+ T cell-like functions of the innate NK cell. In the BM, hematopoietic stem cells (HSC) differentiate into the common lymphoid progenitor (CLP) under the influence of fms-like tyrosine kinase three ligand, kit ligand (KL), IL-3 and IL-7[268] (Fig.1.13). CLPs further differentiate into NK cell precursor (NKP) stages 2 and 3, accompanied by the expression of the IL-15 receptor[269]. Whereas stage 2 NKP's mainly reside in SLTs, stage 3 NKPs further disseminate into peripheral tissues, such as cord blood. Whereas stage 2 NKPs can still give rise to T cells and DC's, stage 3 NKPs lack this capacity[269-272]. A heterogeneous expression profile of CD7, CD56, C121A, CD127, CD336 (NKp44), and LFA-1 is measured on stage 3 NKP's (Fig.1.13)[269,273-275].

2.4.2. Late stages of NK cell development

Hallmarked by the upregulation of CD94, stage 3 NKP cells differentiate into stage 4 NKP's and undergo functional maturation (section 2.4.1)[270,272,276]. After maturation, NK cells gain the expression of functional receptors expressed in a stepwise order: (1) CD161, (2) CD56, CD94/NKG2A, NKp46, and NKG2D and (3) KIR and CD16[269,272,277]. Activated stage 4 CD56^{br} NK cells predominantly produce cytokines with limited cytotoxic potential. These CD56^{br} NK cells reside in high concentrations at SLT's. Downregulation of surface marker CD56 preludes the development of stage 5 CD56^{dim} mature NK cells. In contrast to their precursors, CD56^{dim} NK cells mainly reside in the peripheral blood and exert cytotoxic cell-to-cell responses[278]. The shift in the functionality of CD56^{dim} NK cells is accompanied by a change in receptor expression (KIR^{hi}, CD16^{hi}, NKG2A^{lo}). The expression of CD94[279], CD62L[280], and CD57[281] is used to differentiate between intermediate stages of mature NK cell development, related to memory-like competences (NK cell stage 6)[282].

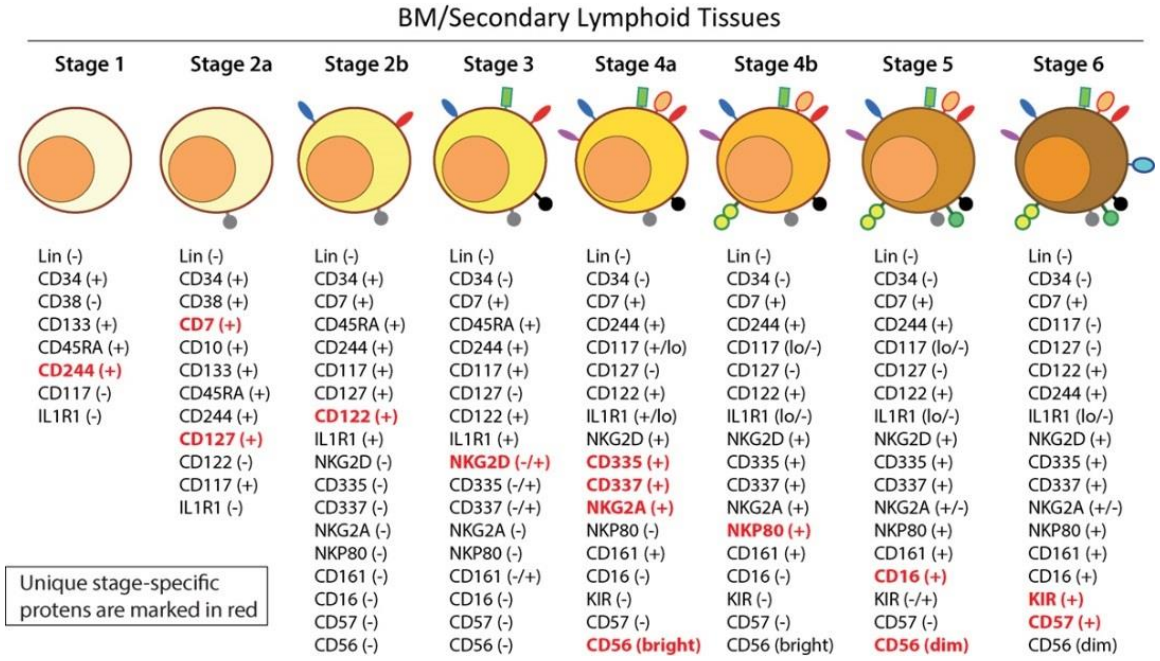

Figure.1.13: Stages of NK cell development in BM or SLTs: *Chronologically, the expression profiles of the various NK cell precursor stages are displayed and compared, markers that appear for the first time during development, or the so-called " developmental stage markers" are highlighted in red (source[283]).*

2.4.3. Sites of NK cell development

The BM contains CD34+ HSC cells and seems to be an essential cradle for NK cell differentiation, as BM ablation abrogates murine NK cell development. BM-derived stromal cells are capable of producing the necessary cytokines to differentiate HSCs into CLPs (FL, KL, IL-3, and IL-7) and NKPs (IL-15)[191,284-288]. Stromal cells, monocytes, DCs, and T cells in the human liver, spleen, and SLTs are also capable of producing similar cytokines, facilitating extramedullary NK cell development[289-292]. In the BM, the stage 2 NKP's comprise less than 1% of the CD34+ HSC population, whereas in the circulating HSC's, 5 to 10% of the cells are stage 2 NKP's. Expression of CD62L, LFA-1, and $\alpha_4\beta_7$ integrin on stage 2 NKPs promotes the migration to SLT[269,270], where all stages of NKPs reside[293,294]. These data propose that BM-derived stage 2 NKPs migrate to SLT's to develop further (Fig.1.14). Also other anatomical sites such as the liver and uterus contain stage 3 NKP's[274,295-299].

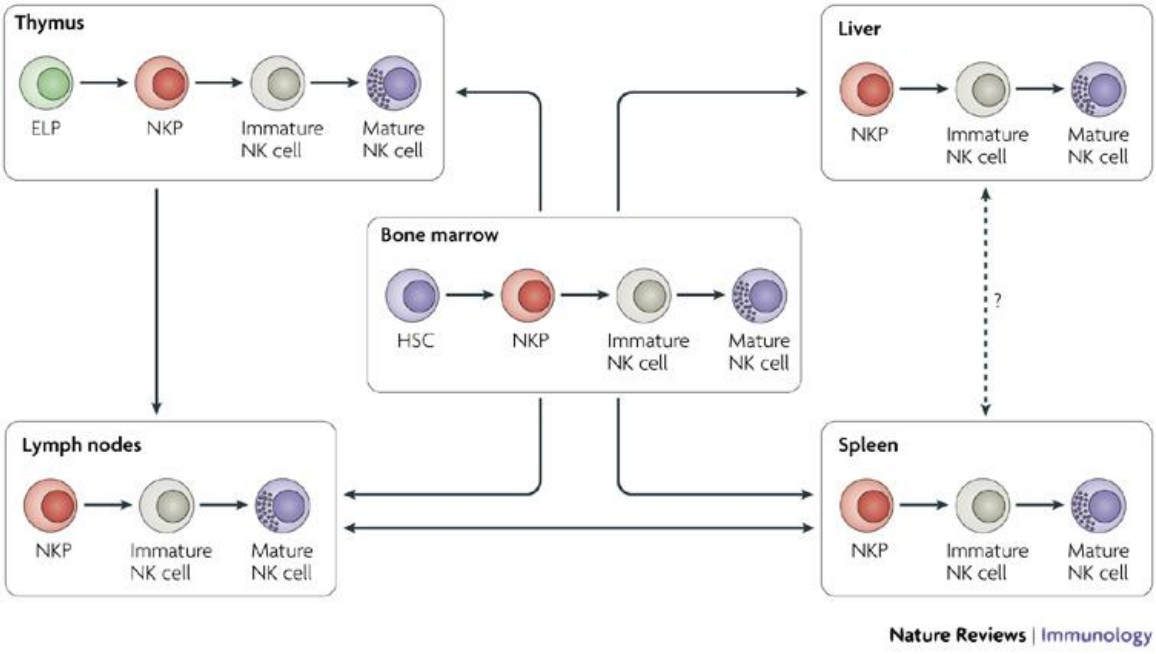

Figure.1.14: Sites of NK cell development: *NK cell maturation occurs in the primary lymphoid tissue (PLT) such as the bone marrow and thymus, but also at different SLTs, such as the liver, lymph nodes, and spleen. In the PLT, NKPs originate and further develop into (im)mature NK cells locally or disperse towards the various SLT's. Within all the mentioned lymphoid organs, NKPs mature into NK cells and migrate to different tissues in between maturation steps. HSC, hematopoietic stem cells; ELP, early lymphoid precursors; NKP, NK cell precursor (source[284]).*

2.4.4. NK cell trafficking and tissue localization

Mature NK cells mostly reside in the peripheral blood (>90%), but can also be found in tissues such as the spleen, lung, liver, mucosal tissues, uterus, pancreas, joints and brain[300]. During viral infection, NK cells respond rapidly and migrate to the site of infection in lymphoid and non-lymphoid tissues[301,302]. Migration of these NK cells is orchestrated by the expression of various chemokine receptors (CCR1, CCR5, CCR7, CXCR3, CXCR4, CXCR6, and CX3CR1), adhesion molecules (macrophage receptor 1 (MAC1) and DNAX accessory molecule 1 (DNAM1) and integrins α2- and α4[303].

2.4.5. Presence of NK cells at the vaginal-cervical and gut-intestinal mucosal surface

The vaginal/cervical mucosa is divided into two parts concerning the anatomical barrier: the upper FGT (endocervix and endometrium) is covered by a vulnerable monolayer of columnar epithelial cells with tight junctions; whereas the lower tract (vagina and ectocervix) is lined by a stratified squamous epithelium in the absence of MALT (Fig.3.1). Between both types of epithelia, there is a cervical transformation zone, a vulnerable location for pathogens to infect the recipient. The composition of the immune system at the vaginal mucosa is very variable between individuals, even when immuno-modulating factors such as inflammation and menstrual cycle are taken into account[304]. The immune cells present at the vagina belong to both the innate (NK cells, pDCs, macrophages, epithelial cells) and the adaptive (CD4+-, CD8+-T cells) system and reside intra- or subepithelial, with immune aggregates in the lamina propria (LP)[66]. In the ectocervix, high concentrations of cytotoxic CD8+ T cells and antigen-presenting cells reside, whereas mainly IgG- and IgA- producing plasma cells were measured in the endocervix. CD56+ cells (NK or CD8+ T cells), were sparsely present in the whole FGT: they are abundantly present in the ectocervical lamina propria and, in a few cases, in the epithelial layer[66,305].

Both the gut and FGT resident NK cells have a similar origin and have a $CD56^{bright}$ CD16- immature phenotype with the expression of NKG2A, strong cytokine production and low cytolytic functions. Vaginal NK cells differ from the other uterine counterparts by the expression of CD16 and lack of CD94(/NKG2A). In chronically progressive HIV-1 infection, a decline in IEL and LP residing NK cells

was measured in the gut mucosa, whereas the IEL NK cells were preserved in HIV-1 controllers. Also after incomplete CD4+ T cell recovery, a higher proportion of IEL and LP NK was demonstrated, although the expansion is believed to be a compensation mechanism for the impaired adaptive immune reconstitution. IEL NK cells can be considered as cytolytic[282] immune cells with the expression of NKp46 and CD57, whereas LP NK cells are rather immuno-regulatory, producing IFN-γ and TNF-α[306,307]. Gut IEL NK cells also express high levels of KIRs and CD16[307] and are therefore able to respond to HLA^{null} target cells in combination with cytokine stimulation (IL-2,-12 or -15)[308]. ILC-3 cells (IEL and LP) are also found at the gut mucosa, producing IL-22 and IL-17[121,309].

2.5. NK cell functionality

2.5.1. Functional education of NK cells

As mentioned before in 2.1, NK cells defend the organism against incoming viral pathogens and malignant cells by constant patrolling of the tissues and peripheral blood. NK cell receptors probe encounters with other cells in search of their ligand. NK cells undergo an education that grants a "license-to-kill" to ensure that these encounters do not result in adverse responses. If the pattern does not meet the requested "safety standards", NK cells remain "hypo-responsive". During maturation, both cell-extrinsic (trans) as cell-intrinsic (cis) interactions between the inhibitory KIRs or NKG2A with their cognate HLA ligands induce and maintain functionality, respectively[310-312]. The strength of the acquired NK cell functionality correlates with the individual affinity between receptor and ligand, as well as the accumulated strength (avidity) of the interactions. As both affinity and avidity vary between the alleles of the highly polymorphic KIR and HLA gene, the intensity of the NK cell functionality differs heavily between individuals[212,313-319]. To summarize, during education a relative equilibrium is set and represents the level of responsiveness in absence, as well as tolerance in the presence of the HLA class I ligand.

The mechanism behind NK cells acquiring or losing functionality is still under debate, as several education models have been proposed (Fig.1.15). It is important to understand that these models do not necessarily exclude each other, but are rather different pieces of a still incomplete puzzle. Both the

licensing and the arming model propose the expression of inhibitory receptors (KIRs) binding self-HLA class I ligands to educate the NK cell. In the licensing model, education uses the same downstream pathway as the inhibiting signal (ITIM)[320], whereas the arming model proposes a distinct cellular pathway[321]. "Licensed" NK cells gain functionality, whereas NK cells educated by the disarming model are already functional before education. Uneducated NK cells remain immature (licensing) or lose their functionality (disarming) in the absence of its ligand or in the presence of continuously activating signals (overstimulation)[322,323]. Also, the co-expression of KIR2DS1 and its ligand HLA-C2 results in hypo-responsiveness, leading to anergic NK cells in chronic stimulation[188,324]. In the rheostat model, the dose-dependency of the receptor-ligand interaction on the strength of NK cell education is discussed. The strength of the educational signal varies between the different KIR receptors. The wide variety of KIR and HLA polymorphisms generates a specific set point of inhibition, reflecting the strength of the NK cell response in the absence of the HLA ligand [212,313,325]. Generally, not one, but the accumulation of all inhibitory signals received through their self-ligands contribute to the setting of this inhibitory threshold[317,326].

Although NK cell education was believed to be a one-time event, NK cells can be "tuned" to the actual HLA environment. Several mouse and human studies demonstrated the acquisition of functionality in the presence of new self-HLA ligands[312,327,328], as well as the hypo-responsiveness in the loss of self-HLA ligands[327,329,330]. To what extent a new self-HLA environment, will result in tolerance adaptation (tuning) or NK cell reactivity (missing-self) is still under debate. However, tolerance towards a changing HLA environment might play a role in the success of reproduction and transplantation[327,331,332].

2.5.2. NK cell activation or tolerance: a balance between inhibiting and activating signals

During surveillance, activated NK cells create an immunologic synapse with the "interrogated" target cell. The formation of an immunological synapse is dependent on the receptor-ligand interactions with the target cell. Each specific receptor-ligand interaction generates an inhibitory (-) or activating (+)

signal of a certain strength. The balance of all these receptor-ligand interactions generate tolerance (- > +) or activation (- < +) of the NK cell (Fig.1.16). To avoid erroneous cytolytic responses, a balanced input of inhibitory and activating signals will result in NK cell tolerance. In healthy conditions, constitutively expressed inhibitory ligands such as HLA class I molecules inhibit auto-immune NK cell responses. In virally-infected cells, the HLA molecules are downregulated to avoid CD8+ T cell recognition. The absence of the HLA ligand generates a lack of inhibitory signals in the encounter with NK cells. The absence of the inhibitory threshold makes these virally-infected cells prone to NK cell activation by any activating signal (missing-self)[333]. In the presence of inhibitory ligands, virus-specific ligands recognized by activating receptors are still able to activate the NK cell if the impact is sufficient to overcome the inhibitory threshold (induced-self)[334-336]. Also the binding of IgG1 or IgG3 antibodies with the FcγIIIA-receptor (CD16) generates an activating signal that will result in antibody-dependent cell cytotoxicity (ADCC)[337,338].

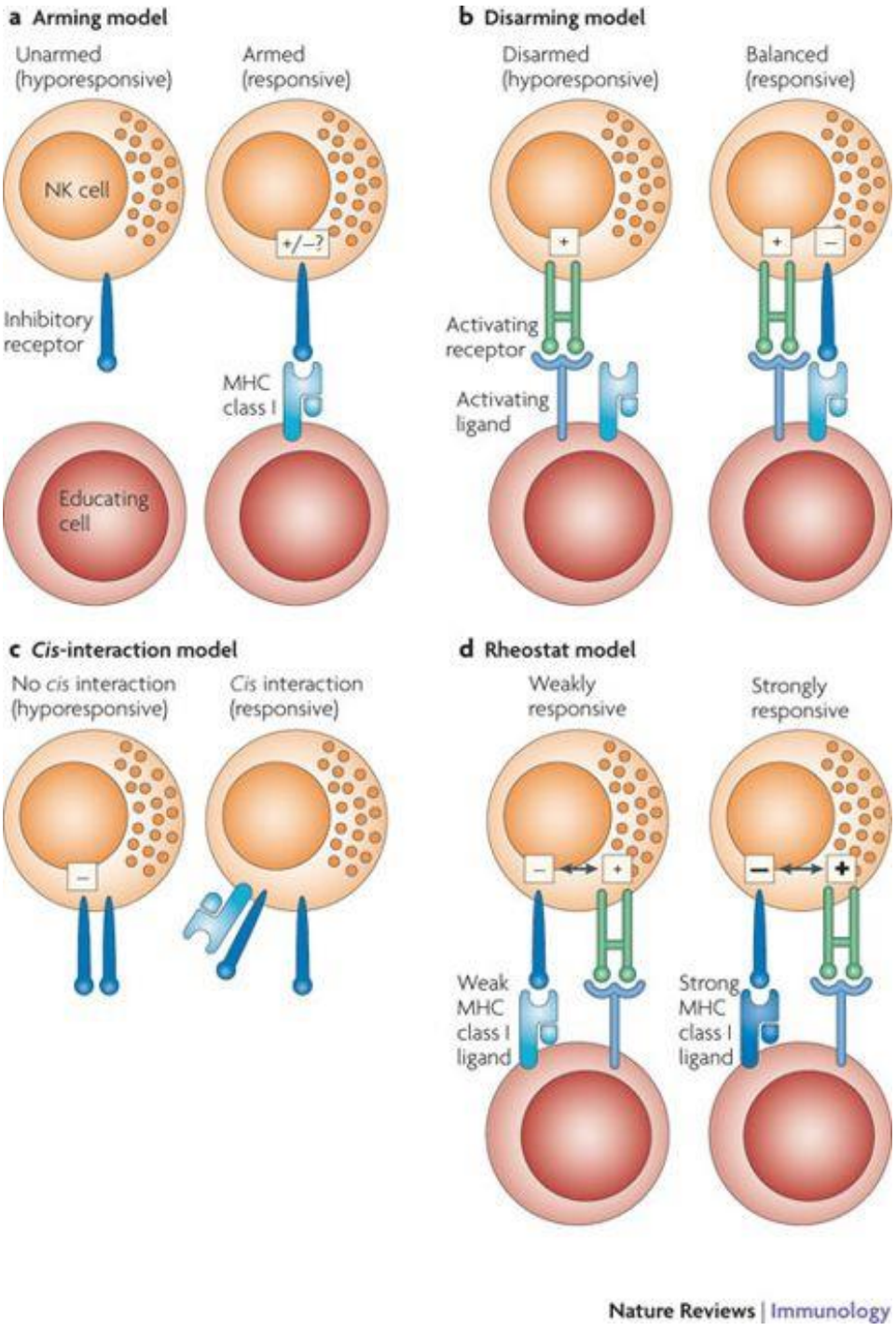

Figure.1.15. Models for the education of NK cells during maturation: *A variety of NK cell education models are proposed to explain the effective NK cell responses and prevention of autoimmunity. In A), the arming and licensing model are displayed as a similar mechanism: granting functionality to NK cells able to bind self-HLA molecules. B) The disarming model explains the hypo-responsiveness of NK cells able to induce an activating bond in the absence of an inhibitory signal. C) NK cells can also be licensed for their functionality if the educational bond is a result of a cis-interaction or a bond with an HLA ligand expressed on the same NK cell. In D), the rheostat model includes the strength of the educational bond to be a parameter for the corresponding NK cell responses (source[339]).*

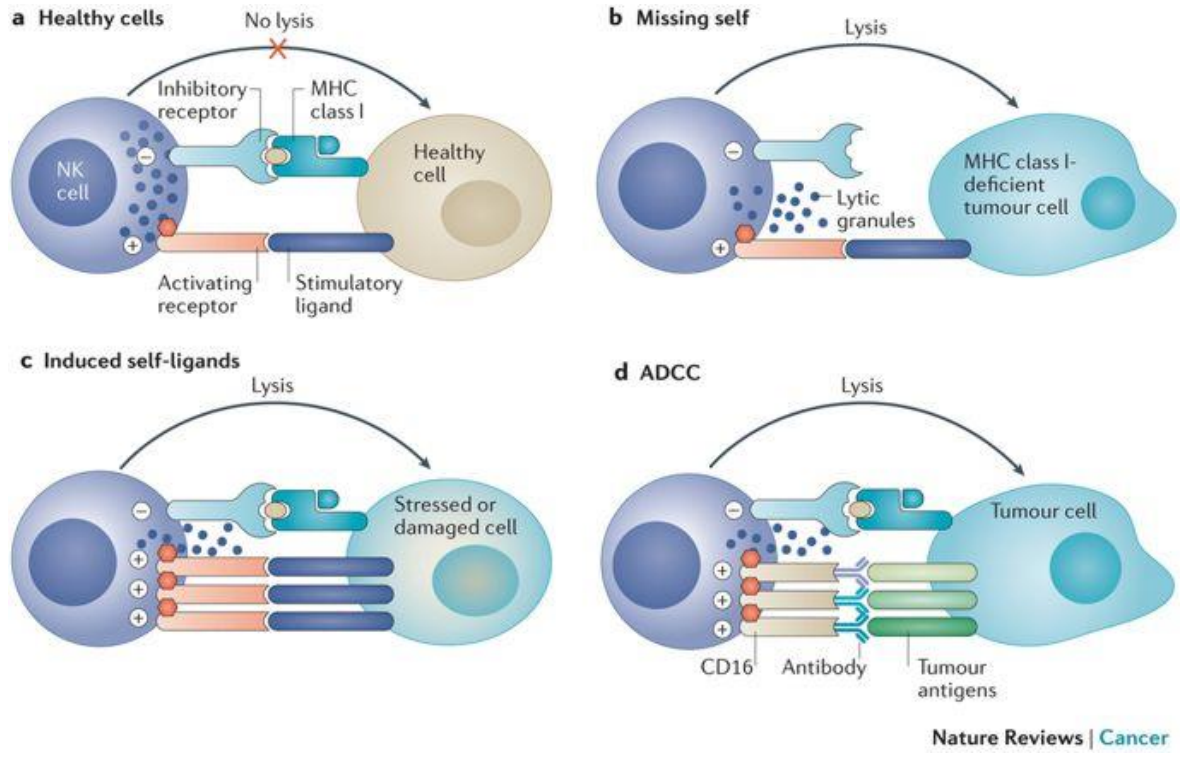

Figure.1.16: Mechanisms of NK cell activation: *Activation of NK cells is based on the balance of incoming activating and inhibitory signals derived from the corresponding receptor-ligand interactions with the interrogated cell. In A), an interaction with a healthy cell results in the tolerance of the NK cell as an equal input of inhibitory and activating signals is dominated by the inhibitory signal to prevent auto-immunity. In B), the absence of the self-HLA ligand (missing-self) results in an NK cell response in combination with an activating signal. In C), in the presence of an inhibitory signal, activation of NK cells can be achieved if the strength of the activating input can overcome the inhibitory input. In D), the strength of the activating input can be increased by the binding of Ab with CD16, described as ADCC (source[334]).*

2.5.3. Degranulation of cytotoxic granules filled with perforin and granzyme-B

Mature $CD56^{dim}$ NK cells are known as the "cytotoxic" NK cells with secretory lysosomes containing perforin and granzyme B. Degranulation of granzyme B and perforin is a cytotoxic pathway common to NK and CTL. The degranulation of NK cells is a gradually orchestrated molecular mechanism induced by activation of the NK cell.

First, an immunological synapse (1) is generated at the site of contact with the target cell. Next, the cytotoxic granules polarize towards the immunological synapse (2) along the redirected microtubule-organizing center (MTOC). At the synapse, the secretory lysosomes dock at the plasma membrane before fusing with the membrane and releasing the content (3). Once secreted, perforin perforates the

cell membrane of the target cell, allowing the influx of granzyme B. Granzyme B activates the caspase pathway, leading to apoptosis of the target cell (Fig.1.17).

2.5.3.1. Formation of the immunological synapse

The immunological synapse between the NK cell and the target cell is composed of two distinct domains, a peripheral-(pSMAC) and central supramolecular activation cluster (cSMAC). The pSMACs form a ring of adhesion molecules and receptors, closely linking the membranes of both cells[340-342]. Also, the cSMAC is the focal point for the polarization, attraction, and secretion of the lysosomes. With this, degranulation of the lysosomes is concentrated close to the target cell membrane, which will result in more efficient cytolysis. Actin is withdrawn from the cSMAC and reorganized at the pSMACs to ensure the flawless fusion of the lysosomes with the plasma membrane[340,341]. An important factor involved in these cytoskeletal rearrangements is the Wiskott–Aldrich syndrome protein (WASp), inducing actin polymerization, nucleation, and branching[343-345].

2.5.3.2. Polarization of the cytotoxic granules to the synapse

Two proteins interacting with WASp, namely WASp interacting protein (WIP) and Cdc42 interacting protein-4 (CIP4), play a role in the polarization of the lysosomes. WIP, associated with the granules, forms a complex with WASp, F-actin and Myosin IIa, and transports the granules to the MTOC via microtubuli[346-348]. At the immunological synapse, the granules come in contact with the MTOC by an interaction between CIP4 and WASp[349]. Once arrived, the MTOC contacts the plasma membrane, bringing the granules in proximity with the synapse with the help of motor proteins adaptor protein three and Rab7[350-352]. The recruitment and exocytosis of the cytotoxic granules is NK cell receptor-dependent. For example, the interaction between CD54 and LFA-1 recruits granules to the synapse, whereas CD16 binding IgG predominantly induces the exocytosis of the granules[353].

2.5.3.3. Docking and fusion of the granules with the plasma membrane

Docking of the secretory lysosomes at the plasma membrane is induced by GTPase Rab27a or Rab27b, both with complimentary activation pathways[354-356]. Rab27a is generally related to the endosomes, but

after NK cell recognition associates with the granules[357,358]. The fusion of the secretory granules to the plasma membrane is catalyzed by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). SNAREs are a generic tool of cells for the exocytic or endocytic transport between membranes. When four SNARE domains across two membranes meet, they form a SNARE complex, fusing both membranes[359]. The formation of these complexes is facilitated by syntaxin 1A, which is released by Munc13-4 after binding with Rab27a[360,361]. Some of the SNAREs are defined as (partially) necessary for fusion, as is syntaxin 7[351] and 11[362], and vesicle-associated membrane protein 7[363].

2.5.4. Production of cytokines and chemokines

In comparison to the cytotoxic CD56^{dim} NK cell subset, the CD56^{bright} NK cells do not express CD16 and have a smaller reservoir of perforin and granzyme-B. CD56^{bright} NK cells do produce cytokines such as IFN- α/γ, TNF-α, IL-6, -7, -10, -12, -13 and GM-CSF; as well as chemokines such as CCL2-5, 8-10 and -26[173,278,364-366]. Therefore, CD56^{bright} NK cells are suggested to primarily exert immuno-regulatory functions. Whereas CD56^{dim} NK cells mainly reside in the peripheral blood, CD56^{bright} NK cells are located in the LNs or other SLTs. With upregulated cytokine receptors such as IL-2 receptor alfa (CD25), the CD56^{bright} NK cells residing in the SLT's are presumably activated by cytokines produced by approximate immune cells[170,171,367]. At the site of infection, cytokine production by NK cells can initiate and amplify the early immune response and the attraction of other immune cells. Depending on the activating signal(s), different cyto- and chemokines are produced too[169].

2.5.5. Antibody-dependent cell cytotoxicity (ADCC)

Besides activating receptors, activation is also achieved when antibodies bind the Fc-receptor expressed on the NK cell membrane. In contrast to the high-affinity FcγRI (CD64) receptor expressed on mast cells and basophils, NK cells express the intrinsic low-affinity FcγRIII (CD16) receptor. Although its low affinity, an allelic variant (158V) has an increased affinity for IgG[368]. Subsequently, the 158V allele has increased anti-tumor activity, as seen in homozygous cancer patients responding to therapeutic monoclonal-Ab (mAb)[369]. CD16 contains an ITAM which will induce an activating signal upon interaction with IgG1 or IgG3[338]. Specifically these Abs, IgG3 and IgG1, were seen to induce strong

ADCC responses in comparison to IgG4 and IgG2 Abs. Hereby, NK cells can be seen as essential mediators of ADCC[370]. In addition, mAbs aid the NK cells by guiding and visualizing the threat, as seen in the treatment of lymphomas with rituximab[371,372]. As CD56^{dim} NK cells predominantly express CD16, ADCC mostly induces the degranulation of the granules. Stimulation of CD16 also induces the production of cyto- and chemokines[373], regulating antigen presentation, maturation of DCs, B cell activation, and plasma cell survival[374].

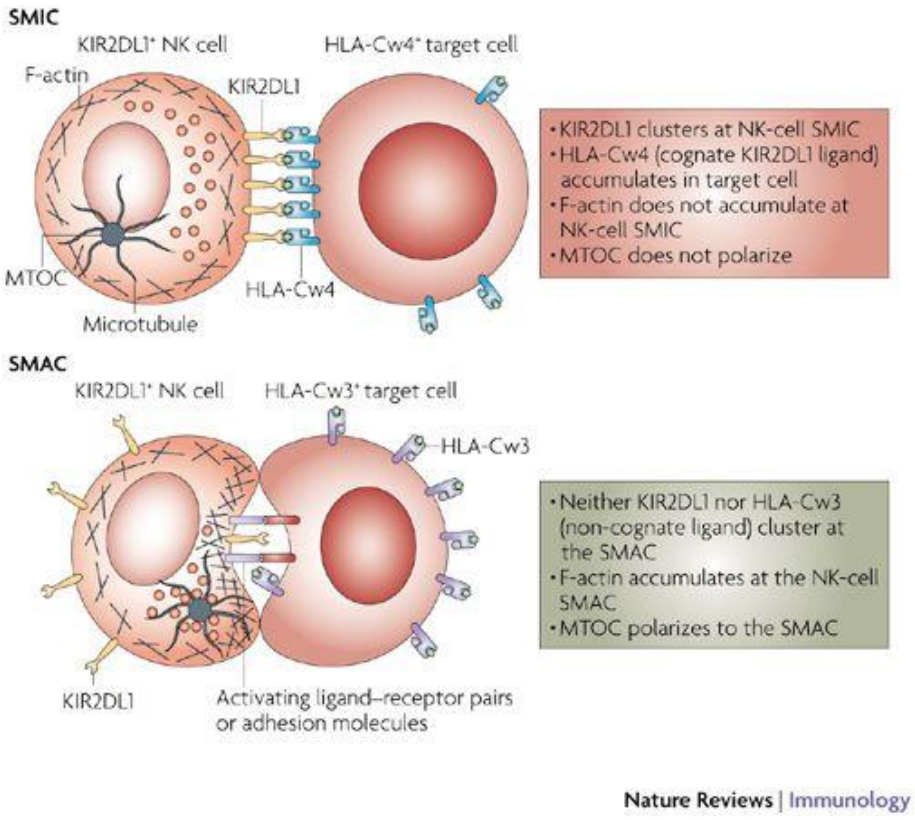

Figure.1.17. The formation of an immunological synapse between NK and target cells: *NK cell tolerance, induced by a KIR-HLA bond, does not initiate the configuration of an immunological synapse. NK cell activation sets in motion a series of cellular kinetics, generating an immunological synapse and the polarization of the cytotoxic granules. Upon activation, F-actin, SMAC, and MTOC modulate the formation of the synapse. MTOC: microtubule organizing center, SMIC: central supramolecular inhibition cluster, SMAC: central supramolecular activation cluster (source [334]).*

2.5.6. Clonal expansion and "memory-like" characteristics of NK cells

As one of the first immune cells capable of responding to a viral infection, NK cells are seen to clonally expand during viral infections such as the cytomegalovirus (CMV) [375-378]. In murine and HCMV, the

expansion of KIR+ and NKG2C+ NK cells, respectively, was observed soon (1 week) after infection[375,379]. In the peripheral blood, the frequency of NKG2C+ NK cells exponentially increased from 1% in seronegative individuals to 50-60% in HCMV patients[377,378]. Clonal expansion of the NKG2C+ NK cells was also observed during a Hanta-virus outbreak, with expansion rates comparable to HCMV infection.

In addition to the clonal expansion of NK cell subsets in the presence of a viral stimulus, certain other characteristics of the adaptive immune system can be ascribed to NK cells[380], such as longevity and immune memory, making them capable of mounting an even faster response against re-exposure to viral agents such as HCMV[376,381]. Firstly, the HCMV expansion phase of NKG2C+ NK cells is followed by apoptosis of the effector NK cells after 1-2 weeks, resembling the contraction phase of the CD4+ T cell response[382,383]. The second piece of evidence for NK cell memory-like functions is found in invertebrates lacking the T cell analogs. Transplantation of parental BM in the offspring (F1) resulted in NK cell-mediated rejection. Interestingly, priming of F1's NK cells with parental BM before transplantation resulted in a faster NK cell rejection of the BM, suggesting NK cells were capable of mounting a "recall" memory-like response[384,385]. NK cells responsed to pre-exposed chemical haptens in mice lacking T- and B- cells, whereas mice lacking T-, B- and NK cells did not elicit any hypersensitivity against the haptens[386]. These hypersensitive NK cells explicitly expressed CXCR6 and resided in the liver[387].

In murine CMV infection, the remaining clonally expanded NK cells in lymphoid and non-lymphoid tissues seem to be long-lived and self-renewing[382]. Besides viral stimuli, NK cells are also able to obtain memory-like capacities after the stimulation by IL-12 and IL-18. *In vitro* stimulation with IL-12/IL-18 led to NK cells producing more IFN-γ upon the ligand-receptor activation of the NK cell[388]. The memory-like functions of NK cells, make them unique within the innate immunity, as other innate immune cells are strictly speaking not capable to "recall" a previously encountered antigen.

Chapter 2 - The role of NK cells in HIV-1 protection: autologous, allogeneic or both?

Review published in *Aids Research and Therapy* on March 18, 2016

DOI: 10.1186/s12981-016-0099-6

Updated version of review to the state-of-the-art literature

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1. Abstract

NK cells are part of the innate immune system and kill virally infected or transformed cells. They become activated as a consequence of an imbalance between incoming inhibitory and activating signals mediated by receptor-ligand binding with the target cell. The traditional KIRs, which are expressed at the cell surface of NK cells, scan potential target cells in the search for their ligand: the highly variable HLA-class I molecule. The absence of HLA ligand binding lowers the NK cell activation threshold ("missing self" theory) by which an additional activating signal suffices to induce NK cell responsivity.

During HIV-1 infection, a beneficial disease outcome is associated with some of these HLA class I and KIR genes, suggesting that autologous NK cell responses pressurize HIV-1 during infection. In response, HIV-1 adapts itself and uses its accessory HIV-1 proteins to impair NK cell functioning. Consequently, an increased aberrant NK cell subset and disturbed DC crosstalk are common aspects seen during chronic HIV-1 infection.

At the same time, NK cells also seem to play a role in protection against HIV-1 transmission, possibly by a competent response prior to HIV-1 dissemination. A different KIR repertoire is seen to be associated with a slower disease progression compared to those associated with protection against HIV-1 transmission. Therefore, these distinct NK cell responses are suggested to be regulated by different activation pathways. An interesting NK cell activation pathway associated with protection against heterosexual HIV-1 transmission is the presence of an iKIR/HLA mismatch between index and recipient partner. HLA-incompatibility between both partners would render the cell-associated HIV-1 transmission vulnerable to alloreactive NK cell responses. Evidence in HIV-1 vertical transmission also suggests a role for alloreactive NK cells in HIV-1 protection.

The objective of this review is to summarize the knowledge about these alloreactive and autologous NK cell responses concerning susceptibility to HIV-1 transmission on one hand and progression after infection on the other.

2. Introduction

Since the start of the HIV-1 epidemic in the late 1970s and early 1980s, 78 million people became infected, and about 39 million people have died from this infection. In 2014, HIV-1 was considered one of the most widespread viruses, with 37 million infected people globally, with prime endemic areas situated in South and East Sub-Saharan Africa[62]. However, HIV-1 sexual transmission efficacy is remarkably low compared to other viruses (0.01-0.001%) and is shaped by a variety of viral and host characteristics. An essential determinant of HIV-1 susceptibility is the efficiency of the mucosal genital innate immune system as it is capable of inducing an early antiviral immune response against infectious pathogens (reviewed in[5]). Since in most (80%) of the investigated cases a single virus was found to be responsible for sexual transmission[10], adequate protection is much more feasible during this timeframe compared to later stages of infection, when HIV-1 also impairs innate and adaptive immune functioning. According to being part of the innate immune system and in charge of killing viral- and tumor infected cells, NK cells are likely mediators of this early HIV-1 protection. Investigating KIR and HLA, determinants of NK cell activation, in HIV-1 ESN and slow progressors revealed associations with HIV-1 outcome. However, different KIR/HLA associations were related to HIV-1 protection versus disease progression, an observation that accentuates the complexity of NK cell interactions[8]. Furthermore, NK cell-mediated HIV-1 protection against transmission was, in some instances, partially dependent on the donor, suggesting allogeneic NK cell responses mediated HIV-1 protection[11].

3. Natural killer cells

The NK cell is one of the protagonists of the innate immunity, and thereby lack the typical T-and B-cell features. Phenotypically, NK cells are identified by the expression of CD56 and CD16 on the cell membrane[171]. These immune cells can either be cytotoxic ($CD56^{dim}$ NK cells predominantly produce perforin and granzyme B) or fulfill a supportive role ($CD56^{bright}$ NK cells), which secrete IFN-γ, TNF-α, IL-10, IL-13, and GM-CSF[170,172]. Initiation of an NK cell response against virus-infected and tumor cells depends on the balance of receptor/ligand-derived activating and inhibitory signals[173-180]. KIRs with a long (L) cytoplasmic tail (iKIRs) or CD94-NKG2A heterodimers receive inhibitory signals..

KIRs with a short (S) cytoplasmic tail (aKIRs), natural cytotoxic receptors (NCR) (NKp30,-44,-46), CD94-NKG2C/E complexes and the NKG2D dimers receive activating signals. KIRs are further subdivided by the number of extracellular Ig-like domains: 2D or 3D. KIR3DL1 molecules bind HLA-A and –B molecules with Bw4 epitopes[389], while KIR2DL1/2/3 molecules bind HLA-C1 or -C2 epitopes[211]. Despite reasonable homology between activating and inhibitory KIRs, to date, only KIR2DS1 binds the same ligand as its inhibitory counterpart, though with lower affinity[390]. The lack of proof for HLA-aKIR bonds might relate to a higher binding affinity, specifically in the presence of a viral or tumor antigen in the HLA-peptide binding groove[224].

Activation of NK cells is directed by the balance of incoming inhibitory and activating signals (Fig.2.1C). More specifically, mature NK cells in the absence of their self-HLA ligand (missing self) lose their inhibiting threshold and become more sensitive for the presence of activating ligands on the target cell, induced-self e.g., by viral, infection or altered-self e.g., by tumor transformation. Alternatively, NK cells are activated when the magnitude of the activating signal(s) overwhelms the inhibitory threshold[207]. Further research on the " missing self" model revealed a mechanism called "licensing", which takes place during NK cell maturation and influences the functionality of the matured NK cell profoundly. Licensing is allocating functionality to NK cells that are capable of creating an iKIR-self-HLA bond with an HLA-presenting cell during maturation (Fig.2.1A). The absence of such a bond will result in incompetent NK cells, also called "hypo-responsive NK cells". These "rules" will ensure tolerance towards healthy, self-HLA expressing cells; and NK cell responsivity towards peptide-altered or self-HLA deficient viral or transformed target cells[335,336,391-395]. Activating KIRs, on the other hand, were not known to play a role in NK cell education, up until Fauriat et al.[396] demonstrated an inverse NK cell education mechanism directed by the aKIR, KIR2DS1 (Fig.2.1B). Licensing of KIR2DS1^{+} NK cells was, paradoxically, only attained in the absence of the corresponding HLA-C2 ligand, whereas the presence of the HLA-ligand generated hypo-responsive NK cells. This newly identified NK cell education mechanism complements the inhibitory counterpart in preventing autoimmunity and in recognition of altered HLA class I molecules[397,398]. It is essential to understand that NK cell education is not a permanent fixation, but a constant adaptation of NK cells to

their current cellular environment, meaning that NK cells which were once hypo-responsive can become functional again and vice versa[328,399].

4. Autologous NK cell activity vs. HIV-1 infected cells

4.1. Influence of HIV-1 infection on NK cell activity

4.1.1. The presence of the CD56neg NK cell subset during HIV-1 infection

In healthy subjects, NK cells are divided into two major subgroups: $CD56^{bright}$ (10% of total NK cell population) and $CD56^{dim}$ (90% of total NK cell population) NK cells; though a third subgroup is present in lower frequencies: the $CD56^{neg}$ NK cell subset[400]. During acute HIV-1 infection, a pathological NK cell redistribution from $CD56^{dim}$ towards $CD56^{neg}$ NK cells is observed. Whereas $CD56^{neg}$ NK cells in healthy individuals display "$CD56^{dim}$-like capacities[401], during HIV-1 infection, this redistribution is accompanied by an aberrant $CD56^{neg}$ NK cell functioning[402]. The decrease in cytotoxic capabilities of $CD56^{neg}$ NK cells is the result of the reduced expression of activating receptors (aKIRs and NCRs), increased expression of inhibitory NK cell receptors (iKIRs) and reduced secretion of IFN-γ, TNF-α, and GM-CSF[403-406]. The redistribution is directly correlated with viral replication despite the inability of HIV-1 to infect NK cells. NK cells are affected by high and chronic viremia, impairing their anti-viral functioning which contributes to rapid disease progression[402] and suppression of viral replication by antiretroviral therapy (ART) is associated with restoration of the normal NK cell subset distribution, indicating that the impact of HIV-1 on NK cell distribution is reversible[407]. *In vitro/vivo* administered IL-2 has also been shown to contribute to the restoration of NK cell subset distributions[402,408].

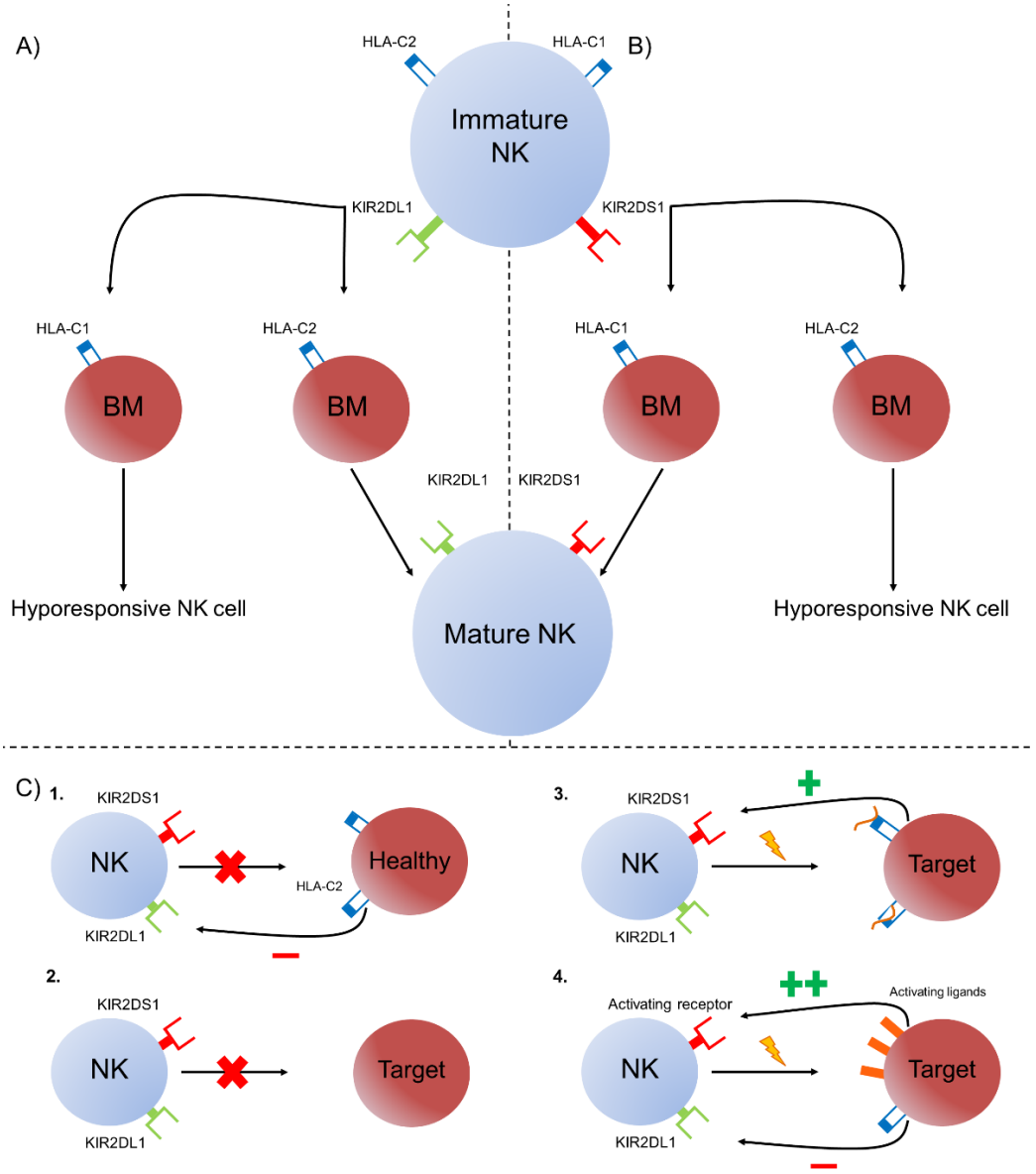

Figure.2.1: NK cell education and activation pathways: *iKIRs and aKIRs (KIR2DS1) expressed at the immature NK cell membrane can mediate NK cell education. In the case of education by iKIR (A), a bond between iKIR and self-HLA is necessary to develop fully functional NK cells, whereas its absence abrogates NK cell education. In contrast, in aKIR mediated education (B), the absence of the HLA-ligand is necessary for the licensing of immature NK cells, whereas its presence will generate hyporesponsive NK cells. KIR-HLA interactions also play a pivotal role in the activation of NK cells (C). Tolerance is mediated by the presence of an iKIR (Inh-R) -HLA bond (red minus) and the absence of an activating impulse (C.1.) or solely by the absence of an activating signal (C.2). However, NK cell activation can be provoked in the presence of viral/tumor peptides in the HLA class I binding groove of target cells, increasing the HLA class I-binding affinity of aKIRs ("altered self") (green plus) compared to the iKIR affinity (C.3). Additionally, an increased amount of activating NK cell receptors (Act-R) binding their non-HLA class I ligands (Act-L) ("induced self") (two green pluses) can overcome the inhibitory threshold (green plus) (C.4).*

4.1.2. HIV-1 impairs the crosstalk between NK cells and DCs

Mature dendritic cells DCs play a pivotal role in the regulation of the immune response. Insufficient DC maturation, resulting in an aberrant adaptive immune response, can be averted by NK cell-mediated i) recruitment of iDCs to the site of infection using chemokines, ii) lysis of pathogen-infected cells providing a source of antigens and iii) production of pro-inflammatory cytokines (IFN-γ and TNF-α)[409-411]. During infection, NK cells are also responsible for the lysis of immature DCs to prevent the suboptimal antigen presentation by these iDCs to the adaptive immune system, also known as DC-editing. On the other hand, both conventional (cDCs) and pDCs stimulate NK cells by the production of IL-12 and IL-18, or IL-15 and IFN-I, respectively[412-416].

During acute HIV-1 infection, NK-DC crosstalk could be of vital importance for producing an efficient and competent immune response by amplifying the inflammatory potency of the innate and, subsequently, the adaptive immunity. However, HIV-1 harms both cell types. The expanding $CD56^{neg}$ NK cell subset expresses low amounts of NKp30 and exerts impaired TRAIL activity, resulting in a reduced capacity for DC-editing[402,417]. $CD56^{neg}$ NK cells also have reduced production of IFN-γ and TNF-α, resulting in reduced DC maturation[402,403]. On the other hand, HIV-1 infection also directly impacts DC functioning as cDCs produce less IL-12,-15 and -18 during acute HIV-1 infection, leading to less IFN-γ production by NK cells[402]. The chronic phase of HIV-1 infection is characterized by an increased secretion of IL-10, which induces the generation of tolerogenic DCs using direct (obstruct maturation)[418] and indirect (switch DC-editing from iDC to matured DC)[419] mechanisms. In contrast, exposure to high concentrations of HIV-1 results in the release of alarmin/HMGB1 by pDCs, which in crosstalk with NK cells, triggers the expression of TRAIL on both pDCs and NK cells[420]. In summary, HIV-1 infection impairs NK and DC function and, as a consequence, their crosstalk, leading to an increasing rate of functionally iDCs and NK cell populations.

4.1.3. HIV-1 uses accessory proteins to escape NK cell recognition

During acute HIV-1 infection, pDC-secreted cytokines (IFN-α and IL-15) activate, arm, and expand the NK cells in an attempt to constrain viral replication[421]. Throughout the years, HIV-1 has developed

multiple evasion strategies to escape this NK cell recognition, suggesting an important role for NK cells in defense against HIV-1[422]. These mechanisms can impair NK cell function and escape recognition by expressing the HIV-1 derived accessory proteins. These intracellular proteins disturb NK cell activity by affecting a wide variety of immunological pathways.

Nef can down-regulate HLA class I molecules on the surface of the infected cell by triggering the accelerated endocytosis of HLA class I molecules in the Golgi complex[423]. Downregulation of HLA class I molecules may prevent recognition by $CD8^+$ T-cells but might render the infected cell vulnerable for NK cell-mediated lysis. However, in-depth analysis revealed that Nef mainly downregulates the $CD8^+$ T-cell receptor ligands HLA-A and, to a lesser extent, HLA-B, while NK cell receptor ligands HLA-C and HLA-E are spared, thus avoiding NK cell activation[424-426]. So Nef-induced HLA class I downregulation results in the partial evasion of T- and NK cell recognition of HIV-1. However, recent studies point to Vpu-mediated HLA-C downmodulation by primary HIV-1 isolates[315,427,428]. This HLA-C downmodulation is accompanied by the corresponding KIR2DL-missing-self like NK cell responses. Other viral proteins are also able to downregulate HLA class I molecules: Tat, by repressing transcription of HLA class I molecules[429], and Vpu by inducing proteolysis of newly synthesized HLA class I molecules[430]. Nef and Vpu also impair NK cell activation by obstructing the expression of HIV-1 upregulated ligands for the activating receptors NKG2D[431] and NKp44[432], as well as co-receptor NK-T-B antigen (NTB-A)[433]. In addition, both Nef[434] and Tat[435] undermine NK cell activation by impairing crucial interactions between DC and NK cells. It is clear that the HIV-1 accessory proteins dampen the "virally infected" phenotype of the host cell and try to mimic the behavior of normal healthy cells in an attempt to avoid recognition by the immune system, including NK cells.

4.2. NK cell activity during HIV-1 transmission and infection

Studying biological samples from HIV-1 ESN subjects allows investigators to identify HIV-1 protective immune mechanisms during transmission. One study in Vietnamese ESNs generated the idea of HIV-1 protective NK cell responses and opened the door for a new field of research focused on NK cells and innate immunity as the potent protagonists in HIV-1 resistance[436,437]. Subsequent research indicated the

importance of KIRs in the HIV-1 mediated functioning of NK cells (summarized in Table.2.1). Also HIV-1 disease progression is affected by certain KIR and HLA molecules and other NK cell receptors, described in the following paragraphs. Noteworthy, when studying ESNs to unravel the plausible protective mechanisms, the HIV-1+ "donor" is, in most cases, not taken into account and only the "recipient" is investigated.

4.2.1. NK cells influencing HIV-1 disease progression

4.2.1.1. KIR+ NK cells slowing down HIV-1 disease progression

A slower progression towards AIDS was first ascribed to the HLA-Bw4-80I genotype (HLA-Bw4 epitope with isoleucine at position 80, evoking strong affinity with KIR3DL1)[438] but was later on associated in combination with the KIR3DS1 gene[439] which was also seen to mediate protection against opportunistic diseases[440]. The relevance of the KIR3DS1/HLA-Bw4-80I genotype on HIV-1 outcome was supported by the expansion of KIR3DS1$^+$ NK cells in primary HIV-1 infected HLA-Bw4-80I$^+$ individuals as well as the efficient suppression of viral replication *in vitro*[188,441]. According to the licensing theory, Pelak et al. demonstrated that the functionality of KIR3SD1$^+$ NK cells was dose-dependent of the licensing via co-expressing KIR3DL1 receptors[442]. These results support the hypothesis of an autologous KIR3DS1-HLA-Bw4-80I bond in the presence of virally derived peptides resulting in a cytotoxic response. However, slower disease progression was also associated with the carriage of KIR3DS1 in the absence of HLA-Bw4-80I, which suggests an HLA-Bw4 independent effect can take place[443,444]. The HLA-Bw4 independency of KIR3DS1 can be related to its new putative ligand HLA-F, of which interaction with KIR3DS1 induced NK cell responses with anti-viral competence[231,232].

In addition, KIR3DL1 is also linked to slower disease progression as slow progressors with licensed KIR3DL1$^+$ NK cells had increased poly-functionality in the absence of KIR3DS1[445]. The highly polymorphic KIR3DL1 alleles can be divided into three expression categories: i) high expressing-alleles, ii) low expressing-alleles, and iii) KIR3DL1 *004, a non-expressing allotype with unknown function. Slow progressors and elite controllers with high expressing KIR3DL1 alleles (KIR3DL1*h)

and HLA-Bw4-80I had increased NK cell functionality[446-449]. Additionally, co-expression of the KIR3DL1*h allele and HLA-B*57 even induced a protective effect against HIV-1 acquisition[450]. The enhanced functionality of these KIR3DL1*h alleles can be explained by another feature of licensing named the "rheostat model": NK cell licensing is dose-dependent based on the amount of iKIR-self-HLA bonds. Stronger iKIR-self-HLA bonds will lead to more robust licensing and result in a stronger reaction in the absence of this bond[331,333]. This mechanism is relevant in KIR3DL1-mediated HIV-1 immune response, as the expression of KIR3DL1 is affected by the presence of polymorphic alleles and copy number variations[442,446]. Another potential NK cell-mediated HIV-1 restrictive mechanism shown in KIR3DL1*h^+/HLA-B*57^+ individuals is the inhibition of viral replication *in vitro* by secreting HIV-1 entry-inhibiting chemokines (CCL4 and CCL5) upon cell contact with HIV-1 infected $CD4^+$ T-cells[451]. Recent epidemiological studies show contradictory associations to slower disease progression, with on the one hand a higher prevalence of inhibitory KIR receptors (KIR3DL1+,-2DL3+ and -2DL5+)[452] and on the other hand the activating Bx haplotype (KIR3DL1/S1+ and KIR2DS1+)[453] in LTNP cohorts.

Moreover, NK cell expressing KIR2DL1-4 receptors generated NK cell responses associated with a slower progression[454-456]. In early HIV-1 infection, Körner et al. measured an increase in the frequency of KIR2DL1^+ or 2/3^+ NK cells in individuals with corresponding HLA-C2 or –C1 genotypes. Also primarily licensed KIR2DL1-3^+ NK cells contributed to NK cell functionality[454]. HIV-1 peptide NK cell responses were also profoundly mediated by licensed KIR2DL3^+/$C1^+$ patients with the support of a more activating KIR phenotype, especially in the presence of KIR2DS2 and KIR2DS5[455]. Similar results were previously described in the Hepatitis C virus (HCV) infection[457]. The HIV-1/HCV protective effect can be explained by the low binding affinity of KIR2DL3 for HLA-C1, compared to KIR2DL2, resulting in a quicker overruling by activating ligands, subsequently leading to NK cell activation.

Additionally, anti-HIV-1 antibodies (Ab) binding the Fc-receptor CD16 on educated NK cells partially overcome their inhibitory threshold derived from iKIR/HLA matches between NK and target cell[6,458-460]. Therefore, anti-HIV-1 Ab derived signals could overcome KIR-HLA interactions resulting in a weak inhibiting result, hence the association with slower disease progression[455]

4.2.1.2. NKG2- receptors associated with slower HIV-1 disease progression

Education of NK cells is not only achieved through iKIR interactions with their self-HLA ligand, but NKG2A can also grant functionality to expressing NK cells[461]. The peptides presented by HLA-E are derived from the same pool as the peptides presented by HLA-class I molecules and could, therefore, present viral peptides to NKG2A+ NK cells[462]. In line with these observations, more NKG2A+ NK cells responded against autologous infected T cells compared to their NKG2Aneg counterpart[463]. Later, the HIV-1 targeted NKG2A+ NK cell responses were confirmed to be triggered by viral peptides impairing the inhibitory NKG2A-HLA-E binding, resulting in an NKG2A missing-self response[462].

Moreover, in primary HIV-1 patients, a lower viral set point was related to a higher proportion of NKG2C+/NKG2A- NK cells compared to uninfected controls, with NKG2C+ NK cells exerting strong anti-HIV activity[464]. HIV exposure to the NK cells might induce a shift from NKG2A to NKG2C expression. The presentation of viral peptides on HLA-E, detrimental for the NKG2A bond, might increase the affinity for NKG2C, similar to what is observed in CMV infection[377-379]. As a result of this, NKG2A+ NK cells could exert missing-self like activation, whereas NKG2C+ NK cells will recognize these infected cells presenting viral peptides.

4.2.1.3. NK cell receptors related to accelerated HIV-1 disease progression

On the contrary, specific KIR/HLA genotypes do not dampen but rather accelerate disease progression. In most cases, the presence of (an) activating KIR(s) is associated with accelerated disease progression, such as KIR3DS1[439,465], KIR2DS2, KIR2DS3[465], and KIR2DS4*001[466]. Regarding KIR haplotypes, similar observations are made, with multiple associations between an activating KIR B haplotype and an accelerated disease progression[465,467]. Also, recent findings associated co-carriage of KIR2DL3 and HLA-C1 with higher viral load and increased mortality rates[468], although other studies suggested that this co-carriage was favorable in HIV-1 and HCV infection[455,457].

4.2.2. NK cell protection against HIV-1 transmission

ESNs are the living proof for the existence of HIV-1 protective mechanisms, in which the discovery of HIV-1 protective KIR and HLA geno-/haplotypes seem to suggest a role for NK cell responses in HIV-1 resistance. Firstly, Ravet et al. reported an increased KIR3DS1/3DL1 ratio in ESNs[469]. In addition, an increased proportion of ESNs were KIR3DS1 homozygous in relation to HLA-Bw4[470,471]. Furthermore, even carriers of KIR3DS1 (hetero- or homozygosity) or HLA-Bw4 separately were seen to be HIV-1 protective[472]. Besides, ESNs had increased KIR3DS1/KIR3DL1 heterozygosity in the presence of HLA-Bw4[469,471-473]. As different alleles of the same gene, the absence of the KIR3DL1 allele automatically means the presence of a KIR3DS1 allele. With this, the latter results indirectly suggest a higher prevalence of the KIR3DS1 in the KIR genotype of ESNs. These results accentuate the role of KIR3DS1 and aKIRs in HIV-1 protective responses as an activating KIR profile is repeatedly seen in ESN cohorts[11,469-473].

While the protective effect of KIR3DL/S1/HLA-Bw4 is well established, KIR2DL receptors remain understudied concerning HIV-1 resistance. The HIV-1 protective potential of KIR2DL was firstly described by Jennes et al. in an ESN FSW cohort from Côte d'Ivoire. The presence of KIR2DL2/3 heterozygosity in the absence of HLA-C1 and KIR3DL1 in the absence of HLA-Bw4 was elevated in the ESN FSW cohort, which relates the protective effect of ESNs to a lowered NK cell activation threshold in the absence of the inhibitory signal[473]. In a Senegalese ESN cohort, Jennes et al.[11] confirmed the presence of the homozygous KIR2DL2 genotype in the absence of its HLA-C1 ligand to be elevated in ESNs. Accordingly, both studies verified the elevated prevalence of B haplotypes in ESNs. In vertical transmission, the homozygous KIR2DL3-HLA-C1/C2 genotype of the child is associated with HIV-1 protection[474], which opposes these findings.

HIV-1 outcome	Receptor/Ligand		Mechanism	Ref
HIV-1 Resistance				
	KIR3DS1/S1	HLA-Bw4	No/less inhibition and increased activation, promoting faster NK cell activation	*470,471*
	KIR3DS1/x	HLA-Bw4	No/less inhibiting and increased activation, promoting faster NK cell activation	*472*
	KIR3DS1/L1		Higher activating ratio, stronger NK cell response	*469*
	KIR3DL1*h	HLA-B*57	"Amplified" education, resulting in stronger NK cell response in absence of ligand	*450*
	KIR3DL1	Bw4	Educated KIR3DL1+ NK cells had increased anti-HIV-1 ADCC mediated cytotoxicity against allogeneic target cells	*459*
	KIR3DL1	HLA-Bw6/Bw6	Absence/lowering of inhibitory threshold, promoting faster NK cell activation	*473*
	KIR2DL2/3	HLA-C2/C2	Absence/lowering of inhibitory threshold	*473*
	KIR2DS4del		Unknown	*unpublished*
	KIR2DL1	HLA-C2	Absence of HLA-C2 expression by allogeneic target cells induces cytotoxicity based on KIR/HLA mismatch	*11*
	Haplotype B/x		Multiple aKIRs inducing stronger response	*11,469-473*
Slower disease progression				
	KIR3DS1	HLA-Bw4-80I	Slower disease progression; Robust expansion during early infection; Inhibition in vitro viral replication	*188,439,441*
			Increase of copy numbers is associated with inhibition of viral replication	*442*
			In vitro production of viral inhibiting chemokines (CCL3-5), preventing HIV-1 entry	*451*
	KIR3DS1	HLA-B*57/58	Lower CD38-expression, increased degranulation and IFN-γ production	*444*
	KIR3DS1		Associated with higher CD4+ T-cell counts	*443*
	KIR3DL1/S1	HLA-Bw4	KIR3DL1-dose dependent-licensed NK cells exert cytotoxicity via KIR3DS1-mediated activation	*446*
	KIR3DL1	HLA-Bw4	Increased polyfunctionality by KIR3DL1 licensed NK cells	*445*
			Educated KIR3DL1+ NK cells had increased anti-HIV-1 ADCC mediated activation	*458*
	KIR3DL1*h	HLA-Bw4-80I	Delayed progression to AIDS, increased degranulation, TNF and IFN-γ production	*446,447*
	KIR3DL1*h	HLA-B*57	"Amplified" education, resulting in strong NK cell response, increased NK cell trifunctionality	*446,448,449*
			In vitro production of viral inhibiting chemokines (CCL3-5), preventing HIV-1 entry	*451*
	KIR3DL1*004	HLA-Bw4	Absence/lowering of inhibitory threshold, because of intracellular expression of KIR3DL1	*446*
	KIR2DL3	HLA-C1	Lower viral load and higher CD4 count associated with HIV-1 specific NK cell responses	*455*
	KIR2DL4		CD4+ T-cell preservation, higher copy number resulting in increased IFN-γ production in SIV-infection	*456*
Rapid disease progression				
	KIR3DS1	HLA-Bw4-80I	Rapid progression, no education of KIR3DS1 expressing NK cells	*465*
	KIR3DS1(/S1)		Rapid progression, robust immune activation accelerating disease progression	*439,465*
	KIR2DS2/3		Rapid progression, robust immune activation accelerating disease progression	*465*
	KIR2DS4*001		High viral load and accelerated HIV-1 transmission, immune activation accelerating disease progression	*466*
	KIR2DL2/3	HLA-C1	Higher viral load and increased mortality	*468*
	Haplotype B/x		Rapid progression, robust immune activation accelerating disease progression	*465,467*

Table.2.1: KIR and HLA genotypes related to HIV-1 outcome: *Summarization of the KIR, HLA, or KIR-HLA haplo-/genotypes associated with HIV-1 protection and disease progression towards AIDS. HLA class I genotypes associated with altered HIV-1 outcomes are not included as its function is not solely relevant for NK cell responses but also $CD8^+$ T cell responses.*

4.2.3. Interpretation of HIV-1 protective and disease progressive correlations

Protection against HIV-1 transmission is associated with an activating KIR profile, whereas slower disease progression is linked to the presence of strongly licensed iKIRs. On the other hand, a detrimental disease outcome was encountered in the presence of aKIRs, in contrast to its effect on HIV-1 transmission. These observations suggest that different receptor-ligand strategies mediate NK cell immunity resulting in protection against HIV-1 transmission or against disease progression.. The data represented in this review can be assembled into a hypothesis: if the early pro-inflammatory NK cell burst does not suffice to induce HIV-1 protection, NK cells in possession of a B haplotype will paradoxically accelerate HIV-1 spread and disease progression by attracting target cells and

contributing to chronic immune activation. On the other hand, NK cell responses by individuals with the inhibitory haplotype A will be less capable of preventing HIV-1 transmission, due to lower cytotoxicity, but will be able to dampen HIV-1 spread and disease progression once HIV-1 mediated HLA class I down-modulation tends to take place or in the presence of anti-HIV-1 Ab. However, the KIR3DS1/HLA-Bw4-80I combination is associated with slower disease progression as well as HIV-1 protection, which is not explainable with the hypothesis mentioned above. Extrapolating the KIR2DS1-licensing theory to KIR3DL1^{-}/S1^{+}/Bw4^{+} NK cells will result in hyporesponsive NK cells. In the situation of disease progression, this might induce a less pro-inflammatory environment, with a slower progression towards AIDS as a result. Regarding HIV-1 transmission, KIR3DS1/HLA-Bw4 NK cells might mediate an immune response according to the "missing ligand" hypothesis, in which even hyporesponsive NK cells can become cytotoxic in the presence of an activating ligand.

5. The benefit of NK cells in haplo-hematopoietic stem cell transplantation, applicable in protection against HIV transmission

Although the debate is still ongoing, cell-associated HIV-1 transmission is believed to be favorable compared to free virion-mediated transmission. *In vitro* and *in vivo* analysis of SIV-transmission efficacy[475-477] confirmed the potential of cell-mediated HIV-1 transmission (reviewed in[70]). The presence of mucosal NK cells, the interpersonal HLA class I variability and the multiple HIV-1 protective associations might suggest a direct NK cell response against incoming allogeneic infected cells. The effectiveness of these "alloreactive" NK cells has already been verified in immunotherapy applied in acute myeloid leukemia and acute lymphoid leukemia using haploidentical-hematopoietic stem cell transplantation (Haplo-HSCT).

5.1. The clinical relevance of alloreactive NK cells in Haplo-HSCT

To increase the availability of stem cell donors in HSCT, clinicians opted to employ haploidentical donors. A haploidentical donor and its recipient share one identical HLA-haplotype and differ at the HLA class I and II locus of the other haplotype. In HLA-identical HSCT, T-cells mediate the graft-

versus-leukemia (GVL) effect, enhance engraftment, and reconstitute immunity. However, T-cells in an allogeneic environment become reactive and need to be extensively depleted prior to haplo-HSCT graft to avoid graft-versus-host disease (GVHD). Remarkably, the GVL effect in T-cell depleted haplo-HSCT was handed over to alloreactive NK cells driven by a KIR/HLA mismatch (Fig.2.2)[478,479]. The large amount of $CD34^+$ stem cells used for transplantation promotes donor-derived immune recovery and helps to overcome graft rejection induced by recipient's anti-donor cytotoxic T-cells (reviewed in[480]). $CD34^+$ stem cells also create a donor-like derived environment in the recipient, resulting in donor HLA-based NK cell education[391,481]. To date, NK cells are capable of remaining alloreactive for more than five years after transplantation[482,483].

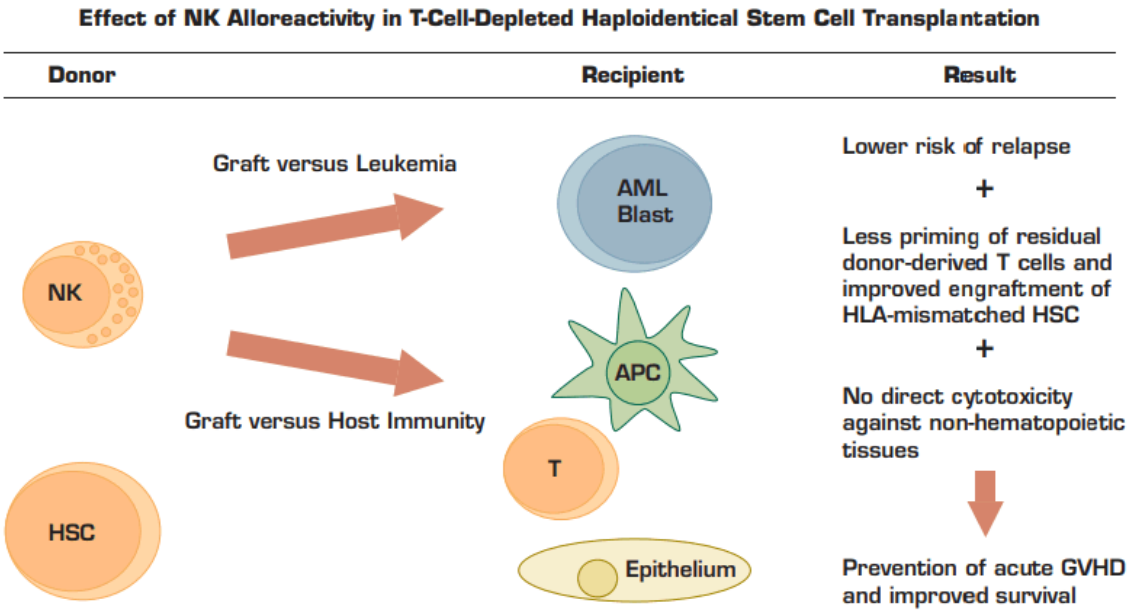

Figure.2.2: The effects of alloreactive NK cells in haplo-HSCT: *NK cell alloreactivity based on the missing-self model, increased the clearing of leukemic cells. As NK cells carried out the anti-tumor effector functions, T cell depletion of the donor graft was permitted, thereby limiting the graft-versus-host disease (GVHD). Combining both features of haplo-HSCT resulted in an improved survival rate and a lower risk of relapse (source[278]).*

5.2. Favorable KIR/HLA interactions in haplo-HSCT

NK cell alloreactivity in haplo-HSCT is only guaranteed in the presence of an iKIR/HLA mismatch between donor and recipient and the corresponding self-HLA present in the donor, which is necessary for NK cell maturation[481]. Also, the absence of the inhibitory CD94/NKG2A was necessary as its ligand,

HLA-E, is expressed on all HLA class-I expressing cells[478,483]. The presence of the activating B haplotype in an AML cohort was strongly related to a low risk of leukemic relapse and prolonged survival[484]. Moreover, different aKIRs have proven their value in haplo-HSCT, as KIR3DS1 and KIR2DS2 were associated with mild acute GVHD and decreased leukemic relapse, respectively[485,486]. KIR2DS1^{+} NK cells have shown to lyse C2/C2 leukemia cells. Signals derived from allogeneic KIR2DS1/HLA-C2 interactions seem powerful enough to override a threshold composed by inhibitory signals generated upon KIR2DL2/3/HLA-C1 or even CD94/NKG2A/HLA-E engagement[483]. Furthermore, KIR2DS1 highly influences the lysis of residual recipient's myeloid-derived DCs (mDCs), decreasing GVHD but also resulting in the uptake of chemokine receptor CCR7, redirecting the NK cells towards the LN[487-491]. Given these results, KIR2DS1 co-expression on donor-derived NK cells is a major contributor to the alloreactivity of NK cells and is considered as one of the more important parameters in calculating the alloreactive NK cell subset size. Besides killing recipient DCs, alloreactive NK cells also kill residual recipient T-cells, preventing host versus graft responses, which results in adequate engraftment[491].

The discovery of alloreactive NK cells as the primary effectors in haplo-HSCT is one of the most significant breakthroughs in the recent history of battling leukemia. Alloreactive NK cells have the potential to improve leukemia therapy further with new, less-invasive and burdensome, techniques.

With HIV-1 transmission, certain parallels can be drawn between HIV-1 and haplo-HSCT protective NK cell characteristics, such as the beneficial effect of an activating B haplotype and the presence of aKIRs. Also, the haplo-HSCT niche precedes HIV-1 in terms of knowledge about alloreactive NK cell responses. Therefore, it is useful to take these results into account to understand the role of alloreactive NK cells in protection against HIV-1 transmission.

6. Alloreactive NK cells in HIV-1 infection

A reasonable amount of research on HIV-1 transmission focuses on the innate immune system, and more specifically, on the relationship of KIR and HLA variability with protection against HIV-1 transmission or disease progression. In most cases, only the recipient's characteristics are taken into

account, whereas NK cells are also able to mediate allogeneic responses, as is seen in haplo-HSCT. In the following paragraphs, we summarized the indications of alloreactive NK cells playing a role in HIV-1 protection in an attempt to provide a clear view.

6.1. Alloreactive NK cells in protection against sexual HIV-1 transmission.

Jennes et al. investigated a cohort of HIV-1 discordant heterosexual couples (dC), one HIV-1^- and one HIV-1^+ partner with mutual exposure, derived from Dakar, Senegal[11]. This study confirmed the association of an activating KIR profile and an autologous KIR/HLA mismatch with HIV-1 protection. On the one hand, the allogeneic KIR/HLA mismatch, KIR2DL1/HLA-C2 recipient and donor HLA-C1/C1, was significantly increased in the HIV-1 dC group compared to the concordant (two HIV-1+ partners; cC) group. On the other hand, the presence of the allogeneic KIR/HLA match: recipient homozygous KIR2DL3 with donor HLA-C1/C2 was significantly increased in the HIV-1 cC group compared to the dC group. In a multivariate analysis, these allogeneic KIR/HLA combinations exceedingly predicted HIV-1 acquisition upon the autologous KIR/HLA combinations, indicating a direct role for the allogeneic infected cells in HIV-1 protection. In theory, the allogeneic KIR/HLA mismatch generates a missing-self context, as in haplo-HSCT, whereby the presence of HIV-1 induced activating ligand expressing allogeneic infected cells induce alloreactive responses by NK cells.

Conversely, an allogeneic KIR- HLA match inhibits NK cell activation as the inhibitory threshold remains. To further investigate the functional capacities of an allogeneic KIR/HLA mismatch, *in vitro* co-cultures between healthy donor NK cells and $CD4^+$ T-cells derived from HIV-1 patients were performed during this PhD (Chapter 4). An apparant increase in $CD4^+$ T-cell death was seen in the presence of an allogeneic KIR/HLA mismatch. These results further support the hypothesis of the presence of HIV-1 protective alloreactive NK cell responses following the missing self-model.

NK cells were also able to kill allogeneic $CD4^+$ T-cells in an anti-HIV-1 Ab dependent manner. In particular educated $KIR3DL1^+$ NK cells from HLA-Bw4 bearing individuals were seen to elicit target cell death[6]. In addition to the results from Jennes et al.[11], these results indicate a beneficial HIV-1 protective effect by educated NK cells. Although HIV-1 ESNs do not possess any anti-HIV-1 Ab, anti-

HIV-1 immunoglobulin G transmission from HIV-1 infected mothers to their child during breastfeeding has been shown to render these children HIV-1 resistant[337]. Therefore, the transfer of anti-HIV-1 Ab during sexual transmission could increase the impact of HIV-1 protective NK cell responses by partially overcoming the inhibitory signals received by allogeneic KIR/HLA matches. Also, educated KIR2DL1^{+} NK cells seemed to benefit from the presence of anti-HIV-1 Ab, but not necessarily in an allogeneic context[459]. These results indicate the beneficial effect of anti-HIV-1 ADCC on protection against HIV-1 transmission in addition to the KIR/HLA mediated NK cell activation.

6.2. Alloreactive NK cells during vertical HIV-1 transmission

HIV-1 infected pregnant women are capable of transmitting the virus to their child during gestation, delivery, or breast-feeding. Mother-child interactions can be interpreted as allogeneic interactions since the child has only one identical haplotype, haploidentical, with the mother. Analogous to multiple studies investigating protection against HIV-1 transmission and infection[11,469-471,473], an elevated presence of an activating KIR profile was observed in "non-transmitting" mothers (HIV-1 protective) compared to transmitting mothers[474]. In multiple studies, vertical transmission of HIV-1 was associated with an allogeneic KIR-HLA match between mother and child[492-495], endorsing the findings of Jennes et al.[11]. Likewise, heterosexual HLA allele sharing was linked to a successful sexual transmission[496]. Vertical and sexual HIV-1 transmission contain similar features as the transmission efficacies for both transmission routes are depending on the quality of the mucosal defense systems. In this regard, it is encouraging to find similar alloreactive NK cell traces associated with HIV-1 resistance.

7. Future perspectives

Mucosal NK cells are present in the female reproductive tract, from the uterus to the vagina, as well as the gastrointestinal tract, and express an immature phenotype (reviewed in[497]). Studying the local maturational, migratory, and cytotoxic capabilities of mucosal NK cells during HIV-1 transmission will help calculate the contribution of mucosal NK cells in HIV-1 protective immune responses. Macaques are an ideal model for these experiments and were already essential for revealing multiple *in vivo* migration routes and immune responses during early HIV-1 infection (reviewed in[498]). Besides, HIV-1

dC couples are very suitable to study the alloreactive mechanism itself on HIV-1 acquisition. Most studies on HIV-1 dC cohorts missed out on investigating NK cell alloreactivity. Habegger et al., for example, looked at KIR and HLA alleles of both partners in an HIV-1 dC cohort without studying the relation between them. If the same data is reanalyzed with attention to allogeneic KIR/HLA relations between both partners, new data can be added to the small amount of information on the role of NK cell alloreactivity in HIV-1 protection. Also, the contribution of anti-HIV-1 ADCC towards NK cell activation is an interesting element that needs to be further explored in allogeneic, but also in autologous context.

8. Conclusion

The HIV-1 epidemic has expanded to become one of the most widespread infectious diseases in the world. Throughout the years, different cohorts have been assembled, consisting of HIV-1 exposed uninfected individuals, ESNs in order to discover and unravel their HIV-1 protective mechanisms in an attempt to develop HIV-1 preventive therapies. In the absence of competent HIV-1 adaptive immune responses and an HIV-1 vulnerable timeframe during and right after transmission, NK cells could be seen as potential HIV-1 restrictive effector cells. NK cells are not only related to protection against transmission but also influence disease progression, although some contradictory results indicate different mechanisms involved in the effect on HIV-1 outcome. The most notable difference was the effect of an activating NK cell profile on the HIV-1 outcome. Protection against HIV-1 infection was associated with a B haplotype and the presence of an activating KIR profile, whereas certain studies associated a B haplotype with a more rapid progression towards AIDS. Importantly, examining the protective effects of HIV-1 dC couples revealed KIR/HLA mismatches between both partners, proposing an HIV-1 protective mechanism orchestrated by alloreactive NK cells, whose usefulness had already been proven in the treatment of leukemia. In summary, an alloreactive NK cell response is mediated i) during the HIV-1 vulnerable timeframe, ii) by an allogeneic KIR/HLA mismatch, triggering the missing self-mechanism, iii) by fully functional NK cells, in contrast to the presence of $CD56^{neg}$ NK cells or the influence of HIV-1 accessory proteins during HIV-1 infection. Still, exact innate immune mechanisms and interactions during HIV-1 transmission and early infection need to be further explored

to situate the potential of NK cells in HIV-1 protection. Future investigations might opt for the use of HIV-1 dC couples or nonhuman primates in search of new insights in alloreactive NK cell responses during HIV-1 transmission. Also, retrospective analysis of HIV-1 dC cohort data is necessary to unravel all the specific components in these NK cell-mediated HIV-1 protective mechanisms. Future research will decide if preventive interventions based on the alloreactive NK cell principle will be one of the possibilities.

Chapter 3 - Scope and outline of the thesis

1. Scope of the thesis

As the lentivirus HIV-1 has only recently infected humans, the arms race between the immune system and HIV-1 is relatively new. On the other hand, primates or other animals already live with the corresponding lentivirus for several thousands of years. As a result, immune responses during SIV infection of primates differ from those observed in HIV-1 infection[499]. Hence, SIV infection in natural hosts does not lead to immune deficiency, whereas HIV-1 infection progresses into the AIDS stage.

Unable to detect the reservoir of latently infected cells[500], the immune system is inadequate to eradicate the HIV-1 infection. Even on a combined ART regimen, the HIV-1 reservoir persists as only the replication active virus is affected by ART. Over time, the progression of chronic HIV-1 infection will lead to the AIDS stage of infection (Chapter 1, 1.6).

In contrast to the progressive immuno-deficiency arising after the systemic dissemination of HIV-1, sexual transmission, as well as acute infection, can be seen as a vulnerable timeframe for HIV-1, since the immune system is intact at that time[5,68]. Evidence for this vulnerable timeframe can be found in the low infection rates of HIV-1, in most cases even established by a single founder viral particle. During HIV-1 transmission, a rapid defense mechanism needs to be present to take advantage of this vulnerable stage. In accordance with the multiple KIR and HLA associations with protection against HIV-1 acquisition (Chapter 2, 4.2.2), NK cells are proposed to interfere at the site of HIV-1 transmission.

2. Hypothesis

2.1. Rationale

Recent genetical studies on ESNs point towards such an innate immune response during these events, generating resistance against HIV-1 acquisition. It was suggested that vaginal NK cells mounted a cytotoxic response against incoming infected CD4+ T cells during or soon after sexual intercourse[11]. The cytotoxic NK cell response may be induced by a missing-self principle, based on the genetic KIR-HLA incompatibility measured between partners of ESN couples (Fig.3.1).

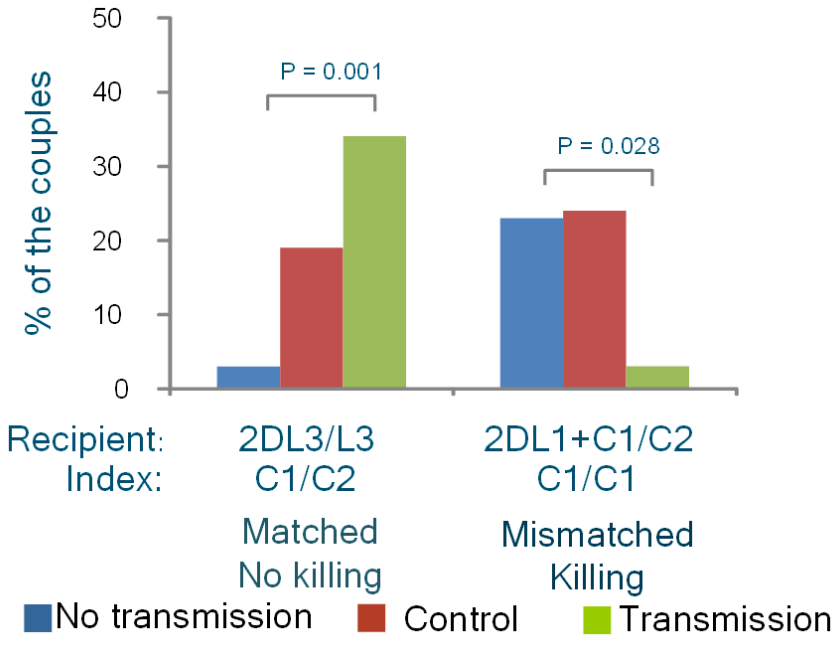

Figure.3.1.: KIR and HLA genotypes of HIV-1 discordant, concordant and healthy couple cohorts: *Prevalence of KIR and HLA genes obtained from both partners in Senegalese HIV-1 discordant (no transmission, blue bar), concordant (transmission, green bar) and healthy (control, red bars) couple cohorts. A KIR-HLA match between index and recipient (KIR2DL3/L3-HLA-C1/C2) was significantly more prevalent in couples with HIV-1 transmission, whereas a KIR-HLA mismatch (KIR2DL1-HLA-C1/C1) was significantly more prevalent in couples without HIV-1 transmission*[11].

2.2. Objectives

To further explore the validity of this theory, we investigated the cytotoxic potential of the allogeneic interactions by recreating these encounters *in vitro*. Based on the missing-self theory, *in vitro* NK cell cytotoxicity should be elevated in the presence of:

1. A KIR-HLA incompatibility between NK cells and CD4+ T cells
2. Activating ligand stimulation, known to be upregulated by HIV-1

In vivo, KIR-HLA incompatibility between stem cell donor and patient-generated missing-self NK cell cytotoxicity against leukemic cells in the treatment of leukemia by Haplo-HSCT (Chapter 2, 5.1). Although NK cells are also able to mount an anti-viral response in autologous conditions, the impact of allogeneic NK cell encounters in a viral context such as HIV-1 are unknown. Therefore, as first, we aimed to observe the **differences in NK cell cytotoxicity against allogeneic CD4+ T cells based on the presence or absence of HLA ligands**. The NK cells (expressing various KIR) represent the

"exposed" partner, while the CD4+ T cells (expressing various HLA ligands) represent the "infected" partner in this *in vitro* model. As each KIR receptor is solely expressed on a subset of the NK cells, more conclusive evidence for the impact of this KIR-HLA incompatibility can be found when measuring the **degranulation by the different KIR expressing NK cell subsets**. In addition, NK cell cytotoxicity by these different KIR expressing NK cell subsets allowed us to **compare the impact of the various KIR-HLA mismatches** (KIR3DL1+/HLA-Bw4-, KIR2DL1+/HLA-C2- and KIR2DL2/3/HLA-C1-) on the strength of the missing-self response. This variety could be of particular interest to verify the HIV-1 protective effect as Jennes et al.[11] specifically associated a KIR2DL1-HLA-C2 mismatch with protection from HIV-1 acquisition. Also, the multiple associations[470,501] of an **activating KIR haplotype (AB)** of the exposed partner were investigated concerning its impact on allogeneic missing-self responses.

Besides the lack of inhibitory input, an activating signal on the infected CD4+ T cells still needs to be present to induce missing-self NK cell responses. In accordance with the anti-tumor activity of allogeneic missing-self NK cell responses, HIV-1 infected cells were suggested to be more prone to NK cell cytotoxicity by the expression of activating NK cell ligands[240]. Therefore, CD4+ T cells were ***in vitro*** **HIV-1 infected** to **measure the impact of HIV-1 infection on missing-self NK cell cytotoxicity**. HIV-1 infection was monitored by the fluorescent staining of the HIV-1 p24 antigen, enabling the comparison of **NK cell cytotoxicity against productively infected, latently-or uninfected cells**. For a more in-depth analysis of the NK cell responses against the HIV-1 infected cells, **activating ligands** expressed on CD4+ T cells were stained and marked for their **contribution to NK cell cytotoxicity** (Table.3.1).

Source	Infected Partner	Co-culture	HIV-1 exposed partner	Objectives
Chapter 4	HIV-1 patient-derived CD4 T cells	--><--	Healthy NK cells	*What is the impact of KIR-HLA incompatibility on NK cell cytotoxicity?*
		Fresh samples		
Parameters	Cell death		NK cell degranulation	
			KIR receptors	*Is KIR expression associated to NK cell activation?*
			KIR haplotype	*Can activating KIRs enhance missing-self responses?*
Chapter 5	*In vitro* infected healthy CD4 T cells	--><--	Healthy NK cells	*What is the impact of HIV-1 on missing-self responses of NK cells?*
		Cryopreserved samples		
Parameters	Cell death			
	HIV-1 related NK cell ligands			*Can HIV-1 related ligands enhance missing-self responses?*

Table 3.1. A scheme explaining the different co-culture models used. *In the table, both the source of the NK cells (HIV-1 exposed partner) and CD4 T cells (Infected partner) used in the co-cultures of both chapters is depicted, as well as these*

samples were used fresh or were cryopreserved before using them in the co-cultures. Within these co-cultures, the different parameters measured are explained. Also the objectives related to these parameters are shown.

3. Thesis outline

In **chapter 4**, *in vitro* allogeneic co-cultures were conducted between NK cells from healthy donors and CD4 T cells derived from HIV-1 patients to provide evidence for missing-self alloreactivity in the context of HIV-1 transmission. **In chapter 5**, CD4+ T cells derived from healthy donors were *in vitro* infected to assure the presence of infected CD4+ T cells and the upregulation of activating ligands. In combination with the staining of activating NK cell receptors, we were able to assess the vulnerability of HIV-1 infected CD4+ T cells for the KIR-HLA incompatible conditions. Finally, **chapter 6** consists of a general discussion on the presented data and our view on future directions in NK cell research investigating protection against HIV-1 acquisition.

Chapter 4 - *In vitro* proof-of-concept of KIR-HLA mismatched allogeneic encounters

Research article published in *Frontiers in Immunology* on July 9, 2018

DOI: 10.3389/fimmu.2018.01588

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Impact of the variable KIR-HLA interactions on NK cell cytotoxicity towards foreign CD4 T cells

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1. Abstract

Background: NK cells are known to mount a response against foreign target cells if the absence of the dominant KIR-HLA interaction significantly lowers the threshold for NK cell activation. NK cells could thus constitute a vital part of the mucosal defense against cell-associated sexually transmitted diseases. Here, we performed a detailed analysis of hitherto unexplored KIR-HLA-incompatible NK cell interactions.

Methods and Findings: NK cells from healthy donors were co-cultured with CD4+ T cells derived from HIV-1 patients, and the KIR-specific NK cell cytotoxicity was measured using flow cytometry. Genotyping of KIR and HLA predicted the KIR-HLA interactions occurring during these 124 allogeneic encounters. KIR2DL1+ NK cells specifically responded most robustly in the absence of their ligand with a 3.2-fold increase in KIR2DL1+ NK cells in the total NK cell response. Moreover, an association between the size of the alloreactive NK cell population and the amount of CD4+ T cell death ($p=0.0023$) and NK cell degranulation ($p=0.0036$) was only present in NK cell donors with an activating KIR haplotype.

Conclusion: We demonstrate differences in the activating effect of KIR-HLA incompatibility, according to the KIR involved, with KIR2DL1 as the strongest responder. An activating KIR haplotype optimized the contribution of KIR-HLA-incompatible NK cells in the total NK cell response.

2. Introduction

NK cells provide a first line of defense against malignant or virally infected cells. This process is orchestrated by the receptor-ligand interactions upon encountering the target cell. The balance between inhibitory[174,176] and activating[175,177-180] interactions will determine the cytotoxic behavior of the NK cell. NK cells mainly interact through receptors of the KIR family, which are categorized by their ability to transmit an inhibitory (iKIR) or an activating (aKIR) signal[173-180]. On the side of the target cell, ligands for each iKIR are found on the HLA class I molecules. Ligands for the KIR2DL1-3 receptors are situated on the HLA-C molecule, binding KIR2DL1 in the presence of an HLA-C2 motif, whereas a C1 motif will bind KIR2DL2 and -3[211]. KIR3DL1 binds HLA-A and -B molecules with a Bw4 motif[389]. In contrast, ligands of activating receptors are generally restricted to tumor- or virally infected cells and are directly expressed on the membrane or as tumoral/viral peptides presented in the peptide-binding groove of HLA-molecules of the target cells[224,228-230,390]. Accordingly, NK cell activation is restricted to encounters with transformed or virally infected cells while tolerating healthy autologous cells.

The absence of the dominant HLA inhibitory signal on the target will bring the iKIR+ NK cell to the brink of activation, with only an additional activating signal needed to push it over the edge into a cytotoxic state. This principle is successfully applied in stem cell transplantation for the treatment of leukemia by the use of haploidentical HLA donors. A pre-defined KIR-HLA mismatch resulted in "alloreactive" cytotoxic NK cell responses against the remaining leukemic cells while leaving the healthy recipient cells unharmed[478,479,502]. As the activating "transformed" aspect thus allowed the selective killing of unwanted cells, it was the avoidance of the inhibitory "donor HLA" threshold that sigbificatly amplified the response, resulting in an improved clinical outcome. As a result of viral infection, cells likewise downregulate their HLA expression to avoid anti-viral adaptive immunity. Nonetheless, viruses such as HIV-1 are able to preserve the expression of HLA-C and, to a lesser extent, HLA-B by viral proteins[423,429,430], hampering NK cell responsivity against these infected cells[424,425].

During sexual HIV-1 transmission, infected donor CD4+ T-cells have been observed to transfer the virus efficiently[70]. As viral HLA modulation only influences the donor's HLA repertoire, it renders itself vulnerable for recipient NK cell responses. Protection against HIV-1 acquisition in dC couples has been related to a genetic KIR-HLA "mismatch" between the partners[11]. These observations highlight the potential of a local[306] protective alloreactive NK cell response triggered by the lack of HLA ligands on the HIV-1 donor-derived cells. However, very little is known about how the different individual KIR-HLA interactions influence the NK cell response against allogeneic CD4+ T-cells[4]. In this study, we examined the various allogeneic KIR-HLA interactions between NK cells derived from healthy individuals and CD4+ T-cells derived from HIV-1 patients. Combining genetic and phenotypical data, we defined the strongest and weakest allogeneic KIR-HLA interactions, as well as their respective contribution to the total NK cell response.

3. Material and Methods

3.1. Study population

NK and CD4+ T-cells were isolated from a total of 62 healthy individuals and 62 HIV-1 patients, respectively. NK cells were isolated from buffy coats obtained from healthy individuals collected at the Blood Transfusion Centre (Rode Kruis-Vlaanderen, Mechelen, Belgium). CD4+ T-cells were isolated from blood samples obtained from HIV-1 patients at the Institute of Tropical Medicine. HIV-1 patient selection criteria was a decent CD4+ T-cell count ($\geq$ 500 cells / mm³). ITM's Institutional Review Board approved the use of HIV-1 patients' blood.. General informed consent was obtained from all participating HIV-1 patients (Policy number 99.002.067).

3.2. *In vitro* co-cultures

3.2.1. Experimental design

NK cells derived from healthy donors were co-cultured with CD4+ T-cells derived from HIV-1 patients. NK cells were always co-cultured in parallel with CD4+ T-cells from 2 different patients to

maximize the amount of possible KIR-HLA combinations. In turn, the CD4+ T-cells of each HIV-1 patient were paired with the NK cells of two healthy donors, resulting in 4 unique NK-CD4 encounters (Suppl. Fig.4.1). In addition, DNA was isolated from left-over PBMCs and used for KIR or HLA PCR genotyping of the donors. With 62 NK- and 62 CD4+ T-cell donors included in the study, this design resulted in a total of 124 NK-CD4 co-cultures. All biosafety procedures to work in a BSL-2 laboratory were applied.

3.2.2. Isolation and cultivation of NK cells

PBMCs were isolated using a density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare Life Sciences) from whole blood. The fresh PBMCs underwent negative magnetic separation to isolate the NK cells (MidiMACS™ Separator, Miltenyi Biotec) with a mean purity of 96,84%. NK cells were incubated in R10 (90% RPMI (LONZA) containing 100 U/ml penicillin, 100 µg/ml streptomycin + 10% fetal bovine serum) and stimulated with 200U/ml recombinant IL-2 (Gentaur) for three days at 37°C, 5% CO_2 to induce proliferation and enhance the cytotoxic potential[257].

3.2.3. Isolation and cultivation of CD4+ T-cells

Fresh PBMCs were separated from HIV-1 patient blood using a density gradient centrifugation (Ficoll-Paque) and were used to isolate CD4+ T-cells using a positive magnetic separation column (MiniMACS™ Separator, Miltenyi Biotec) with a mean purity 98,63%. The CD4+ T-cells were stimulated with 1 µg/ml phytohaemagglutinin (PHA, Remel) and 100 U/ml IL-2 (Gentaur). All CD4+ T-cells were cultured overnight in R10.

3.2.4. Allogeneic in vitro NK-CD4 co-cultures

After these respective pre-incubations, NK cells and CD4+ T-cells were co-cultured in a 96-well Polystyrene plate (U-Bottom, FALCON) and incubated for 4 hours at 37°C and 5% CO_2 (Suppl. Fig.3.1). In the target cell death assay, $500x10^3$ NK cells and $50x10^3$ CD4+ T-cells were co-cultured at an effector:target (E:T) ratio of 10:1. As a negative control, $50x10^3$ CD4+ T-cells alone were cultured in medium. As a positive control, $125x10^3$ NK cells were co-cultured with $50x10^3$ NK cell

sensitive K562 cells at an E:T ratio of 2.5:1. Cell death was measured using 7-Amino-actinomycin D (7-AAD) staining. In the degranulation assay, $250x10^3$ NK cells and $250x10^3$ CD4+ T-cells were co-cultured at an E:T ratio of 1:1. As a negative control, $50x10^3$ NK cells alone were cultured in medium. As a positive control, $125x10^3$ NK cells were co-cultured with $125x10^3$ K562 cells at an E:T ratio of 1:1. NK cell degranulation was detected by measuring CD107a expression, a molecule present on the cytolytic vesicles of NK cells. Fluorescently labeled anti-CD107a (2 µl/well) was added at the start of the assay, and monensin (1µl/well) was added 1 hour into the co-culture to capture CD107a expression at the cell membrane by blocking the intracellular destruction of the vesicles.

NK cell-mediated cell death or CD4 T cell-mediated degranulation were calculated by subtracting the cell death or degranulation in the respective monocultures from the value measured in the co-culture. With this, the remaining value indicates the size of the NK cell-mediated cell death or CD4 T cell-mediated degranulation.

3.3. KIR and HLA genotyping

Genomic DNA was extracted from PBMCs of healthy donors and HIV-1 patients with the use of the DNeasy® Blood & Tissue Kit (QIAGEN®). Using the KIR Typing Kit (Miltenyi Biotec), we determined the presence of 6 inhibitory KIR genes (KIR2DL1,-2DL2,-2DL3,-2DL5(A/B) and -3DL1) and 6 aKIR genes (KIR2DS1, -2DS2, -2DS3, -2DS4 (del/ins), -2DS5 and -3DS1). Using the KIR HLA Ligand kit (Olerup SSP®), we evaluated the presence of the KIR3DL1 ligand, HLA-Bw4, as well as the C2 and C1 motif on the HLA-C alleles, which are ligands of KIR2DL1 and KIR2DL2/3 respectively. NK cell donors underwent both KIR and HLA genotyping, while CD4+ T-cell donors were genotyped for HLA ligands (Suppl. Table 4.1A and B).

Only NK cells that are capable of creating a KIR-HLA bond during maturation will become functionally competent or "licensed" NK cells[336,391,393-395]. According to its HLA genotype, we determined the licensed and unlicensed KIR+ NK cell subpopulations in each NK cell donor. In combination with the HLA genotype of the CD4+ T-cell donor, we determined the possible allogeneic KIR-HLA interactions between licensed iKIR+ NK cells and CD4+ T-cells (Suppl. Table. 4.1C). The

absence of the HLA ligand by the CD4+ T-cell donor resulted in a KIR-HLA mismatch, while the presence of the HLA ligand resulted in a KIR-HLA match. In total, we classified 35 co-cultures with one mismatch and 12 co-cultures with two mismatches.

3.4. Flow cytometric analysis

Measuring the NK cell degranulation after NK-CD4 co-cultures, CD56+/CD3- NK cells and CD3+/CD4+ T-cells were identified in the lymphocyte gate. Subsequently, the frequency of CD107a+ NK cells was measured in the total NK cell population as well as within NK cells that express only one of the four inhibitory receptors (KIR3DL1, KIR2DL1, KIR2DL2/3, and NKG2A). Using Boolean gating, three different Single-iKIR+ NK cell populations were designated: KIR3DL1+ (KIR3DL1+/2DL1-/2DL2/3-/NKG2A-), KIR2DL1+ (KIR3DL1-/2DL1+/2DL2/3-/NKG2A-) and KIR2DL2/3+ (KIR3DL1-/2DL1-/2DL2/3+/NKG2A-) (Suppl. Fig.4.2). The antibodies used in the co-cultures were CD107a-BV421 (BD), KIR3DL1-FITC (Miltenyi), KIR2DL1-APC (R&D), KIR2DL2/3-PE-Vio770 (Miltenyi), NKG2A-PE (R&D), CD3-APC-H7 (BD), CD4-PerCP-Cy5.5 (BD), and CD56-BV510 (BD).

Lymphocytes were gated using FSC and SSC parameters to measure the target cell death in the co-cultures. Within the lymphocyte gate, we identified NK cells as CD3-/CD56+ cells. To optimize the measurement of dead cells, CD4+ T-cells were gated as CD3+/CD4+ cells within all events instead of the lymphocyte gate. Subsequently, the frequency of 7-AAD+ CD4+ T-cells was measured (Suppl. Fig.4.3). The antibodies used were 7-AAD (BD), CD56-PE (BD), CD3-APC (BD) and CD4-BV421 (BD).

All cell cultures were flow cytometrically analyzed by the BD FACSVerse® flow cytometer and the including BD FACSuite® software.

3.5. Statistical analyses

All statistical analyses (Graphpad Prism V.8.1.0) were non-parametrically tested. The non-paired analysis included the Mann-Whitney U test, for paired analysis we applied the Wilcoxon matched-

pairs signed-rank test, correlations were analyzed using Spearman rank correlation, and differences across more than two populations were analyzed using Kruskal-Wallis tests with Dunn's Multiple Comparison Test. Pie chart percentages were based on the median values of each NK cell subpopulation.

4. Results

4.1. Demographic characteristics of the study populations

The distribution of men and women among the NK and CD4+ T-cell donors was comparable. The median age of the healthy NK cell donors was 55 years with an interquartile range (IQR) between 43 and 63 years. The median age of HIV-1 patients was 47 years (IQR: 38-52 years). Within the CD4+ T-cell donors, CD4+ T-cell count (363 cells/mm^3; IQR: 261.5-490) and viral load (92700 cps/ml; IQR: 17900-230074) at the start of ART, as well as the CD4+ T-cell count (745 cells/mm^3, IQR: 566.5-877.8) and viral load (20 cps/ml; IQR: 20-35) at the time of sample collection were collected. The duration of the ongoing therapy (2206 days; IQR: 876-4767) was included as an additional clinical parameter. These parameters were analyzed for their potential to affect the responses in our *in vitro* co-cultures (Suppl. Table 4.3). However, no correlations were found, except for a modest but significant correlation between age of the CD4+ T-cell donors and CD4+ T-cell death (p=0.0053; R=0.2491, data not shown).

4.2. A KIR-HLA mismatch between NK and CD4+ T-cell donors resulted in a significant increase in CD4+ T-cell death and NK cell degranulation

To account for NK cell donor variability, we performed a paired analysis with the NK cell donors displaying one or two KIR-HLA mismatches with one CD4+ T-cell donor and no KIR-HLA mismatch with the other (n=22/62 NK cell donors). Cultures with at least one mismatch showed a clear increase in NK cell degranulation, compared to matched cultures with no mismatch (p=0.0309, Fig.4.1A). CD4+ T-cells demonstrated significantly more cell death in mismatched as compared to matched co-cultures (p=0.0002) (Fig.4.1B).

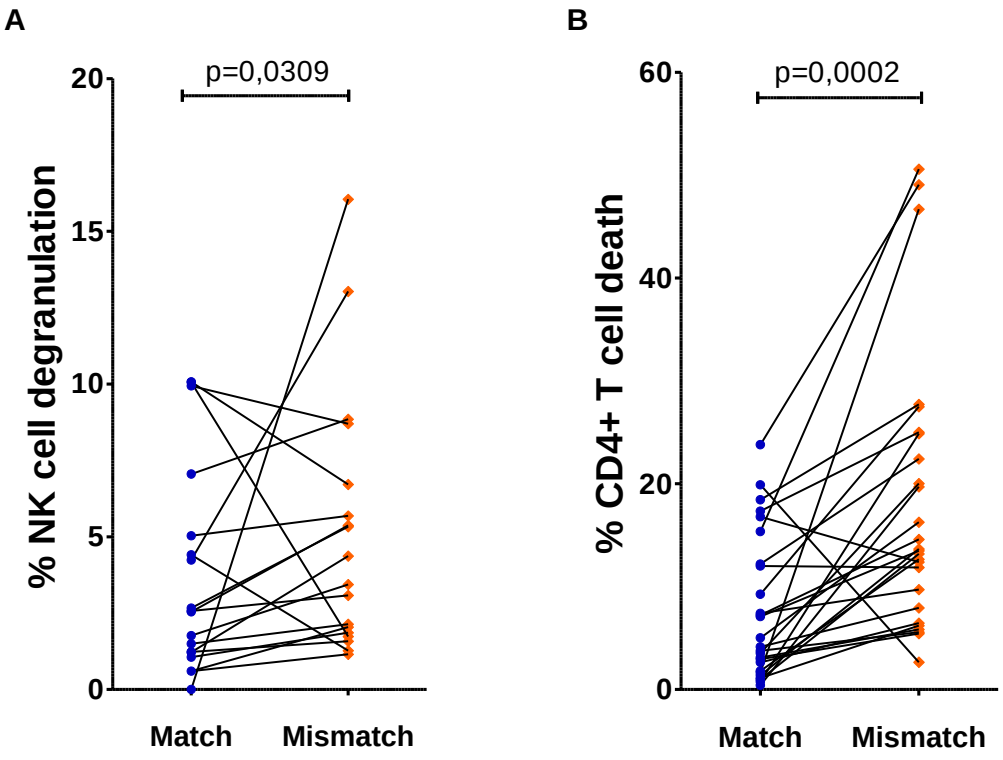


Figure.4.1: Effect of missing-self on CD4+ T cell death and NK cell degranulation: *Both KIR and HLA genotyping of NK cell donors and HLA genotyping of HIV-1 patients resulted in the determination of KIR-HLA mismatches between the NK cells and CD4+ T cells, creating a missing-self context. To exclude NK cell donor variability, we selected the NK cell donors that were put up against CD4+ T cells of 2 different HIV-1 patients that resulted in a KIR-HLA match on the one hand and a KIR-HLA mismatch on the other (n=26). In A), the frequency of degranulating (CD107a+) NK cells (CD56+/CD3-) was compared between co-cultures with (Mismatch, orange diamond) or without (Match, blue dot) a KIR-HLA mismatch. Similarly, in B), the frequency of dead (7AAD+) CD4+ T cells was compared between co-cultures with (Mismatch, orange diamond) or without (Match, blue dot) a KIR-HLA mismatch. For both degranulation and cell death, the shown values are a result of subtraction of the measured value in the respective monocultures. Using a paired (Wilcoxon paired signed ranked test) analysis, a significant increase was seen in a missing-self situation.*

4.3. A KIR-HLA mismatch as an initiator for NK cell degranulation

We next aimed to investigate whether this apparent allogeneic response was indeed due to specific KIR-HLA mismatches. To this end, we performed a similar paired analysis, this time measuring NK cell degranulation by the NK cells expressing those KIRs to which the ligand was absent in the mismatched co-culture. NK cells expressing multiple KIRs were excluded to avoid inhibitory signals received through other KIRs (Suppl. Fig.4.4). Thus, for each NK cell donor, only NK cell

subpopulations expressing either KIR3DL1, -2DL1, or -2DL2/3 (Single-iKIR) were analyzed. Compared to co-cultures where Single-iKIR+ NK cells were exposed to HLA matched CD4+ T-cells, the percentage of degranulating Single-iKIR+ NK cells increased under KIR-HLA mismatched conditions respective to each KIR subtype (KIR3DL1 p=0.0078, KIR2DL1 p=0.0017, and KIR2DL2/3 p=0.0537)(Fig.4.2A). In parallel, the intensity of degranulation (measured by median fluorescence intensity) increased as well (KIR3DL1 p=0.002, KIR2DL1 p=0.0046, and KIR2DL2/3 p=0.0244)(Fig.4.2B).

To determine the contribution of the Single-iKIR+ NK cells to the general NK cell response, we next differentiated the total NK cell degranulating response into segments of Single-iKIR+- and Multi-iKIR+ NK cell subpopulations (blue sectors) (Fig.4.2C). As no additional markers were used, degranulating NK cells not belonging to any of these groups were classified as Non-KIR for comparison. Each pie chart represents a collection of co-cultures where NK cell subpopulations with one of the three KIR-HLA mismatches are highlighted (exploded orange sector). Mismatched conditions were compared to total matched co-cultures (n=14). The contribution of Single-KIR3DL1+ NK cells to the total NK cell response increased 4.4-fold (21.6/4.93%) in the absence of HLA-Bw4 (n=14), while in the absence of HLA-C2 (n=10), the contribution of Single-KIR2DL1+ NK cells to the total NK cell response increased 3.2-fold (26.64/8.4%). The absence of HLA-C1 resulted (n=11) in a 1.4-fold (32.98/24.21%) increase in Single-KIR2DL2/3+ NK cells in the total NK cell response.

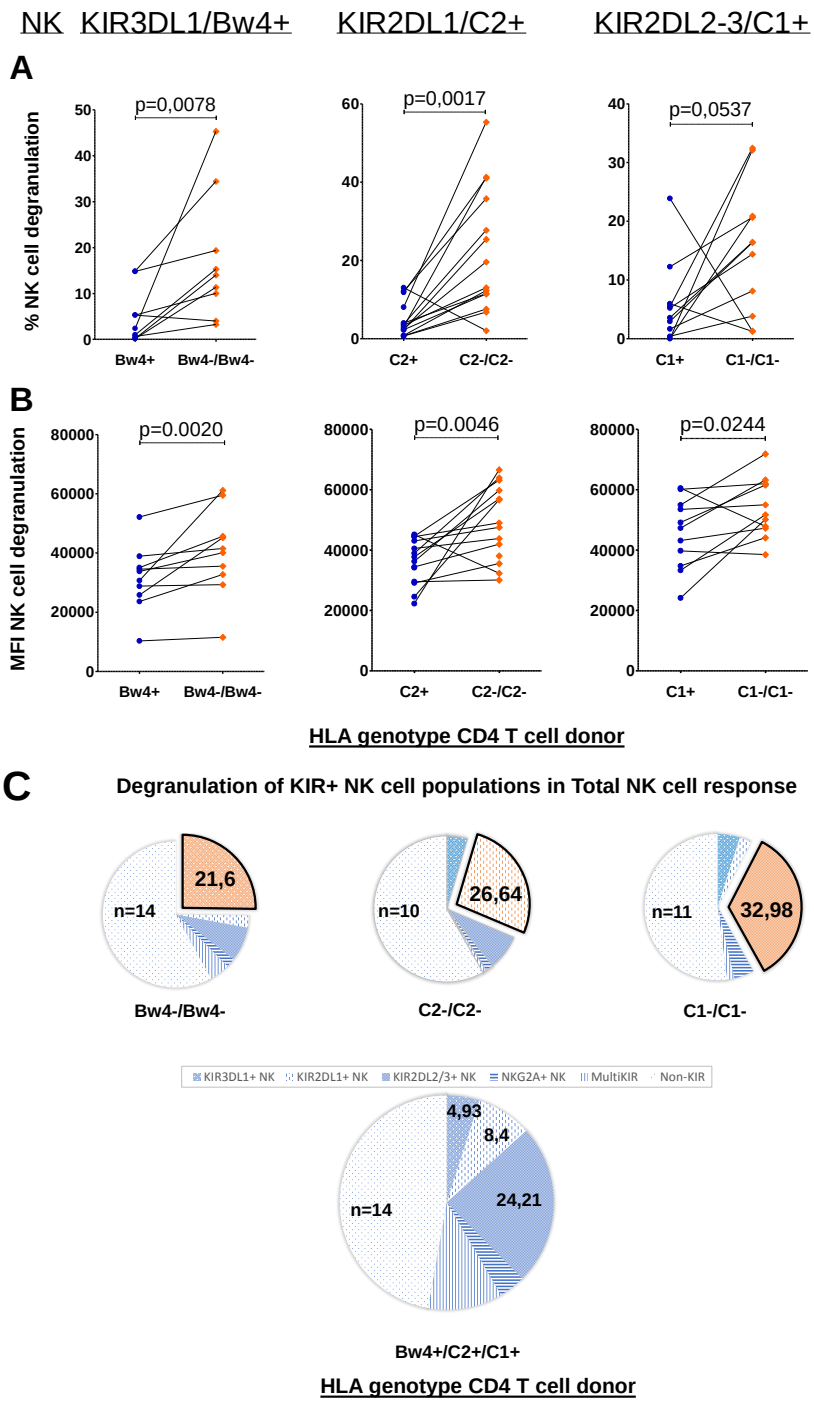


Figure.4.2: Effect of allogeneic HLA genotypes on the functionality of Single KIR+ NK cell populations: *Single KIR+ NK cells were isolated using a boolean gating strategy to evaluate the impact of the absence of self HLA ligands expressed by the CD4+ T cells on NK cell functionality. A first criteria was the presence of "licensed" NK cells (KIR3DL1/Bw4, KIR2DL1/C2, and KIR2DL2-3/C1, above the graphs). A combination with the HLA genotype of the HIV-1 patients (x-axis) resulted in the presence of a KIR-HLA mismatch (orange diamonds) or a KIR-HLA match (blue dots). In graph A), we compared the frequency of degranulating NK cells expressing a single KIR in the context of a KIR-HLA mismatch and match (KIR3DL1/Bw4=9, KIR2DL1/C2=14, and KIR2DL3/C1=11). In graph B), identically to A), we compared the intensity of the*

NK cell response in both the context of a KIR-HLA mismatch and match (KIR3DL1/Bw4=9, KIR2DL1/C2=14, and KIR2DL3/C1=11). Using a paired (Wilcoxon paired signed ranked test) analysis, a significant increase was seen in a missing-self situation. In C), we show pie charts of the total NK cell degranulation response per mismatched condition, divided into segments of Single-iKIR+- and Multi-iKIR+ NK cell subpopulations. The Single KIR+ NK cell population sensitive to their respective mismatch is highlighted (exploded orange sector). The amount of samples used to calculate median values is displayed within the pie chart (n=x).

4.4. The contribution of an activating KIR genotype on NK cell cytotoxicity

NK cells require at least one activating signal to become activated, independent of their iKIR-HLA compatibility. By dividing the previously selected NK cell donors based on their aKIR genes (inhibiting A- and activating B KIR haplotype, AA and AB NK cell donors), we were able to interpret its influence on the various Single-iKIR NK cell populations (Fig.4.3). Paired tests showed significant differences in a KIR3DL1-HLA-Bw4 and KIR2DL1-C2 mismatch condition; however, only when looking at AA NK cell donors. The frequency (Fig.4.3A), as well as the intensity of these responses, (Fig.4.3B) were significantly increased (p=0.0313 for all four tests). No differences were seen regarding Single-iKIR NK cell degranulation in mismatched conditions between AA and AB NK cell donors.

To measure the impact of the KIR haplotype on the various NK cell populations of the total NK cell response, we again differentiated the total degranulating NK cell population into segments of iKIR+ NK cell subpopulations in the context of each KIR-HLA mismatch (exploded orange sector), this time segregating AA and AB NK cell donors (Fig.4.3C). In a mismatch condition, the contribution of Single KIR3DL1+ NK cells to the total NK cell response of AA NK cell donors (n=11) increased 7.5-fold (26.62/3.57%) while in AB NK cell donors (n=3) it did not differ (5.32/5.69%). The contribution of Single KIR2DL1+ NK cells in a mismatch condition resulted in a 2.8-fold (33.95/11.97%) and a 3.4-fold increase (22/6.41%) for AA (n=4) and AB (n=6) NK cell donors, respectively. The contribution of Single KIR2DL2/3+ NK cells of AA (n=4) NK cell donors increased by 2.4-fold (36.15/15.38%) in a mismatched condition, while in AB (n=7) NK cell donors it did.not differ (30.8/29.11%).

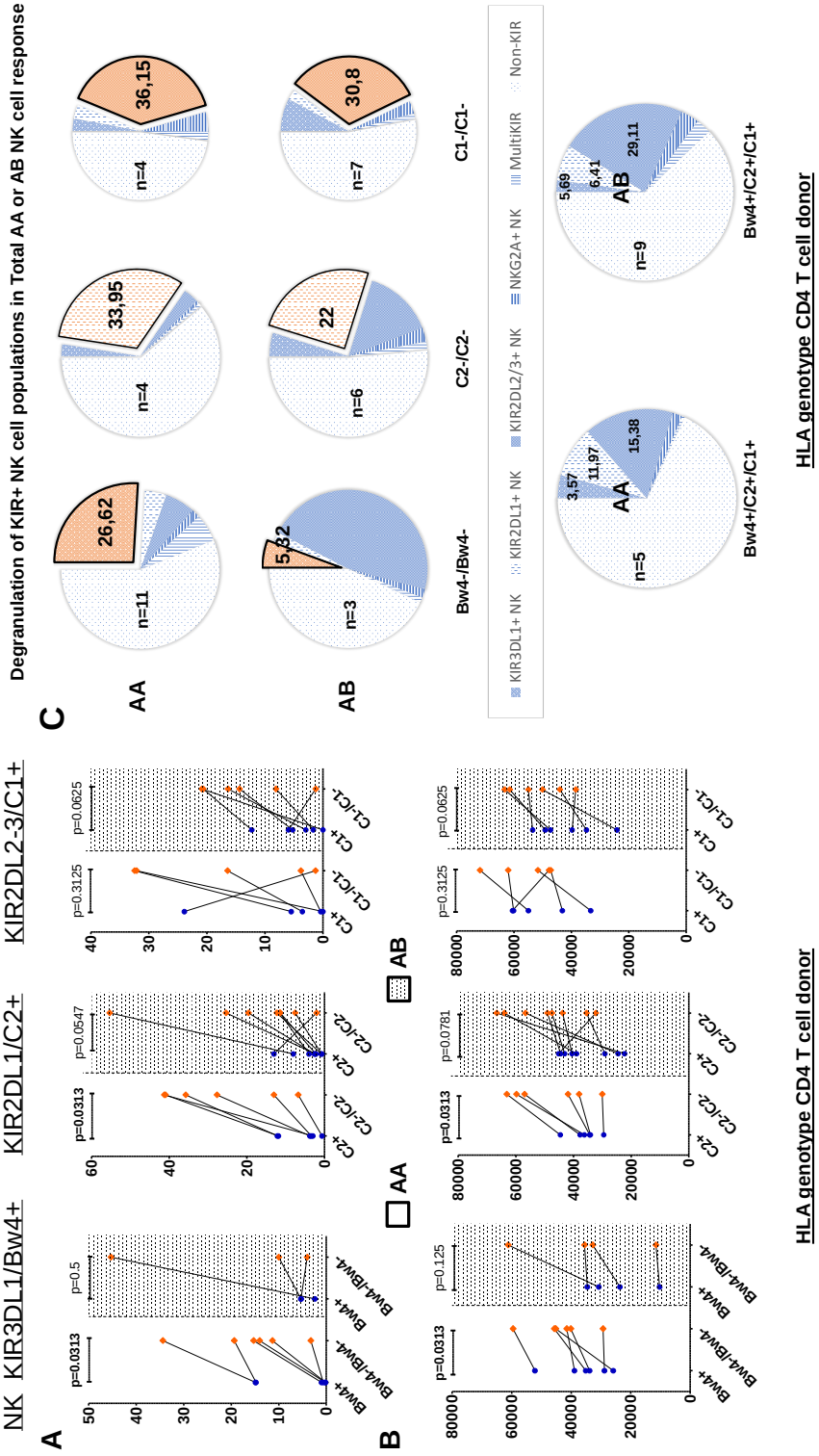


Figure.4.3: Effect of allogeneic HLA genotypes on the functionality of Single KIR+ NK cell populations in AA and AB NK cell donors: *Similar to Fig.4.2., degranulation of "licensed" Single KIR+ NK cells (displayed above the graphs) was compared between KIR-HLA matched (blue dots) or mismatched (orange squares) co-cultures in panel A and B. In addition to Fig.4.2., the NK cell*

donors were divided based on their KIR haplotype, AA or AB (shaded background), and were divided by a dotted line (panels A and B). In the case of KIR3DL1 AA n=6, AB n=3; KIR2DL1 AA n=6, AB n=8 and KIR2DL2/3 AA n=5, AB n=6). In panel A, the frequency of degranulating Single-iKIR+ NK cells is displayed, whereas in panel B, the intensity of the NK cell response (Median fluorescence intensity (MdFI)) is shown. Using a paired (Wilcoxon paired signed ranked test) analysis, a clear increase was seen in a missing-self situation. In C), we show pie charts of the total NK cell degranulation response by AA or AB NK cell donors. The pie chart is divided into segments of Single-iKIR+- and Multi-iKIR+ NK cell subpopulations in the context of each KIR-HLA mismatch (exploded orange sector) and KIR-HLA match (blue sectors). The amount of samples used to calculate median values is displayed within the pie chart (n=x).

4.5. Comparing the intrinsic strengths of the various KIR-HLA mismatch effects

Next, we investigated which Single iKIR+ NK cell population responded the strongest in the absence of their respective ligand. Therefore, we compared the NK cell degranulation and CD4+ T-cell death induced by the different KIR-HLA mismatches (Fig.4.4). To this end, we selected and compared all co-cultures containing one or two KIR-HLA mismatches, regardless of available KIR-HLA matched controls (n=47/124). Significantly more degranulating Single-iKIR+ NK cells were seen in the context of a KIR2DL1-C2 mismatch compared to a KIR3DL1-Bw4 (p=0.0134) mismatch (Fig.4.4A). When looking at the intensity of the NK cell degranulation (Fig.4.4B), the intensity of the KIR3DL1-Bw4 mismatch response was significantly lower compared to the intensity seen in the context of a KIR2DL1-C2 (p=0.0031) and a KIR2DL2/3-C1 (p=0.0009) mismatch. Because the frequency of dead CD4+ T-cells is a downstream event, it cannot be specifically attributed to a single NK cell subpopulation when multiple KIR-HLA mismatches are present. To compare the impact on CD4+ T-cell death, we chose to select only co-cultures with a single KIR-HLA mismatch (n=35/124). Significantly less dead CD4+ T-cells were demonstrated in the context of a KIR3DL1-Bw4 mismatch compared to KIR2DL1-C2 (p=0.0095) and KIR2DL2/3-C1 (p=0.0037) mismatch (Fig.4.4C). No significant differences were seen between KIR2DL1+ and KIR2DL2/3+ mismatched events in all of the three tests (Fig.4.4A-C).

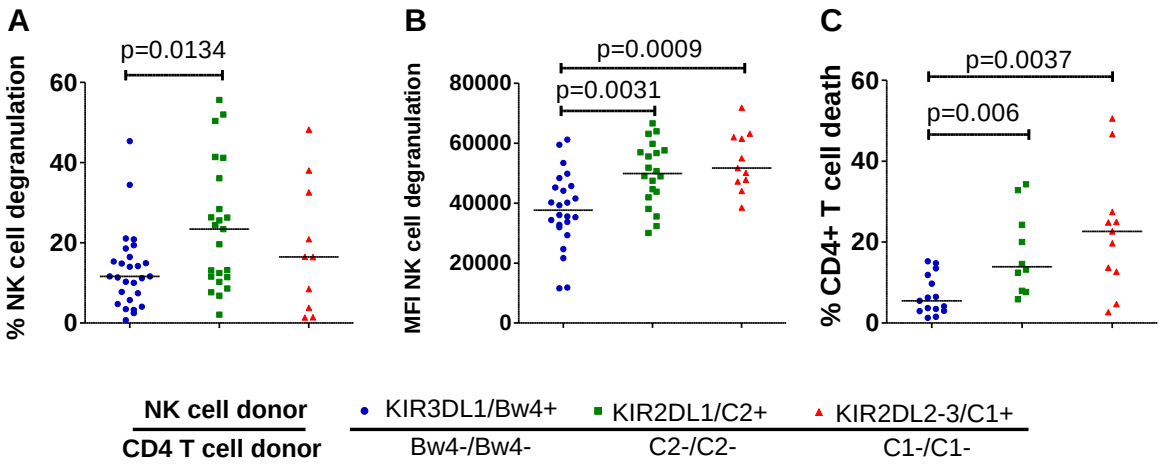
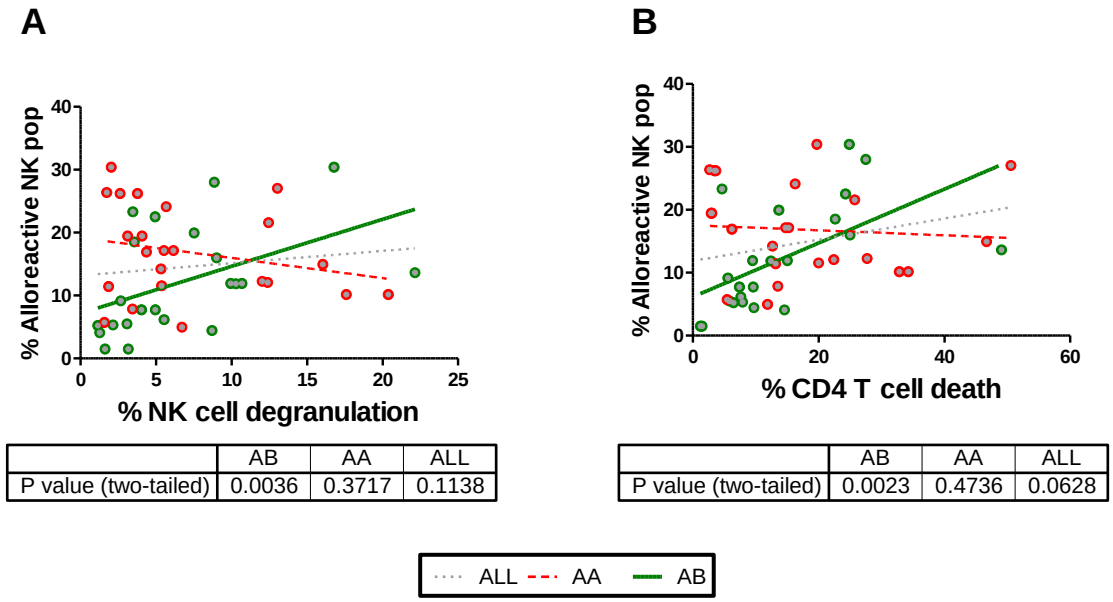


Figure.4.4: Comparison of the different Single KIR+ NK cell populations in the missing-self context: *In graph A), we compared the frequency of degranulating NK cells within each Single-iKIR NK cell population in the corresponding missing-self context. In the case of KIR3DL1 n=27, KIR2DL1 n=23, and KIR2DL2/3 n=11. In graph B), shows the intensity of the degranulation of NK cells in a missing-self context between the different Single KIR+ NK cell populations. In the case of KIR3DL1 n=27, KIR2DL1 n=23, and KIR2DL2/3 n=11. In graph C), we compared the % dead CD4+ T cells in a missing-self context between the different Single KIR+ NK cell populations. In the case of KIR3DL1 n=15, KIR2DL1 n=11, and KIR2DL2/3 n=11. Using non-parametric analysis (Mann-Whitney U test), we demonstrate clear differences between the various Single-iKIR+ NK cell populations.*

4.6. The predictive value of a KIR-HLA mismatch on NK cell cytotoxicity

Although we clearly show the impact of KIR-HLA mismatches on NK cell responsiveness, it is uncertain to what extent the KIR-HLA mismatch catalyzes the total NK cell response against CD4+ T-cells. Therefore, we calculated potential correlations between the frequencies of dead CD4+ T-cells or degranulating NK cells (Fig.4.5) with the pooled frequencies of all Single-iKIR+ NK cells subjected to a KIR-HLA mismatch, irrespective of their degranulation state. No correlations between the frequency of the alloreactive NK cell population and CD4+ T-cell death and NK cell degranulation were seen (NK cell degranulation p=0.1667, CD4+ T-cell death p=0.1). Subsequently, we conducted a similar correlation analysis but with the segregation of the AA and AB NK cell donors. Here, the frequency of mismatched NK cells derived from AB NK cell donors did correlate with NK cell degranulation (p=0,0036; R=0.594) (Fig.4.5A) and CD4+ T-cell death (p=0.0023, R=0.616) (Fig.4.5B).



	AB	AA	ALL
P value (two-tailed)	0.0036	0.3717	0.1138

	AB	AA	ALL
P value (two-tailed)	0.0023	0.4736	0.0628

Figure.4.5: Correlations between CD4+ T cell death and NK cell degranulation with the frequency of NK cells based on KIR-HLA interactions and KIR profile: *We compared CD4+ T cell death and NK cell degranulation in different KIR-HLA interactions with the presence (green circle) or absence (red circle) of an activating KIR haplotype (AB (n=22) vs. AA (n=23)). In A) and B), clear correlations were seen with NK cell degranulation (p=0.0036, R=0.594, green line) and CD4+ T cell death (p=0.0023, R=0.616, green line) and in the presence of an activating KIR profile. Correlations were analyzed non-parametrically (Spearman's correlation). Additionally, the correlation irrespective of the haplotype is shown as a grey dotted line.*

5. Discussion

In this study, we investigated the different KIR-HLA interactions between NK cells and allogeneic CD4+ T-cells for their impact on NK cell cytotoxicity. Inhibitory KIR receptors and their HLA ligands are relevant inhibiting regulators of NK cell activation. In the absence of their HLA ligand, donor-derived NK cells were capable of eliminating the transformed cells in leukemic patients following Haplo-HSCT. NK cells are also reactive to virally-infected cells, which are capable of transmitting various viruses during sexual intercourse. In the context of HIV-1 transmission, NK cells have been related to resistance as KIR-HLA incompatibility between sexual partners was previously identified as a factor favoring protection against HIV-1 acquisition[11]. Both features suggest a theory wherein a missing-self NK cell response can interrupt the foreign cell-associated viral infection. To further investigate these theories, we first need to understand the various KIR-HLA interactions with

foreign cells, potentially leading to alloreactive NK cell responses. However, such comprehensive knowledge is lacking. Here, we provide a detailed analysis of the intricate balance between KIR+ NK cell subpopulations when exposed to allogeneic CD4+ T-cells derived from HLA-incompatible HIV-1 patients. NK cell degranulation and CD4+ T-cell death increased in co-cultures subjected to KIR-HLA incompatibility. Single-iKIR+ NK cells constituted a significant part of the degranulating NK cell population and were most reactive in a corresponding mismatched condition. Furthermore, of these Single-iKIR+ NK populations, KIR2DL1+ NK cells were intrinsically the most robust responders in the absence of their ligand. A correlation between the size of the mismatched NK cell population and NK cell cytotoxicity was only seen in NK cell donors with an AB KIR haplotype. Although an enhanced cytotoxic effect was absent, KIR+ NK cells derived from AB donors were suggested to be activated more consistently by the absence of an HLA ligand.

Following the missing-self theory, we only selected the licensed (and thus functional) KIR+ populations in every NK cell donor for analysis[393,394]. We demonstrate a profound degranulating NK cell response in the missing-self context, resulting in increased proportions of dead CD4+ T-cells. To measure the effect of a KIR-HLA mismatch in the purest form, we examined the degranulation of NK cells expressing only one iKIR, as they are unaffected by the presence of unrelated KIR ligands. More Single-iKIR+ NK cells degranulated in a missing-self context as compared to its HLA matched condition. Stronger NK cell responses were seen in the absence of the HLA-C ligand (KIR2DL1-3) compared to the HLA-Bw4 ligand (KIR3DL1). The significantly increased proportion of degranulating KIR2DL1+ NK cells within the total response appoint these cells as the strongest intrinsic responders in the missing-self context. Although KIR2DL3 and KIR2DL1 showed a similar impact on the licensing of NK cells[503], the overall stronger ligand affinity of KIR2DL1 might explain the difference with KIR2DL2/3+ NK cells[331,333]. Here, we show that KIR3DL1+ NK cells are less reactive to the HLA incompatible conditions compared to KIR2DL+ NK cell responses. Whether these differences are generated by differences between KIR3DL-and KIR2DL-receptors or HLA-B- and HLA-C-ligands is unknown as both loci are highly polymorphic. Interestingly, Jennes et al[11] associated the genotyped KIR2DL1-HLA-C2 mismatch with protection against HIV-1 acquisition,

highlighting the cytotoxic potential against HIV-1 transmitting cells of these KIR2DL1+ NK cells in the missing-self context. These cells, if licensed, are also strongly polyfunctional and attracted to the bloodstream during acute primary HIV-1 infection[454]. KIR2DL1+ NK cells could, therefore, play a pivotal role in early mucosal immune responses against cell-associated viruses, especially in the context of HLA incompatibility between partners.

Notably, the high allelic diversity of the KIR and HLA genes[219] results in highly variable expression and affinity profiles as well as variety in affinity for each other. As the strength of the KIR-HLA bond during NK cell licensing is determined by the affinity and expression levels of KIR and HLA molecules (rheostat model), the magnitude of the missing-self NK cell response could highly vary due to these highly polymorphic KIR and HLA alleles[331,333,504]. Unfortunately, the extensive repertoire of KIR and HLA alleles and our low sample size of specific iKIR-HLA mismatches made it statistically irrelevant to look into allelic diversity in our experiments. However, deep-sequencing of KIR and HLA alleles in allogeneic KIR+ NK cell responses against HLA-mismatched, or even matched, CD4+ T cells could explain the wide variety in allogeneic NK cell responses in "KIR-HLA similar" conditions.

To further dissect these NK cell responses, we used the genetic KIR data to divide the NK cell donors into inhibitory (AA) and activating (AB) NK cell donors. Correlations between the size of the mismatched NK cell population and the frequency of dead CD4+ T-cells and degranulating NK cells were only seen in the presence of an activating KIR haplotype. These data indicate that KIR+ NK cells in the absence of their ligand still need an activating signal to capitalize on the missing-self situation fully. Unexpectedly, not only the mismatched Single-iKIR+ NK cells but also the (matched) KIR2DL2/3+ NK cell contribution to the total NK cell response was increased in AB NK cell donors. Potentially the weak affinity of the KIR2DL2/3 for HLA-C1[390] is more easily overruled by the activating signals obtained by the aKIRs. Moreover, all but one of these AB NK cell donors contained the KIR2DS2 gene, and as the KIR2DL2/3-antibody also stains KIR2DS2[505], the increase in degranulating KIR2DL2/3 in the KIR3DL1- and KIR2DL1 missing-self context might be associated with cross-reactive staining of KIR2DS2[506]. Nonetheless, to fully understand these cell encounters,

specific staining of the aKIR+ NK cells is necessary. Additionally, Parham et al[507] demonstrated differences in binding strength between HLA-C and KIR2DL receptor licensing, depending on the KIR (A or B) haplotype, which would majorly impact NK cell cytotoxicity in the missing-self context. However, due to the few KIR-HLA mismatches in the specific AA or AB single KIR+ NK cell populations, the impact of the difference in binding strength is not visible, except for the KIR2DL1 mismatch response in AA individuals (strong KIR2DL1-HLA-C2 bond; p=0.0313).

In our data, we calculated that the KIR+ NK cells account for ~40% of the total degranulating NK cell population, and ~80% of these cells express only one single iKIR, making them highly relevant in KIR-HLA regulated NK cell responses. The impact of a KIR-HLA mismatch is visible with a 4.4-, 3.2- and 1.4-fold increase of Single-KIR3DL1+, -2DL1+, and -2DL2/3+ NK cells, respectively, in the total degranulating NK cell response. In AB NK cell donors, however, only the KIR2DL1+ NK cell population increased in a mismatch context. Again, this highlights the strong intrinsic response of this subpopulation. The contribution of the remaining KIR- NK cells, defined as the Non-KIR population, in matched AB NK cell donors is one third lower compared to the matched AA NK cell donors (AA= 57.12%, AB=41.68%). Therefore, the contribution of these mismatches is likely overshadowed by the impact of the activating haplotype on the other matched KIR+ NK cell populations. However, after segregating the donors into AA and AB Single-iKIR+ NK cell populations, too few numbers are present to make clear statements or even interpretations.

A few limitations of this study should be considered. The use of CD4+ T-cells derived from HIV-1 patients creates a more relevant cell population concerning HIV-1 transmission but is accompanied by a low amount of cells retrievable from HIV-1 patients, inevitably leading to the selection of chronic HIV-1 patients who had been receiving ART for a more extended period. ART decreases the activity of HIV-1 in the blood, lowering the number of infected cells and the expression of activating signals. The selected HIV-1 patients did have decent CD4+ T-cell counts, but also low viral loads. As an extended proof-of-concept, however, we primarily investigated the influence of a KIR-HLA mismatch rather than the influence of HIV-1. Therefore, we used KIR-HLA matched co-cultures as a control group instead of co-cultures with healthy controls. Additionally, the lack of commercially available

flow cytometric labels for the activating KIRs limited analysis of the specific impact of aKIRs on NK cells. However, genetic haplotype segregation allowed for a primary distinction between KIR-profiles. Nonetheless, this still resulted in a limited sample size of certain Single-iKIR mismatches, thus limiting our interpretations. We focused on NK cell degranulation, the single cytolytic parameter of KIR+ CD56dim NK cells, to measure the direct cell-associated cytolytic impact of the KIR-HLA mismatch towards CD4+ T cells. Including the measurement of cyto- and chemokines would paint the complete picture on the impact of the KIR-HLA mismatch on NK cell functionality. However, these NK cell functions would rather indirectly impact the cytotoxicity against CD4+ T cells and were therefore not studied. Licensing of NK cells is not only possible through KIR receptors but also the inhibitory NKG2A receptor, in combination with its HLA-E ligand[508]. To what extend KIR or NKG2A control NK cell licensing was recently shown to be directed by an HLA-B dimorphism[461]. Nevertheless, in our study, focusing on KIR-HLA mismatches, we limited to iKIR-HLA licensing. New data, however, describe beneficial HSCT outcome with HLA-E incompatible donors, pointing at NKG2A as potential effector receptor[509].

In conclusion, we describe the allogeneic KIR-HLA interactions between NK cells and foreign CD4+ T-cells, as catalyzed by a missing-self context. We measured a distinct increase in NK cell responsivity in the absence of an HLA ligand. The impact of the KIR-HLA mismatch differed between and was dependent on the type of KIR, with KIR2DL1+ NK cells as the intrinsically most robust responders in the absence of their HLA ligand. The size of the NK cell population only expressing one single iKIR was highly relevant and capable of directing the total NK cell response, depending on the HLA genotype of the CD4+ T-cell donor. However, the size of this population only correlated with CD4+ T-cell death and NK cell degranulation after the selection of NK cell donors with an activating KIR haplotype and in the absence of its HLA ligand. These observations highlight the potential of "alloreactive" NK cell responses targeted at HLA-incompatible CD4+ T-cells. As shown genetically by Jennes et al. in HIV-1-discordant couples[11], we deliver *in vitro* evidence for the cytotoxic impact of HLA incompatibility in encounters between iKIR+ NK cells and foreign CD4+ T-cell

Chapter 5 - *In vitro* observations on HIV specific NK cell responses in various KIR-HLA conditions

Research article was submitted in *Frontiers in Immunology* on September 12, 2019

Under *independent review* on October 2, 2019

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The contribution of missing-self encounters in NK cell cytotoxicity against allogeneic HIV-1 infected CD4+ T cells

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Running title: HIV infection circumvents KIR-HLA incompatibility

Keywords: allogeneic, NK, CD4 T cell, HIV, missing-self, *in vitro*

1. Abstract

Introduction: NK cells recognize and eliminate virus-infected cells. Healthy autologous cells are tolerated by virtue of their HLA class I molecules, binding NK cell's inhibitory KIR receptors. The absence of this KIR-HLA bond allows NK cell activation (e.g., in allogenic encounters). In this study, we investigated whether these missing-self conditions triggered NK cell cytotoxicity against the different infectious subsets of allogeneic HIV-1 exposed CD4+ T cells.

Materials and Methods: Based on their genotype, we co-cultured cells from eight healthy NK cell donors with a "fully licensed" genotype (KIR3DL1+,-2DL1+,-2DL3+ and HLA-Bw4+,-C1+,-C2+) with six healthy CD4+ T cell donors (2 HLA-Bw4-,-C1+,-C2+; 2 HLA-Bw4+,-C1-,-C2+; and 2 HLA-Bw4+,-C1+,-C2-). These six sets of CD4+ T cells were either infected with HIV-1 or left uninfected as controls. Next, NK-CD4 T cell co-cultures were stained with fluorescent antibodies specific for activating CD4+ T cell ligands in combination with markers of cell death to be analyzed by flow cytometry.

Results: Missing-self related NK cell cytotoxicity was enhanced against HIV-1 exposed compared to HIV-1 unexposed CD4+ T cells. Remarkably, the enhancement of NK cell cytotoxicity by HIV-1 exposure was associated with the CD4+ T cell subset not expressing HIV-1 p24, whereas cell death was decreased in productively infected (p24+) T cells that either preserved CD4 (hence CD4+) or had downregulated CD4 (hence CD4-). NK cell responses against p24- CD4+ T cells were strongly affected by a missing-self condition, independent of MIC-A/B and Fas receptor (FasR) expression. In contrast, missing-self NK cell responses against p24+ CD4+T cells seemed present when the infected cell expressed MIC-A/B.

Conclusion: Although HIV-1 exposure was related to increased missing-self triggered NK cell cytotoxicity, the effect was primary restricted to CD4+T cells with no or low virus production (p24-). Productive infection with HIV-1 (p24+) inhibited NK cell cytotoxicity, whereby an activating stimulus such as MIC-A/B is needed to unlock the anti-HIV-1 NK cell responses.

2. Introduction:

NK cells are innate immune cells known for their anti-viral and anti-tumoral activity. The activation of NK cells is regulated by the balance of the various receptor-ligand interactions with the interrogated cell[173]. Healthy cells avoid NK cell cytolysis by expressing HLA class I molecules binding the inhibitory killer Ig-like receptors (KIRs)[174]. On virus-infected cells, downmodulation of self HLA avoids recognition by the T cell receptor (TCR) but also results in the absence of a KIR-HLA bond upon encountering NK cells[207,224]. In this "missing-self" situation, the binding of activating NK cell receptors with stress-upregulated or viral ligands can tip the balance towards a cytolytic response[175,177-180,224]. To what extent the absence of the self-HLA can activate NK cells is calibrated during NK cell maturation[212,313]. In this regard, only NK cells recognizing the self-HLA are "licensed" to exert their functionalities[393-395]. Three KIR-HLA interactions are known to influence NK cell functionality strongly; KIR3DL1 binding HLA-B molecules with a Bw4 motif[389], whereas KIR2DL1 and KIR2DL2-3 bind HLA-C with a -C2 and -C1 motif, respectively[211]. HIV-1 also downmodulates the HLA-B and -C ligands on infected cells[315,427,428], which is associated with anti-viral activity by the corresponding KIR+ NK cells[313,510]. In this autologous context, these studies highlight the potential of missing-self responses in battling HIV-1 infection, but the strength of the missing-self response is restrained in proportion to the residual HLA ligands[510]. In the encounter with an allogeneic target however, a complete missing-self

context can derive from inter-individual differences in the HLA genome. This could be the case at the site of transmission, when an HIV-infected "donor" cell is transmitted into a non-infected "recipient" individual.

The first evidence for a strong missing-self driven NK cell response in allogeneic conditions was seen against leukemic cells. Treatment of leukemia with Haplo-HSCT had a beneficial clinical outcome as the stem cell donor was HLA haplo-compatible (only one allele compatible) with the leukemic recipient. An in-depth investigation revealed allogeneic NK cells to be responsible for the increased clearing of malignant cells, mediated by the HLA incompatibility[478,511]. Likewise, at the site of sexual transmission, an allogeneic encounter between penile/cervical NK cells and vaginal secretion/semen-derived HIV-1 infected cells[70] can protect against transmission. Jennes et al.[11] already reported an elevated frequency of genetic KIR-HLA mismatches between HIV-1 discordant sexual partners, whereas HIV-1 concordance was related to a KIR-HLA match. Therefore, in line with the observations in leukemia, it was suggested that allogeneic missing-self NK cell responses could hamper or even obstruct viral transmission by eliminating the incoming infected cells[4]. We subsequently illustrated that an *in vitro* allogeneic KIR-HLA mismatch significantly increased the frequency and intensity of degranulating NK cells of the corresponding (mismatched) KIR+NK cell population, which increased CD4+ T cell death[512].

Although productively infected T cells inhibit autologous NK cell responses, the absence of an HLA ligand could reverse the tolerance of the NK cell towards the infected CD4+ T cell. Although the absence of an HLA ligand favors NK cell activation, the presence of an activating signal is still necessary to tip over the balance into full NK cell activation. To further unravel the modulators of these HIV related missing-self responses, we also focus on the activating compartment of the NK cell signaling. Recent research has shown that infection of CD4+ T cells with HIV results in elevated expression of ligands for activating

NK cell receptors NKG2D[240]. Also, apoptosis is achieved when the Env-induced FasR[513] ligates with the NK cell's FasL[514]. Here, we investigated to what extent a KIR-HLA mismatch could enhance NK cell cytotoxicity against the different HIV-1 exposed CD4+ T cell subsets.

3. Material and Methods

3.1. Sample preparation and genotyping

PBMCs were isolated from buffy coats obtained from healthy donors at the Blood Transfusion Centre (Rode Kruis-Vlaanderen, Mechelen, Belgium). PBMCs were isolated using a density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare Life Sciences) and were cryopreserved at $50x10^6$ cells/tube in a 90% FBS, 10% DMSO cryopreservation medium. The Institutional Review Board of the Institute of Tropical Medicine granted ethical approval for the use of anonymous buffy coats (Policy number 99.002.067).

Genomic DNA was extracted from PBMCs using the DNeasy® Blood & Tissue Kit (QIAGEN®). KIR genotyping (KIR Typing Kit, Miltenyi Biotec) determined the presence of 5 inhibitory KIR genes (KIR2DL1,-2DL2,-2DL3,-2DL5(A/B) and -3DL1) and 6 activating KIR (aKIR) genes (KIR2DS1,-2DS2, -2DS3,-2DS4 (del/ins),-2DS5 and -3DS1). The KIR HLA Ligand kit (Olerup SSP®) detected the presence of the KIR3DL1 ligand, HLA-Bw4, and the C2 and C1 motif on the HLA-C alleles, ligands of KIR2DL1 and KIR2DL2/3 respectively.

3.2. Experimental design

HLA class I genotyping enabled the selection of "fully licensed"[320,394] NK cell donors (HLA-Bw4+,-C1+ and -C2+) and CD4+ T cell donors (missing 1 of the 3 HLA ligands). KIR genotyping was performed to select suitable NK cell donors containing the corresponding

three receptors (KIR3DL1, -2DL1,-2DL2 or 3). In total, we selected eight NK cell donors with fully licensed KIR+ NK cells (1-8), and six CD4+ T cell donors (A-F), each lacking one of the three HLA ligands (co-cultures with A and B (HLA-Bw4-), C and D (HLA-C2-) and E and F (HLA- C1-)). Before co-culture, half of the CD4+ T cells from each donor were *in vitro* incubated with an HIV-1 HIV-1_{Ba-L} (R5-trophic lab strain), whereas the other half remained unexposed. Finally, NK cells and CD4+ T cells were co-cultured and fluorescently stained to measure CD4+ T cell death in total and subpopulations, according to the expression of membrane CD4 and intracellular HIV p24. To calculate the CD4+ T cell death mediated by their encounter, the nonspecific cell death was taken into account by culturing CD4+ T cells alone. In the NK-CD4+ T cell co-cultures, a combination of the NK cell donors with any CD4+ T cell donor resulted in a missing-self response; co-cultures with A and B resulted in a KIR3DL1 mismatch, co-cultures with C and D resulted in a KIR2DL1 mismatch and co-cultures with E and F resulted in a KIR2DL2/3 mismatch. An additional control co-culture was included, composed of NK and CD4+ T cells from the same NK cell donor, to function as an autologous control (Suppl.Fig.5.1).

3.3. *In vitro* CD4 T cell isolation, cultivation, and infection

Cryopreserved PBMCs were thawed at 37°C and used to isolate CD4+ T cells using a positive magnetic separation column (MiniMACS™ Separator, Miltenyi Biotec). The obtained CD4+ T cells were stimulated with 1 µg/ml PHA (Remel) and 100 U/ml IL-2 (Gentaur) in R10 medium (RPMI from LONZA, containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FBS) for three days at 37°C, 5% CO_2. After activation, $3x10^6$ CD4+ T cells were infected by spinoculation at 1200 x g with the HIV-1_{Ba-L} lab strain at a multiplicity of infection (MOI) of 0.01. After 3 hours, the cell pellet was resuspended in 1 ml 100 U/ml IL-2 R10 and cultured at 37°C, 5% CO_2 for seven days, with medium refreshment every two

days. As a control, $3x10^6$ CD4+ T cells underwent the same protocol, adding R10 instead of the HIV-1_{Ba-L} lab strain.

3.4. *In vitro* NK cell isolation and activation

Cryopreserved PBMCs were thawed at 37°C, and NK cells were isolate using a negative magnetic separation (MidiMACS™ Separator, Miltenyi Biotec). NK cells were subsequently incubated at a concentration of $2x10^6$ cells/ml in R10 supplemented with 200U/ml IL-2 (Gentaur) for three days at 37°C, 5% CO_2.

3.5. *In vitro* CD4 T cell mono-cultures and NK and CD4 T cell co-cultures

After respective pre-incubation, (un)infected CD4+ T and NK cells were washed and resuspended in 100 U/ml IL-2 R10 at a concentration of $2x10^6$ cells/ml. In a 96-well polystyrene plate (U-Bottom, FALCON), 200,000 NK cells and 200,000 (un)exposed CD4+ T cells were co-cultured for 4 hours at 37°C, 5% CO_2. In the co-cultures, we measured target cell death by staining with 7-AAD. Therefore, every unique NK-CD4+ T cell combination was represented in two wells, as we stained with two different antibody cocktails related to target cell death. As a negative control, 200.000 CD4+ T cells were cultured in medium only, representing the CD4+ T mono-cultures.

3.6. Flow cytometric analysis

Each unique NK and CD4 T cell mono- and co-culture was stained by two different antibody cocktails measuring target cell death and stained either inhibitory NK cell ligands (staining cocktail 1), or activating NK cell ligands related to stressed- or virally induced molecules (staining cocktail 2). Subsequently, the various markers in these co-cultures were measured by the flow cytometer FACSVerse® (BD) and analyzed by the FACSuite® (BD) software.

After the initial staining of the membrane markers and cell death, the co-cultures incubated with a lysing and permeabilization solution (IntraPrep Leucocytic Permeabilization Reagent®, Beckman Coulter) before the intracellular staining of HIV-1p24.

For both target cell death assays (Suppl.Fig.5.2), NK cells were excluded by gating on CD56-/CD3+ T cells. In the first approach, we measured cell death in the total CD3+ T cell population. Next, we used the HIV-1_{p24} and CD4 staining to differentiate between HIV-1_{p24-} CD4+ T cells (unCD4+) in HIV-unexposed co-cultures. In the HIV-1 exposed co-cultures, we differentiated the following subsets: HIV-1_{p24-} CD4+ T cells (CD4+ p24-), HIV-1_{p24+} CD4+ T cells (CD4+ p24+) and HIV-1_{p24+} CD4- T cells (CD4- p24+). In these subsets, cell death was measured as the average of both cell death assays for increased accuracy. Within the different HIV-1_{p24} populations, expression of HLA-Bw4, HLA-E, FasR (staining cocktail 1) or MIC-A/B and ULBP (staining cocktail 2) was measured. Cell death was measured in the various NK cell ligand expressing T cell populations. Antibodies used in staining cocktail 1 are CD56-APC Vio770 (Miltenyi), CD3-Viogreen (Miltenyi), CD4-Vioblue (Miltenyi), 7-AAD (BD), Kc57-FITC (HIV-1p24, Beckman Coulter), FasR-APC (BD), HLA-Bw4-PE Vio770 (Miltenyi) and HLA-E-PE (Biolegend). Staining cocktail 2 contained CD56-APC Vio770 (Miltenyi), CD3-Viogreen (Miltenyi), CD4-Vioblue (Miltenyi), 7-AAD (BD), Kc57-FITC (HIV-1p24, Beckman Coulter), MIC-A/B-PE (Miltenyi) and ULBP-3-APC (R&D). The antibodies used are also enlisted in Supplementary Table 5.1.

3.7. Statistical analyses

All statistical analyses were performed in GraphPad Prism (v8.1.0) with the significance level set at $p < 0.05$. Differences between co-cultures were analyzed using either a Friedman test with Dunnett's multiple comparison test when investigating the KIR-HLA mismatch effect,

or a Wilcoxon matched-pairs signed-rank test to investigate the HIV-1 effect. A Two way ANOVA was performed to compare the different NK or CD4 T cell subsets.

4. Results

4.1. NK cell-induced CD4+ T cell death

The NK cell-induced CD4+ T cell death was calculated by subtracting the cell death in the CD4+ T cell mono-cultures from that in the NK and CD4+ T cell co-cultures. The obtained "net" CD4+ T cell death was compared between the autologous and "mismatched" (A-F) co-cultures, as well as between the HIV-1 unexposed and the different subsets of HIV-1 exposed CD4+ T cell populations. An average of the CD4+ T cell donors lacking the same HLA ligand was calculated to increase the accuracy of a missing-self response (A+B/2=HLA-Bw4-, C+D/2=HLA-C2- and E+F/2=HLA-C1-).

4.1.1. Cell death in the total CD3+ T cell population

First, we measured the cell death of CD3+ T cells in the HIV-1 unexposed and HIV-1 exposed co-cultures. In both the HIV-1 unexposed and exposed co-cultures (Fig.5.1A and B), a significant difference was measured in cell death between KIR-HLA matched (grey) and combined KIR-HLA mismatched co-cultures (blue, green and orange), using the Friedman test. Only in the HIV-1 exposed co-cultures, this difference was associated with the absence of two separate specific HLA ligands (HLA-Bw4- and HLA-C1-) (Fig.5.1B). The ten-fold differences in the level of significance between both conditions point to HIV-1 as an enhancing factor for the missing-self response. This enhancing effect is further analyzed in panel C, where the contribution of HIV-1 exposure to NK cell cytotoxicity in each KIR-HLA condition was measured. In the presence of all HLA ligands, HIV-1 did not affect cell death (Fig.5.1C.1); but if either HLA-Bw4 or HLA-C2 were absent, HIV-1 exposure significantly

enhanced cell death (Fig.5. 1C.2 and C.3). In the absence of HLA-C1, there was also a small but non-significant HIV-1 effect (Fig.5.1C.4).

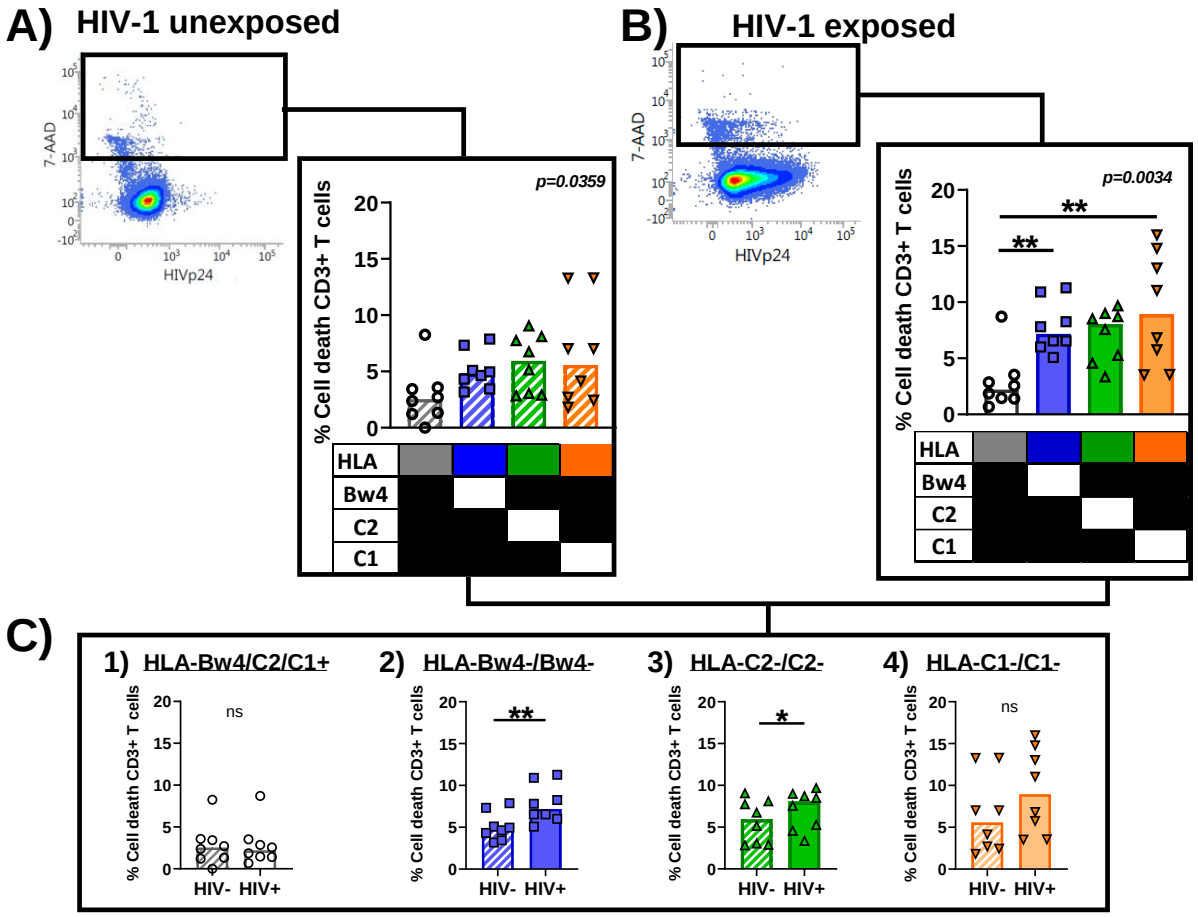


Figure.5.1. The frequency of CD3+ T cell death in the unexposed and HIV-1 exposed NK-CD4 T cell co-cultures: *The overall cell death was measured as the frequency of 7-AAD+ in the CD3+ T cell population. In panel A, CD3+ T cell death in HIV-1 unexposed conditions (hatched bars), and in panel B, in HIV-1 exposed conditions (full bars) were compared between the different KIR-HLA combinations: KIR-HLA matched (HLA-Bw4+/C2+/C1+) (grey hatched in A and full bars in B), absence of HLA-Bw4 (blue bars), absence of HLA-C2 (green bars) and absence of HLA-C1 (orange bars). In the top right corner of the graphs in panels A and B, the p-value of the Friedman test between KIR-HLA matched (grey) and mismatched co-cultures (blue, green, and orange) is displayed. Below the graph, the roster represents the HLA genotype of the CD4+ T cell donors, where the presence of an HLA ligand is blackened. In panel C, cell death of CD3+ T cells with the same of HLA genotype was compared between HIV-1 unexposed (hatched bars) and HIV-1 exposed (full bars) conditions: 1) KIR-HLA matched (grey hatched and full bars), 2) absence of HLA-Bw4 (blue bars), 3) absence of HLA-C2 (green bars) and 4) absence of HLA-C1 (orange bars). All bars represent the median value of 8 data points, which are individually shown. (p-values: * < 0.05; ** < 0.01; *** < 0.001).*

4.1.2. Cell death in the various CD4+ T cell subsets from HIV-1 exposed cultures

In the HIV-1 exposed co-cultures, the CD3+ T cells were divided according to their state of infection. As a first, expression of the intracellular HIV-1 p24 protein was measured as proof of the productive infection of the cell. In addition, p24+ T cells were gated based on the presence or absence of CD4 since the downregulation of CD4 is a phenomenon that indicates a late stage of HIV-1 infection and the budding of virions. In total, the CD4+ T cell population was divided into CD4+ p24-, CD4+ p24+, and CD4- p24+ T cells, with a mean frequency of 66.3, 26.9, and 4.4 of the total T cell population, respectively (Fig.5.2A).

As a control, we measured cell death of the CD4+ p24- T cells in the HIV-1 unexposed co-cultures. When interpreting the graphs, it is essential to realize that the CD4+ p24- T cell population still contains infected T cells, as previously repeatedly demonstrated by GFP reporter viruses, quantitative PCR, and ultrasensitive p24 assays[515-517]. Cell death in the different subsets enabled the comparison of NK cell responses against clearly productively infected T cells (p24+), either still expressing CD4 or with downregulated CD4, and less or non-productively infected T cells (CD4+ p24-).

The CD4+ p24- subset contained the highest frequency of T cell death in the HIV-1 exposed population (Fig.5.2B). Cell death of CD4+ p24- T cells was enhanced compared to the unexposed CD4+ T cell subset (Fig.5.2B), revealing an NK cell activating effect of low-grade HIV-1 exposure. In contrast, NK cell responses against productively infected T cells (p24+), CD4+, and to a lesser extent, CD4-, were inhibited when compared to HIV-1 unexposed CD4+ T cells. Within the p24+ T cell population, an increase in cell death was observed after CD4 downmodulation (Fig.5.2B). These data point to the CD4+ p24- T cell subset as the most vulnerable to NK cell-mediated cytotoxicity within the HIV-1 exposed co-cultures.

In addition, NK cell encounters with CD4+ p24- T cells resulted in strong missing-self responses in the absence of each HLA ligand (Fig.5.2C.1). In contrast, NK cell cytotoxicity against CD4+ p24+ T cells was minimal, and the KIR-HLA mismatch effect was statistically insignificant (p=0.07)

(Fig.5.2C.2). Likewise, no KIR-HLA mismatch effect was observed by NK cells in the encounter with CD4- p24+ T cells (p=0.68) (Fig.5.2C.3).

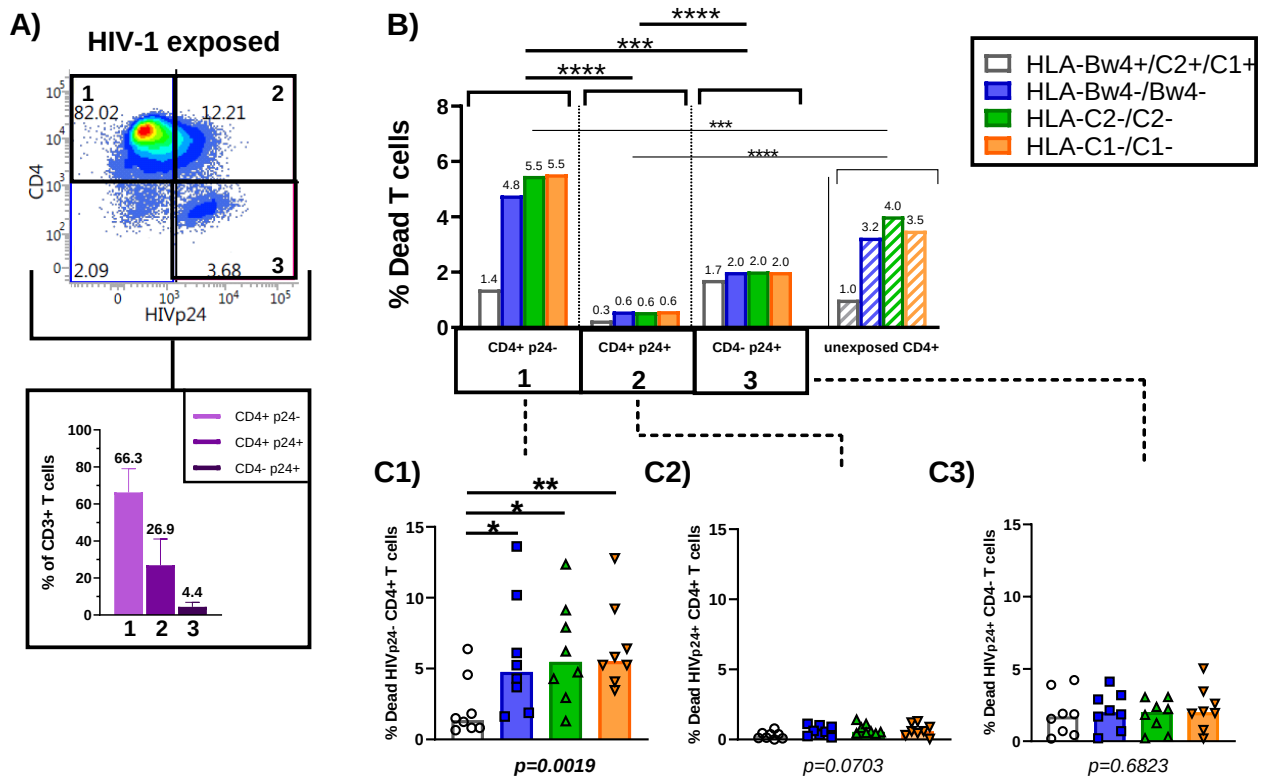


Figure.5.2. The frequency of cell death in CD4+ p24-, CD4+ p24+ and CD4- p24+ subsets of HIV-1 exposed co-cultures: *Within the CD3+ T cell population of the exposed co-cultures; the CD4+ p24- (1), CD4+ p24+ (2) and CD4- p24+(3) subsets were gated as shown by the upper gating graph in panel A. In the lower graph of panel A, the frequencies of CD4+ p24- (1), CD4+ p24+ (2) and CD4- p24+(3) subsets within the CD3+ T cell population are shown with median values on top of the bars. In graph B, the frequency of NK cell-mediated cell death within each of these HIV-1 exposed subsets is shown and compared between these subsets as well as the unexposed CD4+T cell co-cultures (hatched). Median values of cell death are shown on top of the bars. In panel C, the same data as in B is shown, but here cell death within each subset was compared between the NK-CD4 T cell co-cultures with different KIR-HLA combinations: KIR-HLA matched (HLA-Bw4+/C2+/C1+) (grey bar), absence of HLA-Bw4 (blue bar), absence of HLA-C2 (green bar) and absence of HLA-C1 (orange bar). The p-values of these Friedman tests are shown below the graph of each subset, CD4+ p24- (1), CD4+ p24+ (2), and CD4- p24+ (3). All bars represent the median value of 8 data points. (p-values: * < 0.05; ** < 0.01; *** < 0.001).*

4.1.3. Cell death in FasR and MIC-A/B ligand expressing CD4+ T cells

Previously, *in vitro* infection with a CCR5-trophic HIV-1 strain was shown to upregulate the NK cell-activating ligands MIC-A/B and ULBP-3, as well as FasR [240]. Therefore, these three NK cell stimuli were investigated for their potential contribution to NK cell responses against HIV-1 exposed CD4+ T cells (Suppl.Fig.5.3). The analysis strategy is explained in Suppl Fig 5.2A. The frequency of ULBP-3+ CD4+ T cells (mean of 0.08% in each T cell subset) was too low to produce statistically significant results. Within each of the target T cell subsets, defined by expression of CD4 and p24, cell death is shown in the total (read *average*), MIC-A/B, and FasR expressing cell populations (Fig.5.3A). Whereas cell death in the major CD4+ p24- subset was similar in the total and MIC-A/B (+) subpopulation (Fig. 5.3A1), cell death in the p24+ subsets was low in the total population but concentrated in the MIC-A/B(+) subpopulation (Fig. 5.3A2-3). Conversely, there was no enrichment of cell death in Fas-R expressing cells of any of the CD4/p24 subsets. Out of the three investigated NK cell stimuli, MIC-A/B was the strongest mediator of NK cell-mediated cell death against p24+ T cells, whereas cell death of CD4+ p24- T cells was seemingly independent of MIC-A/B expression.

Cell death of MIC-A/B expressing T cells were compared between different CD4+ T cell subsets to verify these differences (Fig.5.3B). The frequency of dead MIC-A/B+ CD4+ p24-T cells was significantly lower compared to the CD4+ and CD4- p24+ T cell subsets. These observations suggest a crucial role for MIC-A/B in anti-p24+ NK cell cytotoxicity, whereas its activating role in NK cell cytotoxicity against CD4+ p24- T cells is uncertain.

Similar to the average cell death, cell death of the MIC-A/B+ CD4+ p24- population was subjected to a KIR-HLA mismatch (Fig.5.3B). Also cell death of MIC-A/B+ CD4+ and CD4- p24+ T cells was increased in the absence of an HLA ligand, which was not evident in the total cell death of these subsets (Fig.5.3A2-A3). However, while MIC-A/B expression was not a prerequisite to activate NK cells against CD4+ p24- T cells, MIC-A/B expression on p24+ T cells was seemingly necessary to generate anti-HIV-1 responses. However, the KIR-HLA mismatch effect in the p24+ subsets was

statistically insignificant (Fig.5.3B), probably due to a wide range of MIC-A/B+ cell death in the p24+ T cell subsets (0 to 30-40%, data points not shown).

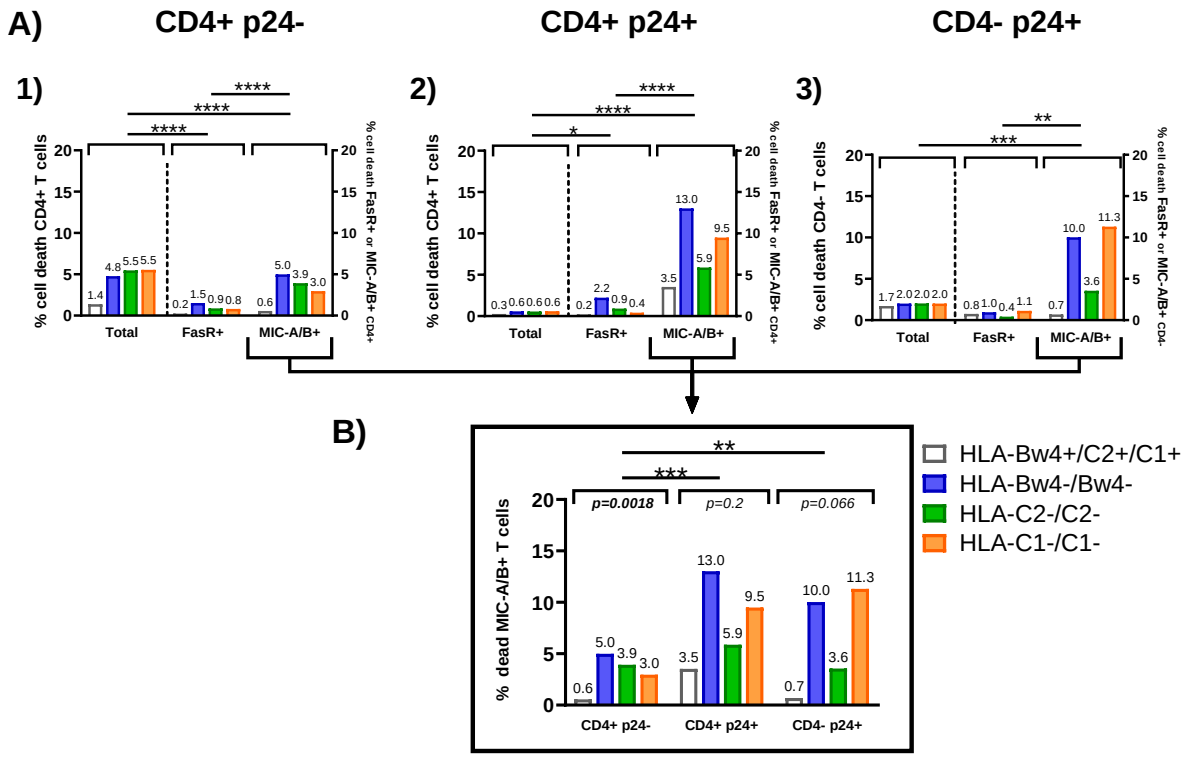


Figure.5.3. The frequency of cell death within the HIV-1 exposed T cell subsets expressing activating receptor FasR and ligand MIC-A/B: *The population of cells expressing FasR and MIC-A/B were gated in each HIV-1 exposed T cell subset and were subsequently evaluated for their frequency of cell death. In panel A, the different graphs show the cell death of each subset; 1) CD4+ p24-, 2) CD4+ p24+ and 3) CD4- p24+. Within each graph of panel A, the frequency of cell death in the total population (left from the dashed line) is depicted on the left y-axis, whereas cell death of FasR and MIC-A/B expressing populations (right from the dashed line) is depicted on the right y-axis. Median values of cell death are shown on top of the bars. In graph B, the frequency of cell death in the MIC-A/B+ fraction of each CD4/p24 subset was compared. Cell death of MIC-A/B+ T cells was compared within each subset between KIR-HLA matched (grey bar) and KIR-HLA mismatched (blue, green, and orange) co-cultures. The p-value of these Friedman tests is shown in graph B above the various subsets.). Median values of cell death are shown on top of the bars. All bars represent the median value of 8 data points. (p-values: * < 0.05; ** < 0.01; *** < 0.001).*

5. Discussion

Transmission of HIV-1 occurs predominantly through sexual intercourse, with both cell-associated and cell-free virus having a role in these events[5]. Consequently, eradication of the allogeneic cell-

associated virus[70] could weaken or even inhibit the transmission of HIV-1. Recently, NK cells were proposed to kill these cells harboring HIV-1, since KIR-HLA incompatibility between sexual partners was associated with protection from HIV-1 transmission[11]. As HLA class I ligands are inhibitors of NK cell functionality, the absence of KIR-HLA interactions induce a cytotoxic missing-self response. Accordingly, we confirmed the *in vitro* NK cell-activating potential of missing-self responses against allogeneic CD4+ T cells, initiated by KIR expressing NK cells[512]. Nevertheless, the absence of an inhibitory signal still needs to be accompanied by an activating signal to elicit these missing-self responses fully. *In vitro*, productively infected (p24+) CD4+ T cells express a variety of activating NK cell ligands[240]. To what extent these activating ligands or the stage of infection influences these missing-self responses is still unknown. Therefore, we investigated the NK cell cytotoxicity in various KIR-HLA combinations with HIV-1 exposed and infected CD4+ T cells.

In a first attempt to assess the impact of HIV-1 on missing-self responses, the overall cell death was compared between HIV-1 exposed and unexposed conditions of the CD4 T target cells. Although the effect of a KIR-HLA mismatch was independent of HIV-1, the presence of HIV-1 did seem to augment the missing-self response. The enhanced NK cell-cytotoxicity upon exposure to HIV-1 was seen in the absence of each HLA ligand, though statistically insignificant when lacking HLA-C1. In contrast, exposure to HIV-1 did not influence NK cell mediated cell death in autologous co-cultures. Therefore, the exposure of HIV-1 was only able to enhance NK cell cytotoxicity in the absence of an HLA ligand.

In vitro infection by HIV-1 generated various exposed CD4+ T cell subsets, according to the expression of intracellular p24 and membrane CD4. Of these subsets, CD4+ p24- T cells were more sensitive to NK cell-mediated cell death than unexposed CD4+ T cells. In contrast, NK cell cytotoxicity against CD4+ p24+ and, to a lesser extent, CD4- p24+ T cells was inhibited. A possible explanation for this -at the first view- paradoxical effect is that exposure to the HIV-1's gp41 protein induces the expression of the activating NKp44L on p24- bystander CD4+ T cells[518,519], whereas p24+ T cells are known to downregulate this ligand[432]. Unfortunately, fluorescent antibodies targeting the NKp44L are commercially unavailable, and this hypothesis could, therefore, not be verified. Another

striking difference was the presence of missing-self responses against CD4+ p24- T cells, whereas cell death of p24+ T cells was seemingly unaffected by a KIR-HLA mismatch. In general, HIV-1 exposure and potential low-grade infection, but not abundantly productive infection of HIV-1, may make CD4+ T cells more prone for (missing-self) NK cell responses.

NK cell responses against productively infected (p24+) T cells are known to be impaired by HIV's upregulation of inhibitory ligands such as HLA-E[426], and downregulation of activating ligands such as MIC-A[520]. These NK cell inhibiting features were observed on the CD4+ p24+ T cell subset (Suppl. Fig. 5.3) and could subsequently limit the amount of (missing-self) NK cell responses, as observed. Correspondingly, the upregulation of MIC-A/B and reduced expression of HLA-E on CD4- p24+ T cells (Suppl. Fig. 5.3) could explain the increase in NK cell cytotoxicity compared to CD4+ p24+ T cells. However, since HLA-E expression was comparable on CD4+ p24- and CD4- p24+ T cells, the discrepancy in NK cell cytotoxicity between both subsets suggests an additional inhibiting feature by p24+ T cells. Additional inhibiting features on the surface of HIV-1 infected T cells could be the downregulation of NKp30[521] and NKp44L[432] as well as the presence of the inhibitory HLA-C ligand[315]. According to the anti-tumor hypothesis, the absence of an HLA ligand is expected to sensitize the target cells for missing-self responses. Nevertheless, HIV-1 seems to be able to compensate for the lack of inhibiting HLA signals and minimize the amount of missing-self NK cell responses against p24+ T cells.

However, when p24+ T cells expressed MIC-A/B (mean of 3.25% with SEM 0.65 of CD4- subset, Suppl.Fig.5.3), NK cell cytotoxicity did increase in the absence of an HLA ligand. In contrast, the absence of an HLA ligand on CD4+ p24- T cells sufficed to induce NK cell cytotoxicity, irrespective of MIC-A/B stimuli. With this, we demonstrate that in addition to a missing-self condition, NK cells need an activating signal such as MIC-A/B (e.g., stress-induced) to overcome HIV-1's inhibition threshold. Notably, MIC-A/B related NK cell cytotoxicity seemed highly variable between NK cell donors and could, therefore, have interfered with statistical significance (Suppl. Fig.5.4). Potentially, the variability of the MIC-A/B stimulus could be related to a functional polymorphism of the NKG2D gene. In cancer treatment, the prevalence of high or low functional NKG2D polymorphisms impacts

the outcome[522]. In HIV-1 conditions, this NKG2D dimorphism should further be investigated for their role in anti-HIV-1 NK cell responses. As the receptor of MIC-A/B, NKG2D is expressed on virtually every cell and can, therefore, be an excellent companion co-expressed with KIR on the surface of the NK cell. The functional NKG2D haplotype could be another important determinant for the hypothesis besides the KIR-HLA mismatch. In general, although not reaching statistical significance, missing-self responses seem to be present against p24+ T cells if an activating ligand such as MIC-A/B, but not FasR, is expressed.

Semen-derived SIV+ CD4+ T cells can transmit the virus *in vitro* and seem to be productively infected at all stages of the infection[477]. Therefore, targeting the productively infected (p24+) subset in the seminal CD4+ T cell population would have implications on the transmission rate of HIV-1. Here, we show that the absence of an HLA ligand favors NK cell cytotoxicity against these productively infected CD4+ T cells in the presence of MIC-A/B. Unfortunately, the expression level of MIC-A/B on semen-derived cells is unknown. It has been shown that MIC-A/B expression on *in vitro* HIV-1 infected p24+ T cells can be triggered by cytokine stimulation or damage to the DNA[523], but can also be inhibited by HIV-1's Nef protein[431,524]. Expression of MIC-A/B on semen-derived cells is likely dependent on the state of disease and inflammation, as well as the Nef-effectivity[524-526]. Also the low-grade infected cells in the p24- CD4+ T population can contain a replication-competent virus and should also be certified as a potential transmitter of HIV-1[527]. Unfortunately, we were not able to investigate whether the NK cell cytotoxicity against the CD4+ p24- subset is directed to the uninfected or low-grade infected T cells.

HLA-Bw4 and HLA-C are known to be downregulated by HIV-1[427,428,510], although this was not detected in the same degree or measured in the experiments, respectively. Moreover, to what extent the HIV-1_{Ba-L} strain downmodulates HLA-B and HLA-C is still unknown. In this regard, these data depict as first the expression profiles of HLA-Bw4 by the HIV-1_{Ba-L} strain, suggesting no or limited HLA-B downregulation (Suppl. Fig.5.3). HIV-1 mediated downmodulation of HLA-B and HLA-C could induce NK cell cytotoxicity by generating a missing-self like encounter[510]. Therefore, future

experiments should investigate the extent of HLA-C downmodulation in HIV-1_{Ba-L} infected T cells to estimate their impact on NK cell cytotoxicity.

In this study, a few limitations should be considered. The high allelic variability of both KIR and HLA results in differences measured in similar missing-self responses. Due to this high allelic variability and the low amount of NK cell donors, we could not take allele specificity into account. Another limitation of our findings is the weak results perceived by measuring NK cell degranulation. This could be due to the use of cryopreserved instead of fresh PBMCs[512]. Although cryopreservation has been documented to decrease the cytotoxic capacities of NK cells[528,529], the *in vitro* cytotoxic capacity can be retrieved when rested overnight[528]. Therefore, NK cells were pre-incubated with IL-2 for three days. Nevertheless, weak cytolytic data were obtained and not included in the manuscript.

In conclusion, we confirmed the *in vitro* NK cell cytotoxic potential of a KIR-HLA mismatch against HIV-1 unexposed as well as exposed CD4+ T cell populations. Within the HIV-1 exposed population; however, the most frequent CD4+ p24- T subset was more sensitive than HIV-unexposed CD4 T cells, while the minor productively infected subsets were remarkably less sensitive. Although NK cell cytotoxicity against productively infected is largely inhibited, missing-self responses are observed in the presence of MIC-A/B, which could suggest a beneficial effect of missing-self responses during HIV-1 transmission. However, in general, NK cell cytotoxicity against p24+ T cells remains limited even in the presence of an allogeneic KIR-HLA mismatch.

Chapter 6 - General discussion

As an innate immune cell, NK cells are responsible for the cytolytic responses against virally-infected or malignant cells. Whether the NK cell becomes active during these encounters is determined by the balance of receptor-ligand interactions with the interrogated cell[173]. On healthy cells, the constitutively expressed HLA class I molecules function as major inhibitors by interacting with the KIR receptor on the NK cell[173]. The absence of this HLA ligand will make the target cell vulnerable for NK cell cytotoxicity, also known as the "missing-self" response. As KIR and HLA genotypes evolve independently of each other, the presence of an HLA ligand for each expressed KIR is not guaranteed in each individual[316,505]. Therefore, a fail-safe mechanism is introduced to avoid auto-immune responses generated by these missing-self conditions. This fail-safe mechanism implies the necessity of a successful KIR-HLA interaction before NK cells are "licensed" to become fully functional[394]. During viral infection, downregulation of HLA occurs as a viral defense mechanism to avoid the HLA class I recognition by CD8+ T cells, but at the same time, it favors NK cell activation[322]. In addition to missing self, NK cell activation requires an activating signal to elicit cytotoxicity[173,530]. This activation balance ensures the specific recognition of malignant or virally-infected cells, whereas healthy cells are tolerated (Chapter 2, 2.4).

Also, HIV-1 infection is known to downregulate the HLA class I molecules. Infection of CD4+ cells with HIV-1 lab strains revealed selective downregulation of HLA-A and B molecules, whereas HLA-C was preserved, thereby avoiding both CD8+ T and NK cell immunity[424]. In addition, HIV-1 infection is seen to be detrimental to the functionality of autologous NK cell responses[404,405,407]. However, recent research shows that HLA-C, as well as the non-classical HLA-E, are also downregulated by primary isolates of HIV-1 patients. Missing-self NK cell responses could still help to control the established HIV infection. Furthermore, both epidemiological and *in vitro* studies were able to depict specific characteristics favoring anti-viral NK cell responses.

Besides autologous NK cell responses, allogeneic NK cell responses are also suggested to be protective from vertical[474,531] and sexual HIV-1 transmission[11]. In resemblance to the mechanism of NK cell alloreactivity in Haplo-HSCT[478] (Chapter 2, 5.1), protection against HIV-1 acquisition was suggested to be induced by a missing-self condition between the host's NK cells and infected cells. As the major infection route, sexual transmission of HIV-1 takes place at the genital mucosa (vaginal/penile) and

approximate lymph nodes[5] where its success rate is strongly dependent on the effectiveness of the defensive layers. As a member of the innate immune system, NK cells located at the genital mucosa[131] could directly interact with the cell-associated virus during transmission (Chapter 1, 1.5). Within this vulnerable timeframe, a potent NK cell response could restrict HIV-1 progression or even prevent transmission.

1. The impact of KIR-HLA incompatibility on allogeneic NK cell cytotoxicity

Formerly, allogeneic missing-self NK cell responses were only investigated in the light of their anti-tumor capacity[478,479]. The impact of these responses in an anti-viral context, however, remained unknown, until Jennes et al. revealed an association between KIR-HLA incompatibility of sexual partners and protection against HIV-1 acquisition[11]. Here, it was suggested that NK cells acquired anti-viral capacities by the absence of the HLA ligand, preventing further HIV-1 transmission. Other research also suggested decisive KIR-HLA interactions at the site of HIV-1 acquisition. In line with Jennes et al. hypothesis[11], HLA compatibility between donor and recipient was seen as a risk factor for sexual and vertical transmission[492-495]. During transmission, the influx of HLA compatible cells was suggested to interact with genital NK cells by the dominant inhibiting KIR-HLA interaction, favoring HIV-1 acquisition. To further substantiate this hypothesis, we first examined the absence of an HLA ligand as leverage to induce *in vitro* allogeneic NK cell cytotoxicity against CD4+ T cells derived from HIV-1 patients (Chapter 4). In the second set of experiments, NK cells were incubated with *in vitro* HIV-1 infected CD4+ T cells to investigate the anti-viral effect of these missing-self responses (Chapter 5).

In the first place, we are the first to provide *in vitro* proof of missing-self NK cell responses against allogeneic CD4 T cells in the context of HIV-1. In our experiments, the absence of an HLA ligand was related to an increase in the degranulation of the corresponding KIR+ NK cell population (Chapter 4). Although expected, the latter result provides clear evidence for the missing-self response as the driving force behind the observed increase in overall NK cell cytotoxicity (Chapter 4, 4.3). Especially NK cells expressing one KIR were susceptible to the missing-self condition. Potentially, an

additional KIR could inhibit the NK cell by binding its corresponding HLA ligand expressed on the target cell. Furthermore, allogeneic NK cell cytotoxicity against CD4+ T cells from HIV-1 patients was seen in the absence of each HLA ligand (HLA-Bw4, HLA-C2, or HLA-C1). These results are in line with the hypothesis postulated by Jennes et al., whereby we confirm that KIR-HLA incompatibility between "donor" and "recipient" induces NK cell cytotoxicity against HIV-1 patient-derived CD4+ T cells. *In vitro*, these missing-self responses (Chapter 4) were present without measurable expression of HIV-1 in the target cells. Therefore, we suggested that these missing-self responses could also be present during infection by other sexually transmitted viruses. In viral infections such as CMV and the hepatitis B virus, both susceptibility and protection against infection are associated with certain KIR haplotypes and should, therefore, be investigated for missing-self like responses during sexual transmission or infection[377,378,382,457,532,533]

2. The individual inhibitory KIRs and the strength of their missing-self responses

In the original epidemiological study, protection against HIV-1 acquisition was explicitly related to the KIR2DL1-HLA-C2 mismatch[11]. Therefore, we compared the strength of each KIR-HLA mismatch to assess its impact on the HIV-1 acquisition rate. Between the three primary inhibiting KIR receptors, KIR2DL1 and 2/3-induced missing-self responses were more potent compared to the KIR3DL1 response in the absence of HLA-Bw4. Directly related to the licensing principle (Chapter 1, 2.3), these differences can be explained by the dominant role of HLA-C in self-recognition and education[507,534]. Although licensing by KIR2DL1 or KIR2DL2/3+ has a similar impact on NK cell functionality[503], KIR2DL1+ NK cells were seen as the most robust responders in a missing-self context. Thereby, our *in vitro* data support the hypothesis postulated by Jennes et al[11], as single KIR2DL1 expressing mucosal NK cells would be most susceptible to become activated in the presence of CD4+ T cells lacking HLA-C2.

Alternatively, NK cells are activated by antibodies capable of binding to the CD16 receptor on NK cells with their Fc-domain. The interaction of the Fc domain with CD16 induces an ADCC NK cell

response. Interestingly, Gooneratne et al.[459] suggested that explicitly licensed KIR2DL1+ NK cells were able to induce ADCC upon the interaction of CD16 (Fc receptor IIIa) with anti-HIV-1 antibodies present in the semen of HIV-1 patients. In line with our (Chapter 4, 4.4) and Jennes' results[11], specifically licensed KIR2DL1+ NK cells were associated with protective NK cell responses, be it by ADCC or/and by a missing-self response. Both NK cell activation pathways could be simultaneously active during HIV-1 sexual transmission. In the absence of other activating interactions, the presence of anti-HIV-1 antibodies could function as a catalyzer in these missing-self NK cell responses at the genital mucosa.

Noteworthy, in Haplo-HSCT (Chapter 2, 5.1), missing-self responses are induced by using stem cell donors that share only one instead of two HLA class I and II alleles. Subsequently, no differences in outcome could be determined between the different KIR-HLA mismatches. Along with the outcome of our experiments, it would be interesting to duplicate these experiments in the context of the anti-tumoral activity. With this knowledge, selection of stem cell donors could be improved, so transplanted NK cells elicit more potent missing-self responses, as was seen with the inclusion of KIR2DS1 in donor selection criteria[483,491,535].

3. The influence of an activating KIR haplotype on NK cell cytotoxicity

Besides release from HLA-KIR inhibition, an activating signal is necessary to provoke an NK cell response. In Chapter 4 (4.6), the impact of aKIRs was measured at the genomic level, as fluorescently labeled Ab staining aKIRs were commercially unavailable. NK cell donors with an activating (AB) or inhibiting (AA) KIR haplotype were distinguished, whereas only one donor contained a BB haplotype. In an attempt to relate KIR-specific cytotoxicity to the KIR haplotype, we correlated the size of the mismatched KIR+ NK cell population with the total NK cell degranulation and CD4+ T cell death for both AA and AB NK cell donors. As expected, a correlation was only significant in the presence of an AB KIR haplotype. This correlation suggests that iKIR+ NK cells need a co-expressing aKIR to fully benefit from the KIR-HLA mismatched situation and generate a missing-self

response. The exposure to HIV-1 in these experiments was unknown, but in any case, low since most HIV-1 patients were under treatment.

4. The impact of HIV-1 infection/exposure on missing-self NK cell responsiveness

In vitro infection of CD4+ T cells generated a population composed of various exposed subpopulations: CD4+ p24-, CD4+ p24+ and CD4- p24+. As in previous *in vitro* research, the CD4+ p24- subset was described as "uninfected"[240]. However, HIV-1 could still be present in these p24- CD4+ T cells as long as the detection threshold for flow cytometry is not reached[527]. With the use of a GFP reporter HIV-1_{AD8} strain, Lee et al. observed the emergence of new GFP+ cells out of the supposed uninfected GFP- CD4+ population. With this, rather than uninfected, the CD4+ p24- population might resemble a heterogeneous population of infected cells producing low amounts of virions, latently infected and exposed uninfected CD4+ T cells. As suggested by Lee et al.[527], NK cell responses directed to the CD4+ p24- subset might resemble the NK cell responses against early infected CD4+ T cells.

The absence of each individual self-HLA ligand generated a strong missing-self response against this CD4+ p24- T cell population (Chapter 5, 5.2). As the cell death rate was even higher as compared to HIV-1 unexposed conditions, we suggested an additional cytotoxic effect by HIV-1 exposure. MIC-A/B expressing cells were not targeted by missing-self NK cell responses, suggesting that other NK-activating ligands, such as NKp44[518], are upregulated on these HIV-exposed CD4+ p24- cells.

Although an increased clearance of allogeneic CD4+ T cells was present in KIR-HLA incompatible conditions (chapter 4), the impact on the success of HIV-1 transmission was still unclear. In the semen of HIV-1 untreated patients, only 0,2% (range 0.002–16%) of the macrophages and CD4+ T cells contained HIV DNA[70], making the majority of semen-derived T cells by definition uninfected. Therefore, we suggested that the missing-self responses should target the HIV-1 infected cells to have an impact on the transmission rate of the cell-associated virus. In Haplo-HSCT, KIR-HLA incompatibility was seen to enable the specific recognition of leukemic cells, generating a "graft-versus-leukemia" effect and a beneficial outcome[480,481]. In HIV-1 conditions, we predicted similar

effects with an increased clearance of HIV-1 infected cells, recognized by the elevated expression of activating NK cell receptors. In chapter 5, CD4+ T cells were superinfected (MOI=0,01) by HIV-1_{Ba-L} to guarantee the exposure and infection by HIV-1. After 7 days in culture, the HIV-1 exposed cells could be divided into CD4+ p24-, CD4+ p24+ and CD4- p24+ subsets. Being productively infected, the p24+ T cell subset could be seen as the population of interest when investigating the HIV-1 protective capacity[477]. In vitro infection with HIV-1 has been shown to elevate the expression of activating NK cell ligands MIC-A/B and ULBP-3 [240], which became our "prime candidates" to catalyze the missing-self response against HIV-1 infected cells.

However, in contrast to the anti-tumor capacities of missing-self NK cell responses[480,481], reduced clearance of the p24+ T cell population was observed compared to p24- T subsets. NK cell cytotoxicity against p24+ T cells was actively inhibited and seemingly unaffected by KIR-HLA incompatibility. These results are in line with the resistance to NK cell lysis observed against autologous HIV-1 infected CD4+ T cells[536-538]. Unexpectedly, the missing-self condition in these allogeneic encounters did not suffice to overcome HIV-1's inhibition of NK cell activation. In the literature, inhibition of NK cell cytotoxicity was ascribed to the upregulation of HLA-E, inducing inhibiting signals upon binding NKG2A[426]. As NKG2A was expressed on a minor subset of NK cells (Chapter 4)[539], this HIV-1 restraining effect would only inhibit a portion of the NK cells, and could not explain the observed near-complete resistance to NK cell lysis. Accordingly, the comparable HLA-E expression between the p24- and CD4- p24+ T cells instead suggests a limited impact of HLA-E on the observed resistance to lysis by NK cells. In addition, HIV-1 is also known to downregulate the expression of activating NK cell ligands such as NKp44L[432] and MIC-A/B[431] on infected cells. In line with these results, expression of MIC-A/B on the CD4+ p24+ T cells was not upregulated compared to unexposed T cells.

In various autologous co-culture experiments with *in vitro* infected CD4+ T cells and NK cells, the MIC-A/B stimulation was a necessity for anti-HIV-1 directed NK cell responses[536,538,539]. Also in our data, anti-HIV-1 NK cell cytotoxicity was seemingly restricted to the expression of MIC-A/B on p24+ T cells. Therefore, the differences in MIC-A/B expression between CD4+ and CD4- p24+ T cells

might explain the differences in NK cell-mediated cell death. Most interestingly, missing-self responses were present against p24+ T cells if they expressed MIC-A/B. Because of the low MIC-A/B expression rate, these missing-self like responses were not detected when measuring total cell death. Hereby, we could state that missing-self responses against p24+ T cells were restricted to cells expressing activating ligands such as MIC-A/B, but not FasR. MIC-A/B is recognized by NKG2D, an activating NK cell receptor with known anti-viral[537,540] and tumoral capacities[522,541]. Besides MIC-A/B, NKG2D also recognizes the stress-induced ULBP 1-6 ligands, which emits an equal activating signal upon interaction. Also, the NKG2D can be seen as one of the most prominent NK cell activators during target cell encounters as the receptor is expressed on virtually every NK cell[542].

To what extent HIV-1 infected vaginal/semen-derived CD4+ T cells express MIC-A/B or other activating NK cell ligands is unknown. The expression profile of these ligands on the HIV-1 infected T cells is subjected to the HIV-1's accessory proteins such as Nef and Vpu. Nef's functionality is dependent on the stage of HIV-1 infection and exhibits substantial functional heterogeneity between natural polymorphisms[525,543,544]. Consequently, expression of the stress-induced MIC-A/B ligand on semen-derived CD4+ T cells may be highly variable inter- as well as intra-individual[526,544]. As MIC-A/B is typically downregulated by HIV-1's evasion protein Nef, expression of MIC-A/B on p24+ T cells could indicate a defective or low functional Nef protein. As a result of this, the effectivity of Nef functionality could have a direct impact on the effectiveness of the missing-self response. Unfortunately, there is no previous knowledge concerning the Nef functionality of the HIV-1_{Ba-L} lab strain. Although the observed maintained expression levels of HLA-B, HLA-E, and MIC-A/B could suggest limited functionality of the HIV-1_{Ba-L} Nef protein, new research needs to intensively investigate the functionality of the HIV-1_{Ba-L} Nef and compare these with other HIV-1 lab strains or HIV-1 primary isolates.

5. Conclusion

Besides the multiple associations between autologous NK cell receptors and beneficial HIV-1 outcome in already infected subjects, also an allogeneic NK cell mechanism was described to be protective

against HIV-1 acquisition. Although solely based on genetic data, KIR-HLA incompatibility between sexual partners was suggested to induce an allogeneic NK cell response against vaginal/semen-derived HIV-1 infected cells. If and to what extent KIR-HLA incompatibility is indeed able to mediate these HIV-1 restrictive NK cell responses was investigated by the use of *in vitro* co-cultures, modeling the encounters at the site of transmission.

In vitro, KIR-HLA incompatibility between NK cells and CD4+ T cells was a potent initiator for NK cell cytotoxicity, even in the absence of demonstrable HIV-1 exposure. We dissected the missing-self responses by the various KIR-HLA mismatches in depth. A missing-self NK cell response was characterized by a significant increase of those degranulating NK cells expressing the affected KIR, which expanded to become the main KIR related contributor of the total NK cell response.

As one of the first investigators in this field, we demonstrated differences in the initial strength of the missing-self responses generated by the different KIRs. The absence of HLA-C1 or -C2 resulted in a more robust missing-self response compared to the absence of HLA-Bw4. Specifically, the absence of HLA-C2 induced strong missing-self responses, able to activate more than half of the KIR2DL1+ NK cells. In accordance with these *in vitro* data, this KIR2DL1-HLA-C2 mismatch was also related to protection from HIV-1 transmission. Besides KIR2DL1, an activating AB KIR haplotype was seen to be an additional factor benefitting NK cell cytotoxicity in the missing-self responses. AB KIR haplotypes were suggested to exploit the absence of inhibitory signals fully and enabled increased NK cell activation, which is in line with its HIV-1 protective effect observed in multiple studies[4].

Refining the model by i*n vitro* HIV-1 infection of the CD4+ T cells and the staining of HIV-1 related ligands on the CD4+ T cells enabled the exploration of the productive versus non-productive HIV-1 infection on NK responses in the missing-self conditions. Exposure of HIV-1 was able to induce an additive cytotoxic effect to the missing-self NK cell responses. However, the additive effect was directed to HIV-1 exposed but p24 CD4+ T cells, rather than the productively infected (p24+) T cells. Overall, NK cell cytotoxicity towards productively infected T cells was even strongly inhibited, rendering the absence of a self-HLA ligand useless in the activation of NK cells.

Nevertheless, NK cell cytotoxicity towards productively infected T cells was seen to be related to the expression of the NKG2D ligand MIC-A/B. More importantly, in combination with MIC-A/B, missing-

self responses were observed against p24+ T cells. Interestingly, late CD4- infected T cells were seen to be more prone to NK cell recognition.

In conclusion, *in vitro* missing-self responses against HIV-1 exposed allogeneic CD4+ T cells were present, whereas NK cell cytotoxicity against productively infected CD4+ T cells was still inhibited. With this, the anti-viral effect of allogeneic missing-self responses during transmission will be limited to cell-associated virus expressing activating ligands such as MIC-A/B, but not FasR.

6. Remarks and Future perspectives

6.1. Suggestions for further studies

As innate immune cells, mature NK cells are found all over the body, continually surveilling in search of virally-infected or malignant cells. The cytotoxic activity against these target cells is based on the presence of ligands for the NK cell receptors, expressed on the surface of these interrogated cells. The mechanism behind the repertoire of expressed NK cell receptors tends to be a surrogate of the previous encounters and the local environment[314]. In our experiments, we used blood-derived NK cells in an attempt to recreate allogeneic encounters during sexual HIV-1 transmission, whereas our HIV-1 protective mechanism should be induced by mucosal NK cells. To what extent differences between mucosal and blood-derived NK cells would influence functionality is unknown. Mucosal NK cells are instead observed as immature with a cytokine-producing functionality, whereas blood-derived NK cells have strong cytolytic capacity (Chapter 1, 2.2.5)[194,306]. Nevertheless, mucosal NK cells in the gut do express KIRs and could, therefore, react to the absence of self-HLA ligands, in line with our hypothesis. To better understand the consequences of a missing-self condition at the genital site of infection, mucosal NK cells should be investigated more extensively, comparing the expression profiles and functionality with blood-derived NK cells. We could also question whether these missing-self NK cell responses are indeed mediated by local mucosal NK cells or are the result of migrating blood NK cells to the site of infection.

When reviewing the literature, HIV-1 dC containing ESNs were only investigated in the context of a strong autologous NK cell response against HIV-1. Whereas HIV-1 dC couples are very suitable to

study the alloreactive mechanisms on HIV-1 acquisition, studies on HIV-1 dC cohorts failed to investigate NK cell alloreactivity. Habegger et al.[472], for example, looked at KIR and HLA alleles of both partners in an HIV-1 dC cohort without studying the relation between them. If the same data is reanalyzed with attention to allogeneic KIR/HLA relations between both partners, interesting data can be added to the small amount of information on the role of NK cell alloreactivity in HIV-1 protection.

6.2. Investigating mucosal NK cell responses

To better understand the NK cell responses occurring at the site of HIV-1 transmission, our rudimentary co-cultures need to be replaced by more refined mucosal-specific assays. For instance, an explant model of the female genital tract[545] could be used to look at IEL NK cell responses against allogeneic CD4 T cells or semen-derived T cells or macrophages.

Furthermore, *in vivo* models such as the human SIV simian model can be used to investigate these missing-self responses. Macaques are an ideal model for these experiments and were already essential for revealing multiple *in vivo* migration routes and immune responses during early HIV-1 infection. By atraumatic inoculation of HIV, the vaginal barriers are left intact and resemble a "natural" influx of semen-derived infected T cells[3,13,78,498,546]. Afterward, cervical and vaginal biopsy samples can be used to investigate the aftermath of the HIV-1 transmission events[78,92,102].

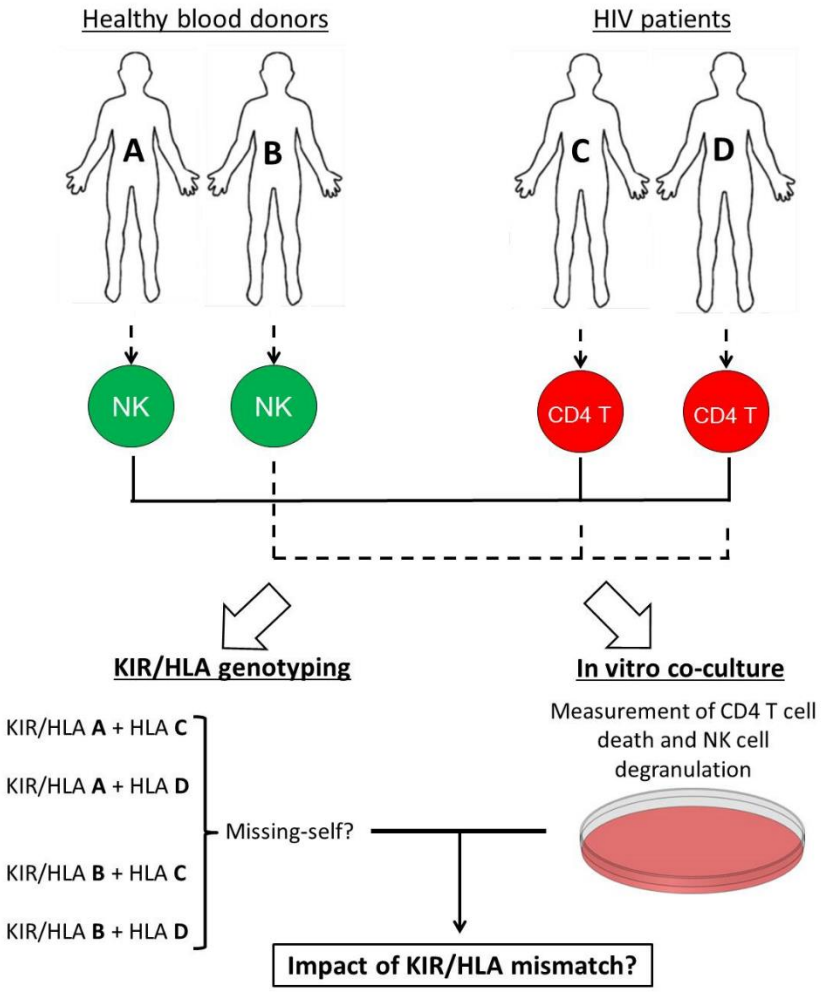
6.3. Future focus points on NK cell studies in HIV-1 infection

Generally, HIV-1's accessory proteins Nef, Tat and Vpu ~~a~~re believed to specifically downregulate the HLA class I molecules linked to CD8+ T cell recognition (mainly A and B), whereas HLA class I molecules binding NK cell's KIR receptors (mainly C) were left unaltered (Chapter 2, 4.1.3). Hereby, HIV-1 can avoid both the cytotoxic CD8+ T and NK cell responses. The emphasis of these studies was on the analysis of the intracellular molecular interactions, and therefore, they made use of genetically modified lab strains to create a controlled functioning of the HIV-1 accessory proteins. Conversely, HLA-C downregulation was witnessed in primary isolates of HIV-1 patients[427]. It was ascribed to Vpu but was also seen to variate highly between patients. Vpu-mediated downregulation of HLA-C was associated with strong CD8+ T cell responses and weak NK cell responses, whereas unaltered HLA-C

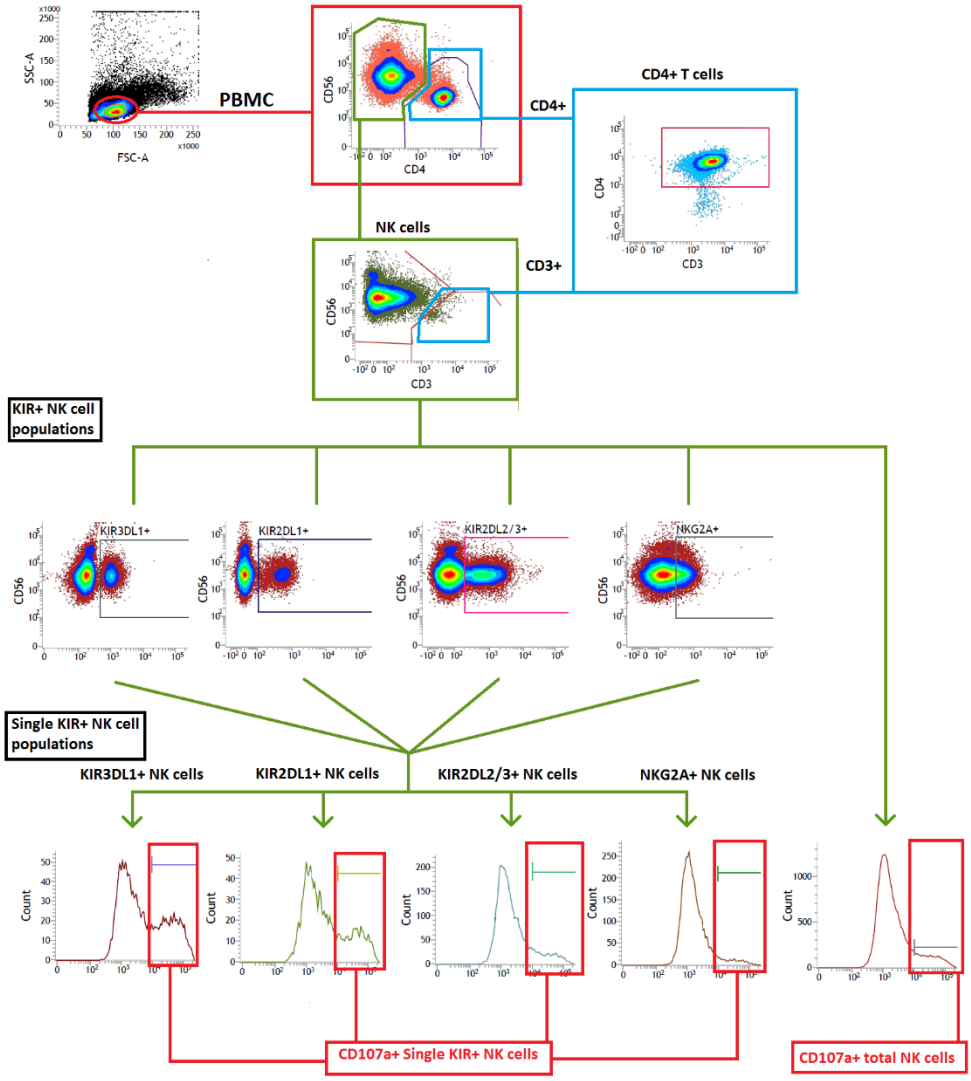
expression was associated with strong NK cell responses[428]. These results show an additional evasion mechanism by HIV-1, which can adapt to the strength of the host's immune system. The effect of these Vpu-modulations on NK cell responsiveness should be investigated in an attempt to aid the control of infection in addition to the conventional ART. To what extent these autologous missing-self responses could have a curative outcome is uncertain. On the one hand, our results indicate that KIR-HLA mismatched NK cell cytotoxicity was lowered by productively HIV-1 infected (p24+) T cells. On the other hand, in contrast to inter-individual KIR-HLA incompatibility, KIR2DL-missing-self responses should specifically target infected cells as the HIV-1 derived Vpu is the mediator for this HLA-C downmodulation.

In addition, HIV-1 accessory proteins influencing NK cell recognition and activation should be extensively investigated for their specific contribution to impairing NK cell functionality. Especially Nef, Tat, and Vpu seem to alter NK cell interactions by upregulating inhibitory ligands and downregulating activating ligands on infected cells. The use of primary isolates or knock-out strains for the various HIV-1 accessory proteins (Nef, Tat, or Vpu) in NK-CD4 T cell co-cultures will reveal the NK cell-inhibiting effects of each protein, or could expose synergistic effects.

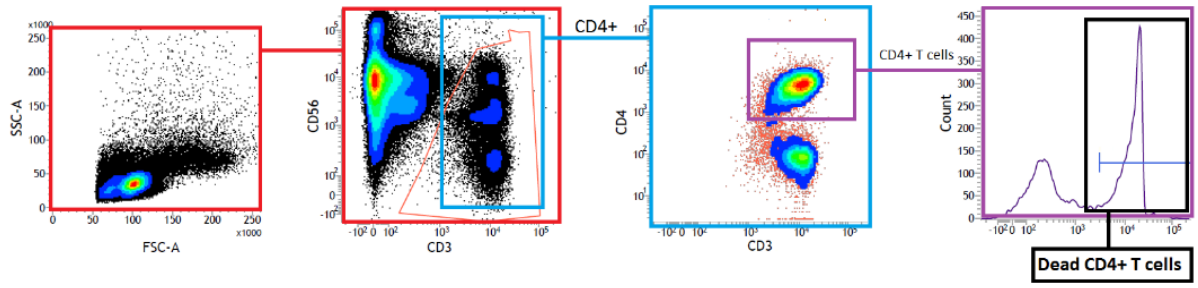
Supplementary figures



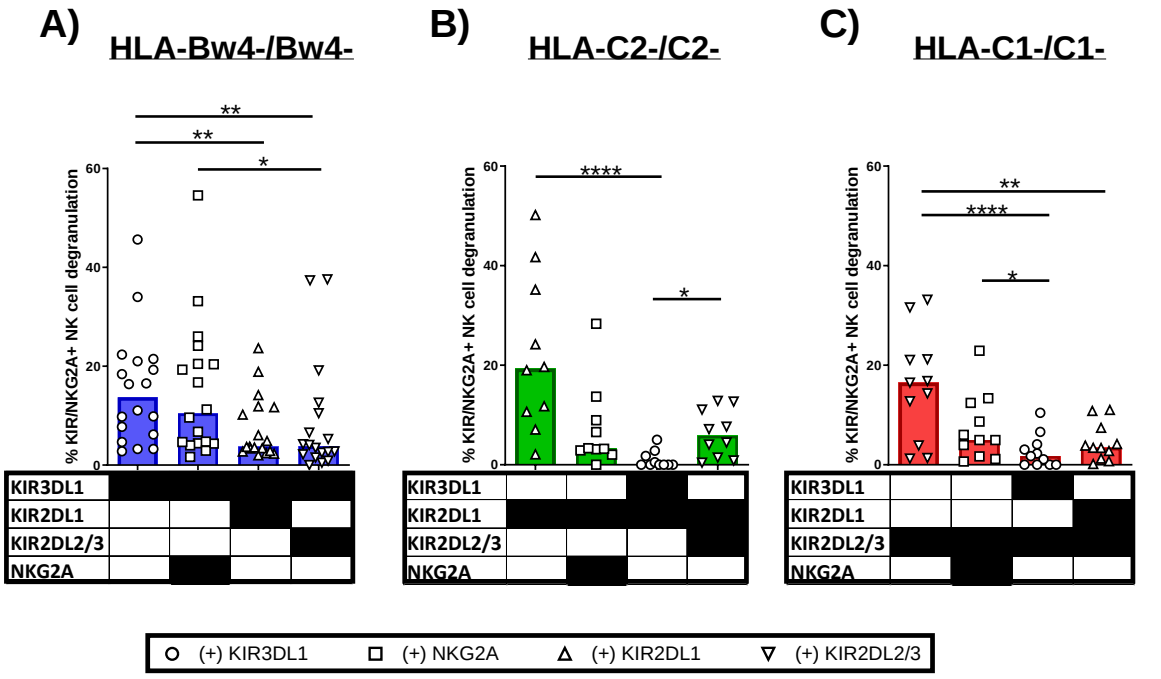
Supplementary figure.4.1: Experimental set-up of the genetic and functional experiments: *In every experiment, we isolated the NK cells from two healthy individuals and the CD4+ T cells of two HIV-1 patients. Each NK cell donor was linked with the two HIV-1 patient, resulting in four co-cultures, to optimize the variety in KIR-HLA interactions, resulting in KIR-HLA (mis)matches with the same NK cell donor. Afterwards, the CD4+ T cell death and NK cell degranulation measured in the co-cultures was ascribed to a KIR-HLA (mis)match and was further used for analysis to determine the impact of these KIR-HLA interactions.*



Supplementary figure.4.2: Template of the flow cytometric analysis of the degranulation of NK cells in the NK-CD4+ T cell co-cultures. *For every parameter, CD4+ T cell death and NK cell degranulation, a different co-culture was used. Here we show the template used to analyze the co-culture set up for NK cell degranulation. Starting from the FSC-SSC graph, based on FSC-SSC and cell density we gated on PBMCs. Next, expression of CD56 and CD4 on PBMCs was displayed and CD56+/CD4- cells and CD56-/CD4+ cells were separately gated. Within the CD56+/CD4- cell population the CD3+ cells were excluded, thus isolating the CD56+/CD3- cells or NK cells. The CD56-/CD4+ and CD56-/CD3+ cells were combined to gate the CD4+/CD3+ cells or CD4+ T cells. NK cells were stained for the expression of KIR3DL1,-2DL1,-2DL2/3 and NKG2A. The KIR+/NKG2A+ NK cells were used in a Boolean gating strategy resulting in the identification of the Single KIR+ NK cell populations (KIR3DL1+/KIR2DL1-/KIR2DL2-3-/NKG2A-; KIR2DL1+/KIR3DL1-/KIR2DL2-3-/NKG2A-; KIR2DL2-3+/KIR3DL1-/KIR2DL1-/NKG2A-; NKG2A+/KIR3DL1-/KIR2DL1-/KIR2DL2-3-). Within the Single KIR+ NK cell populations, we measured the frequency of degranulating KIR+ NK cells. We also measured the frequency of degranulating NK cells in the total NK cell population.*

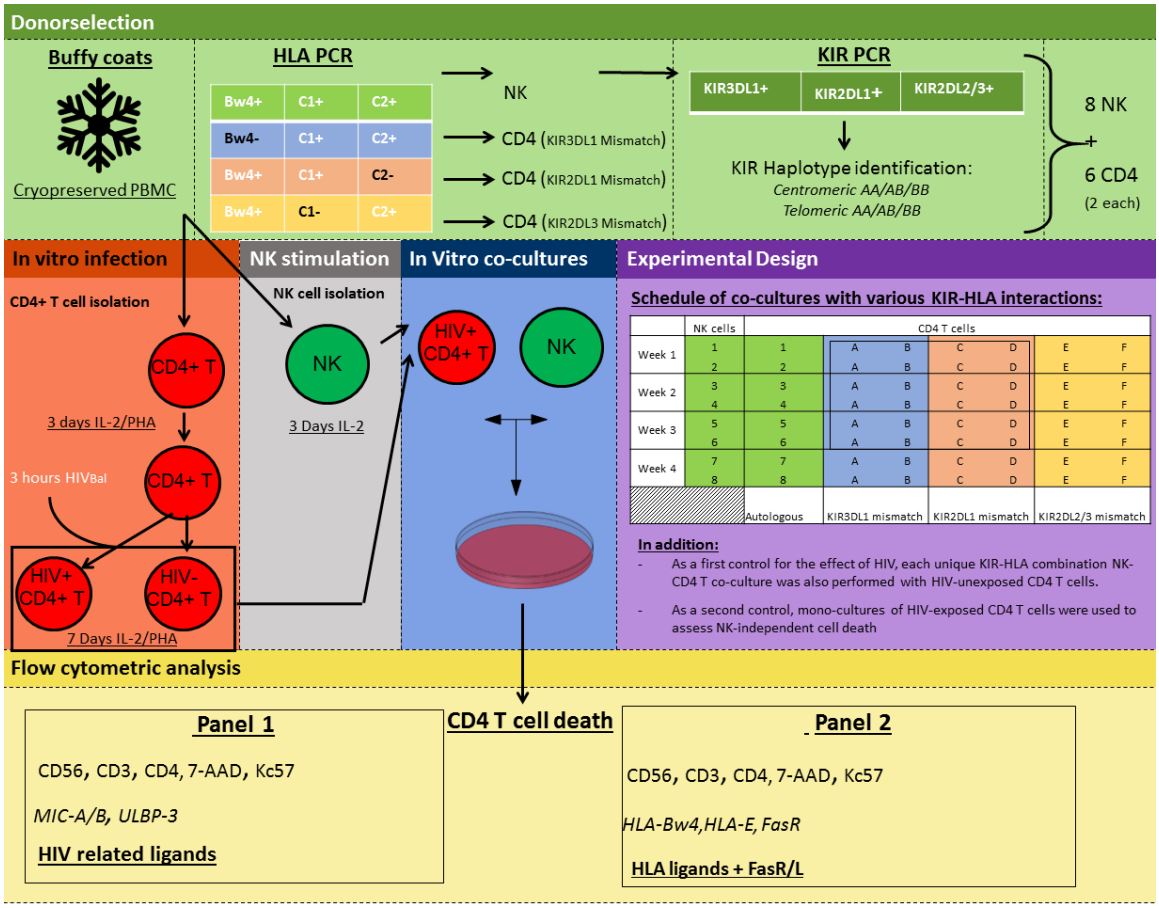


Supplementary figure.4.3: Template of the flow cytometric analysis of the CD4+ T cell death in the NK-CD4+ T cell co-cultures: *For every parameter, CD4+ T cell death and NK cell degranulation, a different co-culture was used. Here we show the template used to analyze the co-culture set up for CD4+ T cell death. Starting from the FSC-SSC graph, we selected all events present, as dead CD4+ T cells do not remain in the general PBMC gating, in which mostly living cells reside. Next, all cells were divided based on the expression of CD56+ and CD4+. Within the CD4+ cell population, we isolated the CD4+ T cells as CD4+/CD3+ cells. Within the CD4+ T cell population, the brightly 7-AAD+ cells were considered and counted as dead CD4+ T cells.*

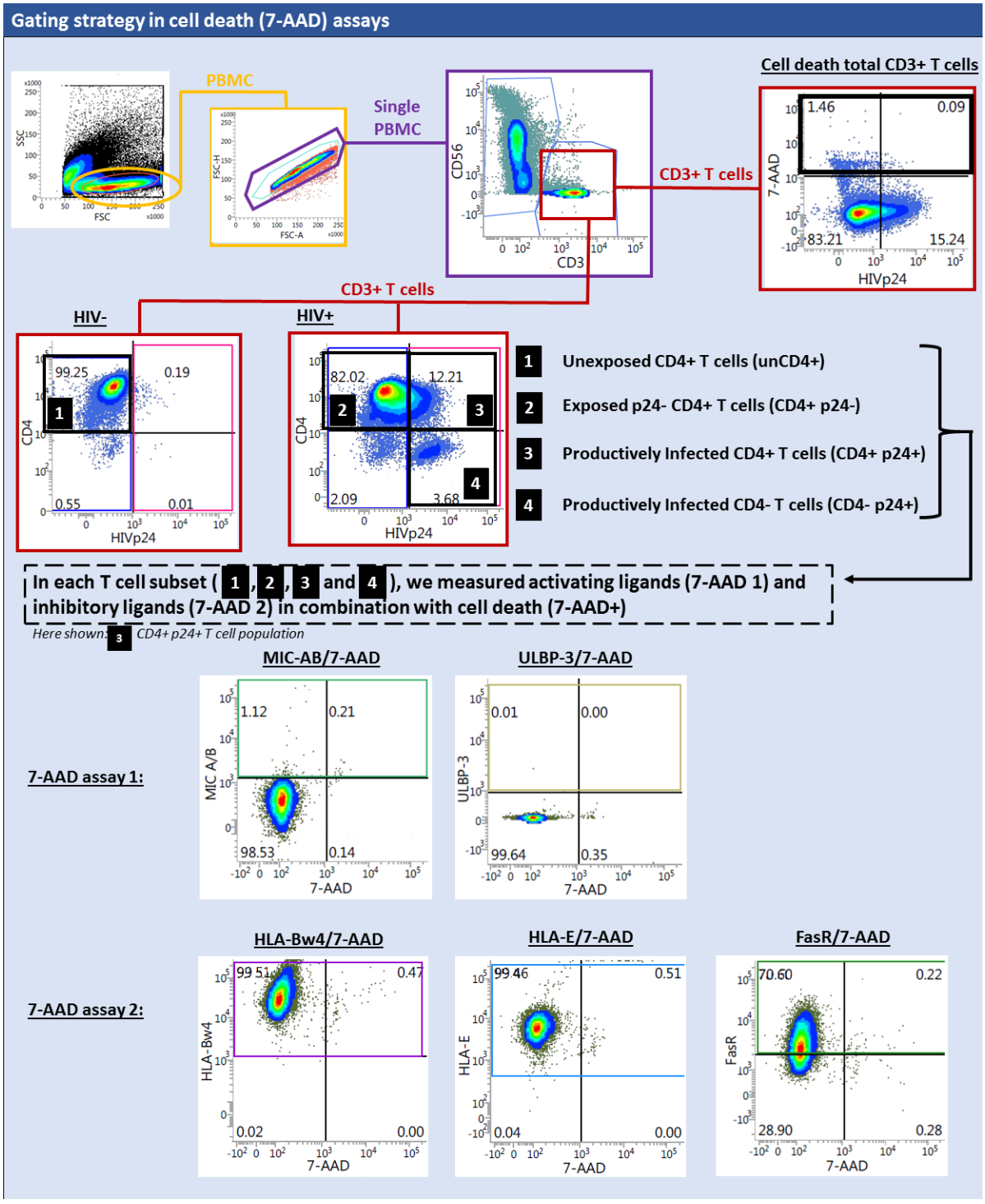


Supplementary figure.4.4: Degranulation of NK cells expressing one KIR (Single-iKIR) or two KIRs in the absence of an HLA ligand: *Each graph represents the degranulation of KIR/NKG2A+ NK cells in the absence of a specific HLA ligand: HLA-Bw4-/Bw4-(A, blue), HLA-C2-/C2- (B, green) and HLA-C1-/C1- (C, red). Respective to the absent HLA, degranulation of the corresponding Single-iKIR+ NK cells was compared with the NK cell subsets expressing an additional KIR or NKG2A: KIR3DL1, KIR2DL1 and KIR2DL2/3 in graph A, B and C respectively. Below each graph, the KIR/NKG2A expression by the NK cells in the column above is displayed, with a blackened box representing the expression of the*

*corresponding KIR/NKG2A. In the legend, the plus symbol between brackets refers to the NK cells expressing more than one KIR/NKG2A (for example: KIR3DL1+KIR2DL1 expressing NK cells). (p-values: * p=0.0332, **p=0.0021, ***p=0.0002,****p<0.0001).*



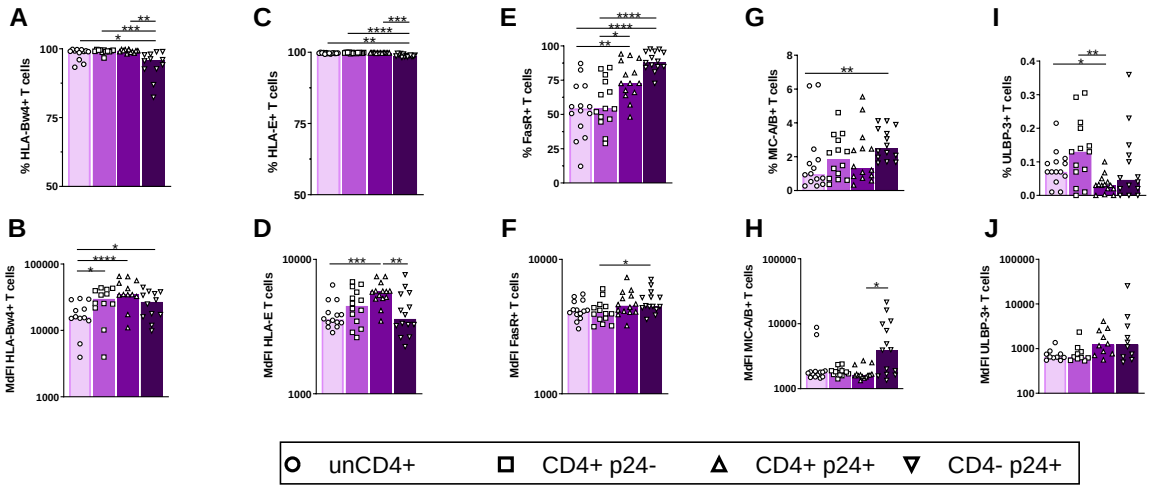
Suppl. Fig.5.1: Schedule of the experimental procedures from cryopreservation to FCM analysis: *PBMCs were isolated from buffy coats, using Ficoll separation. After DNA extraction, PBMCs were HLA genotyped and classified as NK cell donor or CD4 T cell donor depending on the presence of HLA ligands. NK cell donors were selected, based on the presence of the three licensed iKIRs. CD4 T cells were selected based on lack of one of the 3 KIR ligands and presence of the two others. Isolated CD4 T cells from the latter donors were infected with HIV-1_{Ba-L} (i.e. HIV-1 exposed) or sham treated (i.e. HIV-1 unexposed). NK cells were isolated from the NK cell donors and stimulated for 3 days with IL-2. NK cells from every NK cell donor was separately co-cultured with autologous CD4 T cells as well as the CD4 T cells each CD4 T cell donor, both under exposed and unexposed conditions. After co-culture, 2 different panels were used to dissect the NK-CD4 T cell interactions, measuring CD4 T cell death in HLA- or HIV-related subsets.*



Suppl. Fig.5.2. Gating strategy of CD4+ T cell death analysis: *To measure cell death of the total and subsets of the CD4+ T cells, a gating strategy was developed using FACSVerse® software. After gating the PBMCs, single PBMCs were isolated by plotting the forward scatter in area and height, differentiation the "doublets". Within the single PBMCs, CD3+ T cells were isolated and further differentiated into subsets by plotting for expression of CD4 and HIV-1p24.. Within each CD4+ T subset, ligand expression was measured and plotted with cell death, as measured with 7AAD.*

Antibody staining cocktail 1				
Antigen	*Fluorochrome*	*Excitation (nm)*	*Laser*	*Company*
CD56	APC-Vio770	510	Violet	Miltenyi
CD3	Viogreen	785	Red	Miltenyi
CD4	VioBlue	421	Violet	Miltenyi
7-AAD	PerCP	650	Blue	BD
HIV-1p24	FITC	520	Blue	Beckman Coulter
HLA-Bw4	PE-Vio770	775	Blue	Miltenyi
HLA-E	PE	690	Blue	Biolegend
FasR	APC	660	Red	BD
Antibody staining cocktail 2				
Antigen	*Fluorochrome*	*Excitation(nm)*	*Laser*	*Company*
CD56	APC-Vio770	510	Violet	Miltenyi
CD3	Viogreen	785	Red	Miltenyi
CD4	Vioblue	421	Violet	Miltenyi
7-AAD	PerCP	650	Blue	BD
HIV-1p24	FITC	520	Blue	Beckman Coulter
MIC-A/B	PE	565-605	Blue	Miltenyi
ULBP-3	APC	660-670	Red	R&D

Supplementary table 5.1.Enlistment of the antibodies used in antibody cocktail 1 and 2: *In the table, the antibodies are enlisted that were used during the experiments. Alongside, additional information concerning these antibodies, such as antigen, fluorochrome, excitation, kind of laser and the company from were these antibodies were obtained.*



Suppl. Fig.5.3. Baseline expression profiles of CD4 T cells in mono-culture: *Mono-cultured CD4 T cells were observed for their expression of various ligands in absence of NK cells. The examined mono-cultures consisted of CD4 T cells derived from CD4 T as well as NK cell donors. In the graphs, each datapoint represents the expression of an individual PBMC donor (6 CD4 T cell donors and 8 NK cell donors). The frequency of cells expressing inhibitory HLA molecules and viral*

upregulated ligands as well as the MdFI of this expressing population were compared between CD4 T cells in the HIV-1 unexposed (unCD4) and exposed mono-cultures (Fig.1). Within the exposed mono-culture, CD4 T cells were further classified as HIV associated CD4 T cell subsets using HIV-p24 and CD4 markers; exposed p24- CD4+ T cells, CD4+ p24+T cells and CD4- p24+T cells. Both the frequencies of HLA-Bw4+ and HLA-E+ were significantly downregulated in the CD4- p24+ T cell subset compared to the other subsets (A and C). The MdFI of HLA-Bw4 and HLA-E on the CD4+ p24+ T cells was significantly increased compared to the unCD4 T cells,and regarding HLA-E also to the CD4- p24+ T cells (B and D). The frequency of FasR+ p24+ (CD4+/-) T cells was significantly increased compared to the unCD4+ and CD4+ p24- T cells (E). Likewise, the MdFI of FasR was upregulated on CD4- p24+ T cells compared to the CD4+ p24- T cells (F). The frequency of MIC-A/B+ CD4- p24+ T cells was significantly elevated compared to the frequency of MIC-A/B+ unCD4+ T cells (G). The MdFI of MIC-A/B was significantly increased on CD4- p24+ T cells compared to the CD4+ p24+ T cells (H). The frequency of ULBP-3 was rather low on all CD4+ T cell subsets, but significantly downregulated on the CD4+ p24+ T cell subset compared to the unCD4+ and CD4+ p24- T cell subsets (I). No differences in MdFI of ULBP-3 were measured between the various CD4+ T cell subsets (J).

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