The immunoregulatory role of HLA-F in human diseases

A dissertation
For the doctoral degree

DOCTOR RERUM NATURALIUM

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2023

Date of oral defense: 03rd of November 2023

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Glossary of terms

ADCC: antibody dependent cell cytotoxicity
AIDS: acquired immunodeficiency syndrome
AILD: autoimmune liver disease
AIH: autoimmune hepatitis
ALS: amyotrophic lateral sclerosis
ALP: alkaline phosphatase
ANA: antinuclear autoantibody
APC: antigen presenting cells
ART: antiretroviral therapy
β2m: beta-2 microglobulin
BKPyV: BK polyoma virus
CIITA: class II major histocompatibility complex transactivator
CCL: C-C motif ligand
CCR: C-C chemokine receptor
CD: cluster of differentiation
CHIKV: chikungunya virus
CMV: cytomegalovirus
CTL: cytotoxic lymphocyte
CTLA-4: cytotoxic T lymphocyte associated antigen 4
CTV: cell trace violet
CV: coefficient of variance
CXCL: C-X-C motif ligand
CXCR: C-X-C chemokine receptor
DC: dendritic cells
DENV: dengue virus
DNA: deoxyribonucleic acid
EBV: Epstein-Barr virus
EBV-HLH: Epstein-Barr virus-induced hemophagocytic lymphohistiocytosis
ECM: extracellular matrix
ER: endoplasmic reticulum
ERAAP: endoplasmic reticulum aminopeptidase associated with antigen processing
ERAD: endoplasmic reticulum associated degradation
ESCC: esophageal squamous cell carcinoma

EVT: extravillous trophoblasts
FC: flow cytometry
GALT: gut associated lymphoid tissue
GBM: glioblastoma
GvHD: graft versus host disease
HadV5: human adenovirus 5
HBV: hepatitis B virus
HCC: hepatocellular carcinoma
HCV: hepatitis C virus
HCMV: human cytomegalovirus
HGF: hepatocyte growth factor
HIV-1: human immunodeficiency virus 1
HLA: human leukocyte antigen
HOM: hepatocyte organoids medium
HPV: human papillomavirus
HSC: hepatic stellate cells
IFN: interferon
Ig: immunoglobulin
INSTI: integrase strand transfer inhibitor
IQR: interquartile range
IRF1: interferon regulatory factor 1
ITAM: immunoreceptor tyrosine-based activation motif
ITIM: immunoreceptor tyrosine-based inhibitory motif
JEV: Japanese encephalitis virus
KC: Kupffer cells
KDDB: KIR and diseases database
KIR: killer cell immunoglobulin-like receptor
LAG-3: lymphocyte activation gene 3
LC1: anti liver cytosol type 1 autoantibody
LCL: lymphoblastoid cell line
LCMV: lymphocytic choriomeningitis
LFA-1: lymphocyte function-associated antigen 1
LILRB: leukocyte immunoglobulin-like receptor subfamily B
LKM: liver kidney autoantibody
LPS: lipopolysaccharide

LSEC: liver sinusoidal endothelial cells
MCMV: mouse cytomegalovirus
MdFI: median fluorescence index
MHC: major histocompatibility complex
MIC: major histocompatibility complex class I chain-related proteins
mRNA: messenger ribonucleic acid
NCAM: neural cell adhesion molecule
NCR: natural cytotoxicity receptor
NFκB: nuclear factor “kappa light chain enhancer” of activated B-cells
NK: natural killer cells
NKG: natural killer group 2 member protein
NKM: natural killer cells medium
NKR: natural killer cells receptors
NNRTI: non-nucleoside reverse transcriptase inhibitor
NPC: nasopharyngeal carcinoma
NRTI: nucleoside reverse transcriptase inhibitor
NSCLC: non-small-cell lung cancer
OC: open conformer
PBC: primary biliary cirrhosis
PBMC: peripheral blood mononuclear cell
PD-1: programmed cell death 1
PLC: peptide loading complex
PNPLA3: patatin-like phospholipase domain-containing protein 3
PSC: primary sclerosing cholangitis
ROS: reactive oxygen species
SH2B3: Src homology 2-B adaptor protein 3
SLE: systemic lupus erythematous
SLO: secondary lymphoid organs
SMA: anti smooth muscle autoantibody
STAT4: signal transducer and activator of transcription 4
TAP: transporter associated with antigen processing
TGF: transforming growth factor
Th1: CD4 T helper cells type 1
TLR: Toll-like receptor
TNF: tumor necrosis factor

TRAIL: tumor necrosis factor related apoptosis-inducing ligand

ULBP: UL-16 binding proteins

UPR: unfolded protein response

VISTA: V-domain Ig suppressor of T cell activation

VSV: vesicular stomatitis virus

ZIKV: zika virus

Zusammenfassung

HLA-F ist ein nicht klassisches HLA-I-Molekül, welches z.B. auf virusinfizierten Zellen hochreguliert wird und somit die stark zytotoxischen natürlichen Killerzellen (NK) aktivieren kann. Die Oberflächenexpression von HLA-F ist streng reguliert und wurde im Zusammenhang mit der Aktivierung von gesunden Lymphozyten beschrieben. Im pathologischen Kontext wurde die HLA-F-Oberflächenexpression bei verschiedenen Viruserkrankungen, Autoimmunerkrankungen und Krebserkrankungen detektiert. Die Expression von HLA-F in Geweben und die Faktoren, die seine Hochregulierung an der Zelloberfläche bewirken, sind bislang unklar. Angesichts seines Potenzials, zytotoxische Immunreaktionen gegen gestresstes Gewebe auszulösen, können diese Faktoren jedoch von großer Relevanz sein.

Das Humane Immundefizienz-Virus 1 (HIV-1) ist eine Virusinfektion mit erheblichen Auswirkungen auf die globale Gesundheit. Ein zentraler Schwerpunkt der HIV-1-Forschung ist das Erreichen einer langfristigen Immunkontrolle der Infektion, um die Behandlung von Patienten zu verbessern. Für die Autoimmunhepatitis (AIH), eine Autoimmunerkrankung der Leber, gibt es außer einer unspezifischen Immunsuppression keine gezielten Behandlungsmöglichkeiten, da hier bislang nicht genügend Informationen über zugrundeliegende Krankheitsmechanismen vorhanden sind.

In dieser Arbeit wurde die Oberflächenexpression von HLA-F, den Stress-Liganden NKG2DL und ihren Rezeptoren auf NK-Zellen von therapienaiven HIV-positiven Personen untersucht. Die Hochregulierung von HLA-F als Marker einer HIV-1-assoziierten Immunaktivierung wurde ebenso wie die phänotypische Veränderung von NK-Zellen festgestellt. In-vitro-Studien zur Abtötung nicht-infizierter aber aktivierter CD4-T-Zellen durch NK-Zellen, was einen möglichen Mechanismus der immunmodulatorischen Regulierung der HIV-1-Infektion durch NK-Zellen darstellen könnte, ergaben, dass die HLA-F:KIR3DS1-Achse, möglicherweise in Kombination mit der NKG2DL:NKG2DL-Achse, an diesem Mechanismus beteiligt ist.

Die Stimulierung von Hepatomzelllinien und humanen Hepatozyten-Organoiden, die aus Proben von AIH-Patienten gewonnen wurden, zeigte, dass die HLA-F-Oberflächenexpression im Lebergewebe möglicherweise durch einen zweistufigen Prozess über eine proinflammatorische Stimulation angetrieben wird, was die breite Gewebeexpression von HLA-F im Kontext von entzündlichen Erkrankungen erklären könnte. HLA-F bleibt ein Stress-Ligand, der weitergehende Untersuchung benötigt und der eine klare Verbindung zu verschiedenen humanen Erkrankungen zeigt, welches seine therapeutische Relevanz unterstreicht..

Abstract

HLA-F is a non classical HLA-I molecule that can be a strong activating trigger for highly cytotoxic natural killer (NK) cells when upregulated on virus-infected target cells. HLA-F surface expression is tightly regulated and has been described upon activation on lymphocytes under healthy conditions. In pathologies, HLA-F surface expression has been described in various viral diseases, autoimmune diseases and cancers. HLA-F expression in tissue and the factors inducing its upregulation at the cell surface are to date unclear but are likely important given its potential to trigger cytotoxic immune responses against stressed tissues.

Human immunodeficiency virus 1 (HIV-1) is a viral infection with major global health impact and achieving long-term immune control of HIV-1 infection is currently a strong focus of research efforts in advancing HIV-1 patient treatment. Autoimmune hepatitis (AIH), an autoimmune liver disease, is lacking targeted treatment options apart from unspecific immunosuppression due to the missing understanding of underlying mechanisms driving disease.

In this thesis, the surface expression of HLA-F, the stress ligands NKG2DL and their receptors on NK cells were profiled on therapy naïve HIV positive individuals. The upregulation of HLA-F as a marker of ongoing immune activation in HIV-1 infection was detected, along with NK cell phenotypic alterations. *In vitro* studies of uninfected activated CD4 T cell-killing by NK cells, a potential mechanism of immunomodulatory regulation by NK cells in HIV-1 infection, revealed that the HLA-F:KIR3DS1 axis is possibly implicated in this mechanism in combination with the NKG2DL:NKG2DL axis.

The stimulation of hepatoma cell lines and human hepatocyte organoids derived from AIH patient samples revealed that HLA-F surface expression in hepatic tissue is potentially driven by a two-step mechanism via pro-inflammatory stimulation, explaining the wide tissue expression of HLA-F during inflammatory diseases. HLA-F remains an enigmatic stress ligand with a clear link to various human diseases, rendering it interesting as a therapeutic target.

Introduction

Introduction to HLA-F

1 The human MHC system

The human major histocompatibility complex (MHC) describes a family of molecules that regulates the immune system by presentation of intracellular or extracellular peptides to immune cells. These molecules are also directly recognized by innate and adaptive immune cells, which make them crucial regulators of the immune system[1].

The human MHC, called the human leukocyte antigen (HLA) complex, is encoded in the p21.3 region of chromosome 6, the most polymorphic region in the human genome[2]. It is divided into two classes: (i) HLA class I (HLA-I), expressed in every nucleated cell under physiological conditions, presenting peptides derived from intracellular proteins to cluster of differentiation (CD)8 T cells[3], and (ii) HLA class II (HLA-II), expressed on specialized immune cells called "professional antigen-presenting cells (APC)", presenting peptides derived from extracellular proteins to CD4 T cells. Notably, HLA-I can additionally present exogenous peptides via a process called cross-presentation[4]. Peptides presented are recognized by adaptive immune cells (CD8 T cells for HLA-I presented peptides, CD4 T cells for HLA-II presented peptides) via their T cell receptor, leading to their activation.

1.1 Classical HLA-I molecules

Highly allele polymorphic with 24548 alleles for 14272 proteins (The Immuno Polymorphism Database, as of May 2023)[5], classical HLA-I molecules (namely HLA-A, -B, and –C) are key players in antiviral and anti-tumor immunity given their general expression in the body. Their high polymorphism combined with the polygeny accounts for the diversity of peptide presentation but also for the difficulty to match recipient and donor in organ transplantations, thus the denomination of "histocompatibility" complex.

Depending on the allotype, classical HLA-I molecules – apart from their role for adaptive immunity– are also used as an immune surveillance tool by innate immune cells, which will be described in detail below.

Composed of three alpha chain domains (α1, α2 and α3) stabilized by a non-covalent association to the non-polymorphic beta-2 microglobulin (β2m), classical HLA-I molecules present a wide range of peptides of 8-10 residues derived from intracellular proteins in the peptide binding groove formed at the junction of the α1 and α2 chains[6]. The substantial variety of peptide binding from

HLA-I is explained by the focus of most allelic polymorphisms on the peptide binding groove. Interestingly, the binding of a peptide is mostly dependent on the interaction with six pockets of anchor residues in the groove, closed by tyrosine residues at the C- and N- termini.

The antigen presentation pathway of classical MHC I molecules is composed of two main steps: antigen processing and antigen presentation **(Fig 1)**[7].

First, a protein from self (mechanism of self tolerance), viral or intracellular bacterial origin is processed by the proteasome. The proteasome is a multicatalytic protease that generates short peptides from ubiquitin-tagged proteins and can be stimulated by interferon (IFN) in order to increase the production of C-terminal hydrophobic residues that are preferentially transported and loaded on HLA-I molecules[8].

Once a peptide is generated, it is transported from the cytosol to the endoplasmic reticulum (ER) via transporters associated with antigen processing (TAP) TAP1:TAP2 heterodimers. This transporter is induced by IFN and exhibits a preference towards hydrophobic or basic C-termini peptides of 8-16 residues.

Once in the ER lumen, the peptide, mainly exhibiting N- terminal extension, is trimmed by the endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP), which has a specificity toward 9-16 amino acids peptides with hydrophobic C- termini.

The ready-to-be loaded peptide is then chaperoned from TAP to the peptide loading complex (PLC) by tapasin. The HLA-I α chain is stabilized in a partly folded configuration by calnexin and upon binding of β2m is dissociated to bind to the PLC via the chaperone calreticulin. The interplay between tapasin and the chaperone ERp57 leads to the loading of high-affinity peptides in the peptide binding groove.

Once the peptide is successfully loaded, the HLA-I:β2m complex is exported to the Golgi and the cell's surface. HLA-I heavy chains not stabilized by β2m and not loaded with a peptide are called open conformers (OC) and can also be exported to the cell surface but are not as stable and are quickly internalized.

In summary, classical HLA-I molecules are ubiquitously expressed molecules whose role is intracellular antigen presentation to CD8 T cells and that can be distinguished from non classical HLA-I molecules, which show different properties of peptide presentation.

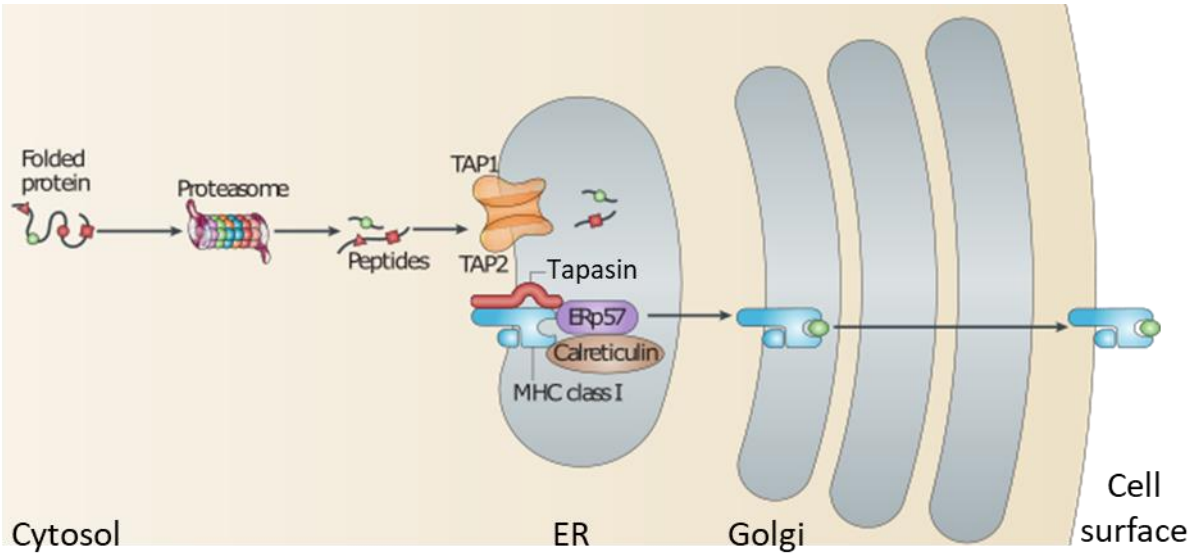


Figure 1 HLA-I presentation pathway. Depicted is the peptide generation, transport to the ER, loading to HLA-I and cell surface expression. Adapted from [7]

1.2 Non classical HLA-I molecules

Contrary to the classical HLA-I molecules described above, non classical HLA-I molecules, named HLA-E, -F, and -G, do not display a high level of polymorphism with only 522 alleles coding for 189 proteins (The Immuno Polymorphism Database, as of May 2023)[5], but are also capable of presenting peptides upon stabilization by β2m as described above. Peptide presentation by non-classical HLA-I molecules displays unusual properties given their lower polymorphism, structure, and their restricted peptide repertoire.

1.2.1 HLA-F

First described in 1990 by Geraghty *et al*.[9] and previously suspected of being an HLA class I pseudogene[10], the HLA-F locus comprises 59 alleles encoding for 11 proteins (The Immuno Polymorphism Database, as of May 2023)[5], which is the lowest allelic polymorphism among HLA-I molecules. *HLA-F*01:01* is the most frequent allele worldwide[11,12]. Interestingly, this unusual level of invariance for an MHC gene has been shown to be conserved in human and primate populations through comparative genetic analyses[13,14].

Unlike classical HLA-I molecules, the expression of HLA-F is not ubiquitous, but displays a tightly regulated, tissue and activation restricted expression.

Data from single-cell transcriptomics show a clear enrichment in lymphoid tissue as well as in various tissues including the liver [15]. However, no single-cell type proteomic data are available to confirm the transcriptomic data, which is important to note given the currently not well elucidated

regulation of HLA-F protein expression. Under healthy conditions, intracellular expression of HLA-F has been shown in resting lymphocytes, fetal liver, adult tonsils and thymus[16,17]. Surface expression on lymphocytes under healthy conditions is rare, but HLA-F surface expression has been linked to activation of T cells, B cells, natural killer (NK) cells, and was detected on Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell line (B-LCL), and extravillous trophoblasts (EVTs)[18–20].

HLA-F regulation is yet to be investigated in detail, but existing data show a large distinction to classical HLA-I molecules. At the transcriptional level, nuclear factor “kappa light chain enhancer” of activated B-cells (NFκB), interferon regulatory factor 1 (IRF1) and class II MHC transactivator (CIITA) are shown to upregulate HLA-F mRNA expression[21]. Surface expression of the HLA-F protein has been shown to be TAP and tapasin independent[17], and HLA-F protein is mainly ER trapped[16,22].

Interestingly, HLA-F can be detected at the cell surface in at least three different conformations with distinct features and binding capacity to NK-cell receptors (NKRs): HLA-F:β2m is peptide loaded and binds to the inhibitory leukocyte immunoglobulin-like receptor subfamily B (LILRB) receptors LILRB1 and LILRB2[11,22]. This structure has been published and in contrast to classical HLA-I displays an unanchored N terminal antigen binding groove created by a mutation (R62W). This mutation allows peptide presentation of longer length with 7 to 30 amino acids, peaking at 12 amino acids, compared to 8-10 amino acids for classical HLA-I molecules[11]. It has also been shown that this conformation can homodimerize at the cell surface of extravillous trophoblasts during pregnancy[20].

HLA-F can be stably expressed at the cell surface without binding to β2m or presenting peptides as so called “open conformer” (OC), with only the heavy chain of HLA-F. This conformation exists both de novo or from HLA-F:β2m conformer losing the interaction with β2m and their presented peptide, as shown during acid pulse experiments[23] and is potentially upregulated during cellular stress, infections, autoimmune diseases and cancer transformation[24]. This conformation binds with high affinity to the activating NKR killer cell immunoglobulin-like receptor (KIR) KIR3DS1[23,25] and with a lower affinity to the inhibitory receptors KIR3DL1/2[23,26] **(Fig2)**. However, its structure has not been solved yet.

The HLA-F heavy chain has also been described in a cross-presentation mechanism via heterodimerization with classical HLA-I heavy chain[27]. The HLA-I heavy chain is used to bind epitope-specific long polypeptides (50 amino acids) while HLA-F stabilizes the HLA-I heavy chain in this peptide-receptive conformation.

Finally, *HLA-F* has been associated with immune tolerance in pregnancy as a fecundability-associated gene in expression quantitative trait loci mapping study[28], as well as in a screening study revealing an interaction between HLA-F and V-domain Ig suppressor of T cell activation (VISTA), a B7 family immune checkpoint [29].

In summary, HLA-F is a tightly regulated molecule likely not involved in classical peptide presentation to CD8 T cells that binds to activating and inhibitory NK receptors depending on its conformation. Its role in eliciting or inhibiting innate immune responses is yet to be investigated in more detail, compared to the other non classical HLA-I molecules.

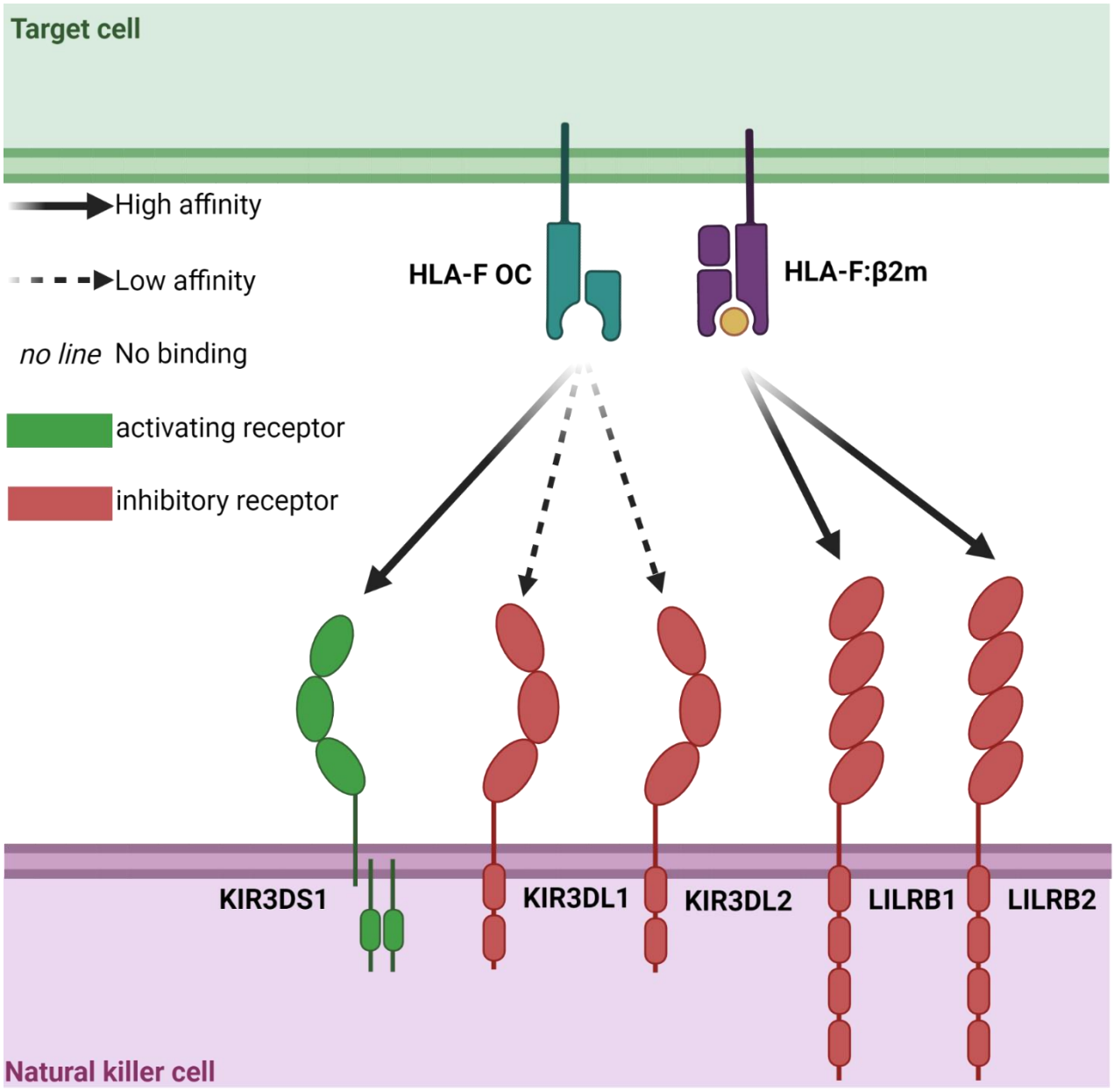


Figure 2 HLA-F conformers and their affinity to NK receptors. Depicted are the HLA-F conformers, the NK receptors binding to them and their affinity. *Created with BioRender.com*

1.2.2 HLA-E and -G

To date more studied than HLA-F, the structures of HLA-E and HLA-G and their presented peptides are much better elucidated[30–33].

HLA-E is ubiquitously expressed, but at a lower level than classical HLA-I and presents peptides derived from leader sequences of HLA-A/-B/-C/-G molecules as well as virus-derived peptides[34–37]. Displaying a low polymorphism with 346 alleles coding for 140 proteins (The Immuno Polymorphism Database, as of May 2023)[5], and two dominant alleles: *HLA-E*01:01* and *HLA-E*01:03*[38,39]. HLA-E binds to the natural killer group 2 member protein (NKG) inhibitory NKG2A:CD94 and activating NKG2C:CD94 receptor complexes, and is the only non classical HLA-I molecule to also present foreign antigen to and trigger CD8 T cells[40].

HLA-G is highly tissue restricted, with expression described in extravillous trophoblast cells, cornea, thymus, nail matrix and mesenchymal stem cells under healthy conditions[41–45]. Under inflammatory settings (infection, tumor microenvironment), HLA-G is expressed on dendritic cells (DC), monocytes and macrophages[46,47]. Displaying a low polymorphism with 117 alleles coding for 38 proteins (The Immuno Polymorphism Database, as of May 2023)[5], its peptide binding groove is highly conserved and most of the allelic variability occurs within the 3' untranslated region, affecting its posttranscriptional regulation[48,49]. Concomitantly, peptides presented by HLA-G are very restricted, deep buried within the peptide-binding groove and because no HLA-G specific CD8 T cells have been described yet, it is unlikely to have a role in classical peptide presentation[50,51]. HLA-G binds to the inhibitory KIR2DL4, and via a conformation of disulfide-linked homodimerized HLA-G:β2m complexes to inhibitory LILRs with a greater affinity than classical HLA-I molecules[52,53].

In summary, non classical HLA-I molecules have a distinct role compared to classical HLA-I molecules. Predominantly, they are not involved in classical antigen presentation (except HLA-E), have specific structural features, a tightly regulated tissue expression (HLA-F and -G), and bind mainly to inhibitory but also activating NK receptor depending on the presented antigen.

1.3 HLA-I-like molecules

HLA-I-like molecules include various MHC encoded (MICs) as well as non MHC-encoded (ULBPs, MR1, CD1, HFE, FcRn, ZAG, EPCR) proteins[54]. Of interest, MHC class I chain-related proteins (MICs) -A/-B, and UL-16 binding proteins (ULBPs) are ligands for the activating NK cell receptor NKG2D. Both families do not present peptides and are independent of β2m. Interestingly, MICs have an architecture of α1/α2/α3 domains, which is highly homolog to HLA-F OCs, while the ULBP

architecture is devoid of α3 domains. This protein family is called “stress ligands” as they are low/non expressed upon healthy conditions and upregulated upon cellular stress, infections, autoimmune conditions, transplantation and tumor transformation[55–59]. In contrast to the structurally close HLA-F, MICs are highly polymorphic proteins with MICA displaying 531 alleles coding for 280 proteins and MICB displaying 244 alleles coding for 47 proteins (The Immuno Polymorphism Database, as of May 2023)[5]. MICA polymorphism has been particularly associated with autoimmune disease, cancers and viral infections[60].

In summary, classical, non classical and HLA-I-like molecules have very distinct expression patterns and function in immunity including antigen presentation and activation or inhibition of cytotoxic immune cells.

1.4 Rationale

Despite being an HLA-I molecule, HLA-F is unlikely to have a primary role in peptide presentation. Prior studies have shown that HLA-F can be a strong activating trigger for highly cytotoxic NK cells when upregulated on virus-infected target cells *in vitro*[23].

HLA-F expression in tissue and the factors inducing its upregulation at the cell surface are to date unclear but are likely important given its potential to trigger strong cytotoxic immune responses against stressed tissues. Control of stressed lymphocytes by cytotoxic effectors such as NK cells is well documented in murine studies but has not yet been investigated in the context of HLA-F.

Viral infections are controlled by cytotoxic immune responses and have been associated with HLA-F surface expression. Given its interaction with cytotoxic cells, it is important to understand if the expression of HLA-F in the context of the inflammation triggered by the viral infection is protective (leading to a rapid control of immune inflammation and viral spread) or detrimental by inducing the excessive killing of activated lymphocytes.

Autoimmune diseases are multifactorial, trigger various immune pathways, including the cytotoxic immune response and have been associated with HLA-F expression. Tissue expression data of HLA-F in these pathologies are scarce, but association studies demonstrate a strong association with the cytotoxic activating receptor *KIR3DS1*. The HLA-F:KIR3DS1 axis is important to investigate given its potential to worsen acute tissue damage in autoimmune diseases targeting specific tissues. Nonetheless, studying HLA-F surface expression in human tissue remains challenging, which is why there is a need to further develop and assess suitable novel culture models of human tissue for these studies. Understanding the role of HLA-F in the context of chronic inflammatory pathologies – driven by both virus infection and autoimmune responses – is

important to gain knowledge on its triggers and study its potential impact on cytotoxic immunity and thus disease course.

2 Hypothesis and aims

The study of the non classical HLA-I molecule HLA-F in human diseases has been mainly restricted to *in vitro* models. The central aim of this study is thus to characterize the dual role of HLA-F in autoimmunity and virus infection in patients in specific disease settings. As disease models, we chose (i) human immunodeficiency virus (HIV)-1 infection given the previous studies showing HLA-F upregulation on HIV-1 infected cells *in vitro*, and (ii) autoimmune hepatitis (AIH) – an autoimmune disease targeting the liver - as prior work has shown an induction of HLA-F in liver tissue in the setting of viral hepatitis.

HIV-1 is a viral infection with major global health impact and achieving long-term immune control of HIV-1 infection is currently a strong focus of research efforts in advancing HIV-1 patient treatment. Autoimmune hepatitis (AIH) is lacking targeted treatment options apart from unspecific immunosuppression due to the missing understanding of underlying mechanisms driving disease. While *in vitro* data show HLA-F upregulation and activation of NK cells via KIR3DS1 in HIV-1, no data on HLA-F surface expression in HIV-1 infection *ex vivo* are reported. Likewise, HLA-F expression in autoimmune liver disease has not been studied to this date.

Usage of a therapy-naïve HIV-1 patient cohort and hepatocyte organoids derived from AIH patients allowed me to study HLA-F for the first time in the specific disease contexts of a viral infection and autoimmune liver diseases using patient-derived samples.

Based on prior studies and literature on HLA-F, two hypotheses were formulated for this thesis work:

1) HLA-F surface expression on primary CD4 T cells induced by the inflammation driven by HIV-1 infection results in recognition and killing by KIR3DS1+ NK cells.

2) HLA-F surface expression on human hepatocytes is linked to inflammatory stimuli associated with AIH disease.

Therefore, the aims of this thesis were:

1. To examine the expression of HLA-F, NKG2DL and their NK receptors in a cohort of therapy-naïve HIV-1 individuals.
2. To assess the role of HLA-F in the killing of activated, non-infected CD4 T cells by NK cells.

3. To comprehensively assess HLA-F surface expression in hepatocyte organoids derived from AIH patients.

Work on HIV-1 infection led to the co-authorship “Host KIR/HLA-C genotypes determine HIV-mediated changes of the NK cell repertoire and are associated with Vpu sequence variations impacting downmodulation of HLA-C”[61] and work on HLA-F autoimmunity in the liver led to the co-authorships “The co-inhibitory receptor TIGIT regulates NK cell function and is upregulated in human intrahepatic CD56bright NK cells”[62] and “GOAT: Deep learning-enhanced generalized organoid annotation tool”(submitted to bioRxiv)[63]. A manuscript resulting from the study of HLA-F in HIV-1 infection is currently in preparation.

Chapter 1: The role of HLA-F in HIV-1 infection

1 HLA-F in viral infection

Similar to the stress ligands MIC-A/-B and ULBPs, HLA-F mRNA and protein expression have been shown to be upregulated in viral infections, affecting various cell type, as described in **Table 1**. Notably, these publications used cell culture, *ex vivo* samples and organoid culture, and investigated widely different cell types. All published data including studies testing HLA-F OCs showed that HLA-F OC capable of binding to KIR3DS1 were upregulated during viral infection. The most probable upregulated conformer in viral infection is the OC over the HLA-F:β2m, which is more prone to downregulation by immunoevasins targeting the PLC. Antibodies distinguishing HLA-F conformers are currently missing in order to fully decipher the HLA-F conformation in viral infections.

Viral pathogen	HLA-F mRNA/protein expression	Cellular origin	KIR3DS1-Fc binding	Cell type	Reference
HIV-1	mRNA	Activated lymphocytes	Yes	activated CD4+ T cells	[23]
HCV	surface expression	Hepatic	Yes	Huh7.5, mouse hepatocytes	[64]
HadV5	surface expression	Intestinal epithelium	Yes	human intestinal organoids	[65]
JEV	mRNA /intracellular and surface expression	amniotic, brain microendothelium	not tested	AV-3, WISH, ECV, HBMEC	[66]
BKPyV	surface expression	Kidney tubules	Yes	RPTEC/TERT 1, kidney biopsies	[67]

Table 1 HLA-F expression in viral infections. HIV-1: human immunodeficiency virus 1, HCV: hepatitis C virus, HadV5: human adenovirus 5, JEV: Japanese encephalitis virus, BKPyV: BK polyoma virus, mRNA: messenger ribonucleic acid, KIR3DS1-Fc: Killer cell immunoglobulin-like receptor 3 domains short tail – fragment crystallizable, OC: open conformer.

Additionally, Kumar *et al*. showed in 2014 that JEV infection and tumor necrosis factor (TNF) TNFα stimulation induce HLA-F mRNA expression in an NFκB-dependent manner[68]. This was demonstrated by reversing HLA-F mRNA expression by blockage of NFκB using the inhibitor Bay 11-7085 and shRNA targeting of the p65 subunit.

It is also interesting to note that two association studies linked *HLA-F* polymorphism with disease outcome and viral load (**Table 2**).

Viral pathogen	HLA-F association	Observation	Reference
ZIKV DENV CHIKV	−1610 C (rs17875375), +1383 G (rs17178385), +3537 A (rs17875384)	Increased complications	[69]
HBV	HLA-F*01:03	Decreased level of HBV DNA	[70]

Table 2 Gene association studies of HLA-F in viral infections. ZIKV: zika virus, DENV: dengue virus, CHIKV: chikungunya virus, HBV: Hepatitis B virus, DNA: deoxyribonucleic acid.

In summary, HLA-F expression has been reported in various viral infections and shown to be linked with triggering the activating NK-cell receptor KIR3DS1. NK-cell mediated immune responses are critical in the control of viral infection and –as is increasingly reported- virus-associated inflammation. Thus, investigating the influence of HLA-F surface expression in the context of the immune response to viral infection is of high relevance.

2 The immune response to viral infection

The human immune system is a dynamic, interactive, and complex network of cellular and humoral processes that ensure the protection against transformed self (tumors) and non-self hazards (pathogens such as bacteria, viruses, parasites, and fungi). These processes are executed by two separated group of cells: (i) innate immune cells, broadly scattered in the human body, whose main function is immune surveillance, early and broad detection of hazards and triggering of a fast and broad response. The innate response is composed of inflammatory cytokines and complement proteins as well as cellular effects from several families of innate immune cells. This response induces the inflammation process that leads to the recruitment of more innate immune effectors and export of pathogen antigen by specialized innate cells to secondary lymphoid organs (SLO) to trigger the priming and activation of adaptive cells. Innate immune cells finally lead to the active recruitment of adaptive cells to the inflamed tissue[71]. (ii) Adaptive immunity is composed of specialized immune cells responding with a delay and capable of inducing a strong immune response in response to a specific antigen. The adaptive immune response is composed of antibodies, inflammatory cytokines as well as cellular effects from several families of adaptive immune cells.[72]

It is crucial to understand that the dichotomy innate/adaptive immunity is very porous, as both group of cells are interdependent to trigger and sustain an effective immune response to most pathogens. Increasing data show adaptive features in innate cells as well as innate features of adaptive cells[73,74]. Nonetheless, the designated cell type of innate immunity for controlling virus infection is the highly cytotoxic NK cell, which is activated within hours after virus infection.

2.1 Natural killer cells

As innate immune cells of the lymphocytic lineage, NK cells account for 5-15% of the circulating lymphocytes[75] and their crucial role in early detection of pathogens[76] and control of viral infections has been long established[77]. The term “natural killer” cells is derived from observation of direct cytotoxicity, although broader involvement in inflammation and homeostasis has been since then abundantly described, including description of adaptive features[78–81].

NK cells are defined by their expression of the neural cell adhesion molecule (NCAM, CD56) and lack of expression of the T cell co-receptor CD3 and can be divided in two subsets: The $CD56^{bright}$ $CD16^{dim/neg}$ and $CD56^{dim}$ $CD16^{pos}$ cells[75], exhibiting different function-related NK receptor distribution as well as cytotoxicity potential and inflammatory cytokine production[82]. Literature reports $CD56^{dim}$ $CD16^{pos}$ cells as mature NK cells (terminally differentiated upon expression of CD57) highly cytotoxic and expressing KIRs, while $CD56^{bright}$ $CD16^{dim/neg}$ are less abundant in peripheral blood, less mature, but high producers of IFNγ and TNFα upon triggering by cytokines[83,84].

NK cell mediated cytotoxicity (reviewed in [85]) can be triggered via three main mechanisms: (i)The triggering of death receptors Fas and death receptor (DR) DR4/DR5 on target cells by their respective death-receptor ligands on NK cells (FasL and TRAIL) [86]. A second mechanism is (ii) the antibody dependent cell cytotoxicity (ADCC) ,mediated by the FcγRIIIa receptor (CD16) upon triggering by an IgG1/3 Fc domain bound to the target cell[87,88]. (iii) The third mechanism is controlled through the balance of activating and inhibitory signals from NK receptors: NK group 2 (NKG2), Killer Ig-like receptors (KIR), Leukocyte Ig-like receptor (LILR), Natural cytotoxicity receptors (NCR), immune checkpoint families and DNAM-1. ADCC and activating signals lead to exocytosis of cytolytic granules at the immunological synapse containing perforin, granzyme (and granulysin in humans), leading to the expression of CD107a at the surface of the degranulating cell, which can thus be used as a marker for NK cell degranulation[89]. The effective translocation of granzyme and/or the triggering of death receptors lead to the initiation of caspase signaling in the target cell. This signaling ends with the activation of caspase-3, leading to apoptosis, thus detection of active caspase-3 is a marker to determine the apoptotic potential of live cells.

In summary, NK cells are innate immune cells implicated in control of viral infection via a multitude of mechanisms. The involvement of NK receptor responses in viral infections has been extensively studied given their high immune pressure on viruses, driving viral escape mechanisms targeting NK cell immune evasion.

2.2 NK receptors in viral infections

NK receptors are composed of an Ig-like or C-type lectin-like extracellular domain, a transmembrane domain promoting clustering and, in the case of activating receptors, an adapter protein mediating downstream signaling. Balance between activating and inhibitory receptors is crucial to regulate NK cell function. NKG2, KIR, NCR, LILRB, DNAM-1 and CD16 signaling is mediated by two mechanisms[85]: The activating signal is initiated via the phosphorylation of immunoreceptor tyrosine-based activation motives (ITAM) on the associated adaptor protein DAP12/CD3ζ/FcεRIγ or YINM tyrosin-based motive on DAP10. Inhibitory signal is mediated via the phosphorylation of immunoreceptor tyrosine-based inhibitory motives (ITIM) or KIEELE motif present on the intracellular domain on inhibitory receptor. ITAM phosphorylation leads to the binding of the SH2 domain of downstream effector molecules, YINM phosphorylation leads to the recruitment of phosphatidylinositol 3-kinase (PI3-K) while ITIM phosphorylation recruits the SHP-1 tyrosine phosphatase, which prevents cellular activation, showing that the downstream signaling acts like an ON/OFF switch with strong interactions between the activating and inhibitory signaling. It is important to note that the expression of receptors is stochastic within a given individual, leading to a diversity in the individual NK populations and responses[90,91]. All NK receptors can be categorized depending on their interaction with HLA-I molecules[92].

2.2.1 HLA-I interacting NK receptors

The KIR family is expressed in mature NK cells, as well as in some populations of CD4 and CD8 T cells as well as NKT cells. This family is comprised of two groups of receptors and nomenclature depends on the number of extracellular Ig-like domains, namely two domains (KIR2D) and three domains (KIR3D). Among both groups, activating and inhibitory KIRs exist: while activating KIRs have a short cytoplasmic tail (S) and are associated to DAP12 to mediate signaling via ITAMs, inhibitory KIRs have a long cytoplasmic tail (L) containing ITIMs. In general, KIR2Ds bind to HLA-C and KIR3Ds bind to the Bw4 motif on HLA-B (and some HLA-A allotypes), except for KIR2DS3 (unknown ligand), KIR2DL5 (binds to CD155) and KIR2DL4 (binds to HLA-G)[93–95]. Given their specificity for HLA-I molecules, inhibitory KIRs are critical for “missing self” recognition.

Despite binding to classical HLA-I molecules, KIRs also bind to non-classical HLA-I (KIR2DS4 with HLA-G; KIR3DS1, KIR3DL1 and KIR3DL2 to HLA-F OCs). A recent paper combining a CRISPR/Cas9 knockout strategy with KIR-Fc staining showed that tested receptors containing a D0 domain (KIR3DS1, KIR3DL1, KIR3DL2, KIR2DL4, and KIR2DL5) but not those lacking it (KIR2DL1, KIR2DL2, KIR2DL3, and KIR2DS4) bind to heparan sulfates, which are

glycosaminoglycans expressed at the surface of most cells[96,97]. KIRs display a peptide dependency, highlighting their role in the control of viral and bacterial infections[98–101].

Among this receptor family, KIR3DS1 and KIR3DL1 are of particular interest: encoded as two different alleles from the same locus and showing high homology in their extracellular domains. *KIR3DS1* was the first KIR associated with HIV-1 control in a study published by Mary Carrington's laboratory in 2002[102]. This study showed that donors possessing *KIR3DS1* and HLA-B harboring the Bw4 motif (HLA-Bw4) and an isoleucine at position 80 (I80) were associated with a slower progression to acquired immunodeficiency syndrome (AIDS). Because a KIR3DS1 ligand was not described at the time, and KIR3DL1 was shown to bind to HLA-I molecules harboring the Bw4 motif (either HLA-A or -B) with a modulated strength depending on polymorphism at the position 80[103,104], it was long assumed that KIR3DS1 will bind to the same ligands. Despite data showing only limited interactions between KIR3DS1 and Bw4[105,106], studies in HIV-1[107,108], HCV[109,110] and KSHV[111] revealed a better viral clearance upon possessing both molecules. Even more interesting*, KIR3DS1* expression without Bw4 was shown in the first study by Mary Carrington`s lab to be potentially associated with a faster progression to AIDS, although this did not reach significance. KIR3DS1 studies in HIV-1[112], HBV[113,114], human papillomavirus (HPV)[115], EBV-induced hemophagocytic lymphohistiocytosis (EBV-HLH)[116,117], BKPyV[118] and DENV[119] showed both viral susceptibility and clearance, but do not account for HLA-F surface expression, as the binding of KIR3DS1 to HLA-F was only discovered in 2016. Recent studies in HIV-1 and HCV showed that upregulation of surface expressed HLA-F leads to a polyfunctional response of KIR3DS1+ NK cells with cytokine production, degranulation and inhibition of viral replication, resulting in a high interest in studying HLA-F in viral infections as a functional "induced-self" mechanism[23,64]. Moreover, KIR3DL1: HLA-Bw4 association, which is inhibitory for NK cells as explained above, has been shown to be protective against HIV[107,108,120,121] while KIR3DL1 without HLA-Bw4 has been shown to increase susceptibility to DENV[119].

The NKG2 family is expressed in a large proportion of NK cells and binds to non classical HLA-I and HLA-I like molecules. It is comprised of the activating heterodimer CD94/NKG2C that binds to HLA-E, signals through DAP12[122], and is a marker for the highly differentiated subpopulation called "adaptive NK cells"; the activating homodimer NKG2D binding to the MHC-I like molecules MICA/B and ULBP, signals through DAP10 and the inhibitory heterodimer CD94/NKG2A binding to HLA-E[123], whose signaling prevails over that of NKG2C[124,125]. Interestingly, although HLA-E possesses low polymorphism compared to classical HLA-I, the two major *HLA-E* alleles present a different subset of peptides, and it has been shown that the binding of HLA-E to its NK receptors is sensitive to the presented peptide[126,127]. NKG2D, characterized by its ubiquitin-dependent

endocytosis upon engagement with its ligands, is a major activating receptor controlling NK cell regulatory function[128].

NK cells also express LILRB1 (CD85j), an inhibitory receptor that broadly recognizes HLA class I molecules and possesses a high affinity for the HLA-F:β2m conformer[22,129]. This receptor, CD94/NKG2A and the inhibitory KIRs are linked to the "missing-self" recognition of NK cells. The tolerance in NK responses is maintained by the balance between activation and inhibition. The downregulation/lack of HLA-I molecule surface expression observed in viral infections is a common immune evasion mechanism to avoid recognition by CD8 T cells. HLA-I downregulation induce a loss of inhibitory NK receptors signaling binding to HLA-I, leading to the killing of target cells in the presence of activating NK receptor ligands.

2.2.2 NK cell receptors not binding to HLA-I

While CD16 triggering is sufficient to induce NK activation on its own[130], critical co-activating signals are delivered by the receptors 2B4, DNAM-1, and the NCRs (NKp30, NKp44, NKp46)[130,131]. Immune checkpoint receptors (PD-1,CD96,LAG-3,TIM-3,TIGIT) are absent on resting NK cells and are upregulated upon stimulation of NK cells, and are a critical regulatory element driving peripheral tolerance while being targeted for immune escape in tumors and viral infections[132].

In summary, NK receptors are varied, mediating the "missing self" response mainly via inhibitory KIRs, capable of binding HLA-F OCs as well as the stress ligands MICA/B and ULBPs, and the ADCC response is capable to activate NK cells on its own. In addition, those receptors, although being expressed on innate cells, have been associated with adaptive features on NK cells.

2.3 Adaptive features of NK cells

Adaptive features of NK cells are of importance given their immunomodulatory function and their interplay with adaptive immune cells. Adaptive features for NK cells are listed in **Table 3**. First, and most importantly for this thesis is the reported control of activated T cells, mostly CD4 T cells, that has been described both in human and mice, in healthy conditions and pathogen infection, and is almost exclusively shown to be detrimental to T cells. Although IFNγ production as well as the co-stimulatory OX40 ligand expression by NK cells can promote T cell priming and differentiation[133,134], most studies showed that NK cells are capable of killing CD4 T cells. CD4 T cell killing by NK cells has been proven to affect activated but not resting CD4 T cells, and more specifically expanded effector as well as regulatory cells depending on the condition. Reports

elucidating the mechanisms involved in CD4 T cell killing are conflictual: while some studies show that NKG2D:NKG2DL is the main axis, others show that TNF-related apoptosis-inducing ligand (TRAIL) or a combination of activating receptors are the main triggers of the cytotoxicity towards CD4 T cells. While most murine studies showed CD4 T cell killing by NK cells during acute and chronic pathologies, human data showed that this killing mechanism can take place in healthy conditions upon stimulation. HLA-F upregulation in activated (and stressed) CD4 T cells, its binding to an activating NK receptor, and proof that NK cells are capable of killing CD4 T cells via HLA-F:KIR3DS1 have been already shown in the context of HIV-1 infected CD4 T cells[23].

The second interesting feature is the specific recognition of peptides by NK cells either directly or indirectly: NK cells can specifically recognize viral peptides as shown in mouse cytomegalovirus (MCMV) and human cytomegalovirus (HCMV), and have been shown to recruit and cluster but not trigger inhibitory KIRs upon presentation of certain epitopes by HLA-C. This feature is opposed to HLA-F recognition as only conformers without peptide are recognized by KIR3DS1[11,23].

Regarding the existence of distinct long-lived effector subpopulations, CD94/NKG2C+ NK cells are a highly differentiated population upregulated in CMV infection that is shown to provide memory-like responses in CMV but also after exposure to other pathogens. Additionally, CXCR6+ liver resident NK cells have been described as antigen-specific memory cells to multiple pathogens, proving that NK memory can be antigen specific.

Interestingly, surface expression of exhaustion markers upon chronic stimulation via activating markers has been described, which is similar to existing mechanisms in T cells.

NK adaptive features	Observation	Reference
Control of activated CD4 T cells	NKG2D/LFA-1/TRAIL mediated killing of autologous activated CD4 T cells	135
	Activated CD4 lysis by NK cells can be detrimental in LCMV infection	136
	TRAIL dependent killing of activated CD4 T cell during chronic MCMV	137
	Suppression of DC-primed antigen-activated autologous CD4 T cells, blocked by the rapid induction of HLA-E on CD4 T cells	138
	NKG2D mediated killing of activated antigen-specific CD4 T cells in chronic GvHD	139
	NKG2D/NKp46 mediated reduction of Treg expansion during tuberculosis infection, preferentially expanded but not freshly isolated cells	140
	NKG2DL are upregulated on expanded activated T cells, rendering them susceptible to NKG2D mediated killing	141
	Recently primed but not resting T cells are susceptible to NK killing	142
Specific recognition of peptide	HLA-C presented peptides can antagonize KIR2DL2/KIR2DL3 inhibition	143
	MCMV MHC-like protein m157 is recognized by Ly49H and confer host protection	144
	adaptive NKG2C+ NK differentially recognize HCMV strains encoding variable UL40 peptide	145
Memory-like adaptive NK cells	expansion of long-lived effector CD94/NKG2C+ NK cells during CMV infection	146–149
	CXCR6+ liver resident NK cells confer antigen specific memory of influenza, VSV, HIV-1 and haptens	150,151
Activating receptor induced checkpoint receptor expression	LAG-3 and PD-1 expression upon chronic stimulation via activating receptor engagement	152

Table 3 Adaptive features of NK cells. NK: natural killer cells, NKG2C/NKG2D: natural killer group 2 member C/D protein, LFA-1: lymphocyte function-associated antigen 1, TRAIL: tumor necrosis factor related apoptosis-inducing ligand, LCMV: lymphocytic choriomeningitis, MCMV: mouse cytomegalovirus, HCMV: human cytomegalovirus, DC: dendritic cells, GvHD: graft versus host disease, NKp46: natural killer protein 46 kilodalton, NKG2DL: natural killer group 2 member D protein ligands, CXCR6: C-X-C chemokine receptor type 6, VSV: vesicular stomatitis virus, HIV-1: human immunodeficiency virus 1, LAG-3: lymphocyte activation gene 3, PD-1: programmed cell death 1.

In summary, adaptive features of NK cells contribute to immune regulation by multiple mechanisms. Among them, the control of activated CD4 T cells as targets in HIV-1 infection is of relevance because this mechanism could lead to the killing of infected but also activated and uninfected CD4 T cells in chronic infection, influencing the progression to AIDS. This mechanism has never been studied in humans in the setting of viral infection and potentially involves the HLA-F:KIR3DS1 axis.

3 Human immunodeficiency virus (HIV) 1

3.1 HIV-1 infection and immune response

HIV-1 is a single stranded positive RNA retrovirus affecting around 38.4 million people worldwide and newly infecting 1.5 million people in 2021[153]. Cross-transmitted from the simian immunodeficiency virus throughout the 1900s[154,155], it is responsible for a chronic infection leading to acquired immune deficiency syndrome (AIDS). Although no curative treatment exists for HIV/AIDS, its life cycle and immunological implications have been extensively studied since it became a public health issue in the 1980s.

Using its envelope (env) glycoprotein, HIV-1 primarily targets CD4 expressing cells, e.g. CD4 T cells and monocyte/macrophages. It also requires a preferential co-receptor for entry, which is either C-C chemokine receptor 5 (CCR5), C-X-C chemokine receptor 4 (CXCR4) or both. CCR5 is expressed on activated T cells, monocyte/macrophages, and DC while CXCR4 is constitutively expressed on T cells. As expressed by their names, both receptors are chemokine receptors: CCR5 binds to C-C motif ligand (CCL) CCL3/4/5 and CXCR4 to C-X-C motif ligand 12 (CXCL12), which are of interest for therapeutic use[156]. As CCR5 is highly expressed in mucosal CD4 T cells, the sexually transmitted virus is almost exclusively CCR5-tropic[157,158], but can later in infection shift to CXCR4 tropism, although this mechanism is not fully understood yet[159]. The central role of CCR5 for HIV-1 is further demonstrated by individuals harboring the CCR5-Δ32 deletion, leading to a lack of CCR5 expression, who have been shown to be almost protected from HIV-1 infection[160,161]. The antiretroviral therapy (ART) drugs of the entry inhibitor family target CCR5 and HIV, but not CXCR4.

Once the virion is bound to its target cell, the viral membrane fuses with the target envelope, releasing the capsid and the pro-virus inside the cytoplasm of the cell. The single stranded RNA is then reverse transcribed into linear double stranded DNA using the infected-cell machinery. This process can be of target by two families of ART drugs: the nucleoside reverse transcriptase inhibitors (NRTI), that prevent DNA elongation, and the non-nucleoside reverse transcriptase inhibitors (NNRTI), that bind to the reverse transcriptase to inactivate it. Uncoating of the

nucleocapsid and nuclear entry of the newly transcribed DNA is then the next step of the life cycle of the virus, leading to integration inside the host genome. This step is targeted by the ART drugs of the integrase strand transfer inhibitors (INSTI) family. Finally, transcription and translation of the viral RNA and proteins happen, including that of the viral accessory proteins that help in immune evasion. One of those accessory protein is Nef, that induces the early loss of CD4 and HLA-I expression. Upon translation and maturation of viral proteins, the virion then assembles and buds from the host cell. This last step is targeted by the restriction factor tetherin (CD317, BST-2) that tethers virions to the cell surface. Tetherin expression is downregulated by the accessory protein Vpu[162]. Viral assembly and maturation are targeted by antiretroviral drugs of the protease inhibitor family, which target the viral protease to inhibit enzymatic cleavage of long viral polypeptides.

The dynamics of infection are tracked with two parameters in peripheral blood: HIV RNA plasma levels, and the CD4 T cell count. HIV-1 first infects mucosal tissue, then it spreads to lymph nodes, where it leads to systemic spread. This first phase, called eclipse phase, is characterized by the first infection of cells, the exponential decrease of CD4 T cell counts, the establishment of the innate immune response and the destruction of the gut associated lymphoid tissue (GALT). This phase ends with the induction of the adaptive immune response, and development of flu-like symptoms, called the acute phase. The early part of the acute phase shows a peak of HIV RNA (10^6-10^7 copies/ml) and the lowest CD4 count (called nadir). Nadir, together with the peak of HIV RNA are used as factors defining the immunological outcome of chronic infection[163]. Once the adaptive CD8 T cell response is triggered, HIV RNA decreases drastically to a level called the set point. The set point is correlated with clinical outcome[164]. Individuals who can control HIV-1 infection without treatment in the long term are called elite controllers and they represent less than one percent of HIV positive individuals. Functional studies have associated polyfunctional HIV-specific CD8+ T cells to the control of infection but most of the factors leading to elite control are still not understood [165,166]. In general, there is a high degree of inter-individual variability in the duration between HIV-1 infection and onset of AIDS symptoms. Large genetic association studies showed that certain *HLA-I* genes as well as the activating *KIR3DS1* (in conjunction with Bw4) are delaying the time between HIV-1 and progression to AIDS.

Among patients interrupting ART after several years of treatment, a clinical phenotype of “post-treatment controllers” who do not show a loss of viral control has been described but not linked to functional data yet, although they show low immune activation[167].

Following the set point, for the vast majority of individuals, HIV-1 establishes reservoirs via latent infection of long lived memory CD4 T cells and other long lived cells[168,169], leading to the chronic phase of infection. Without ART treatment, chronic inflammation takes place. This leads to

exhaustion of the immune system, which aggravate the CD4 T cells count loss overtime and leads to AIDS within 5-10 years on average.

The current main challenge for HIV-1 infection is the lack of treatment for eliminating the HIV reservoirs, as discontinuation of ART generally results in a rebound of viral load within a few weeks [170]. Establishing a vaccination strategy is complicated due to the high rate of variation of the virion, that is estimated at one mutation every few replication events[171]. This high mutation rate leads to 0.6-1% variation per year of the envelope sequence in a single individual, and to an average of 25% between individuals[172–174].

The immune response towards HIV-1 infection leads to a partial control of the viral load mediated by cytotoxic cells, including NK cells, that may be overwhelmed in chronic settings. Immunoregulatory functions of NK cells are potentially important in the disease setting to delay AIDS progression by ameliorating the inflammatory immune response.

3.2 NK cells in HIV-1 infection

Although exuberant CD8 responses and antibody production targeting the envelope protein were historically thought to be the main mechanisms in HIV-1 immune control, NK cells have been shown to bear important functions[175]. Some studies described that NK cells alone are sufficient to mediate viral inhibition when co-cultured with HIV-1 infected CD4 T cells both *in vitro* and *ex vivo*[176], and that HIV-1 peptide variants have been identified in individuals bearing specific KIRs, leading to NK inhibition and reduced elimination of infected cells [177].

HIV-1 infected cells upregulate stress signals and downregulate HLA-I molecules, rendering them sensitive to NK cell killing due to the loss of inhibitory signal (missing-self mechanism) and the triggering of activating receptors (induced-self mechanism). NK cells also mediate ADCC, and produce CCL3/-4/-5 leading to the blocking of CCR5, the viral entry receptor of CCR5 tropic HIV-1 strains[178].

HIV-1 accessory proteins affect NK cells ligands via pleiotropic effects: Nef downregulates ULBPs and MIC-A/B[179], Vpu and partially Nef downregulate PVR (ligand of DNAM-1), and NTB-A[180–182]. Vpr, another late expressed viral accessory protein, leads to the upregulation of the stress ligands MICA/-B and ULBP1-3, which in turn leads to the activation of NKG2D[183,184]. Furthermore, envelope gp41 protein leads to the upregulation of an unknown ligand for NKp44[185,186].

HLA-I classical molecules HLA-A and -B are downregulated by Nef[187], as well as HLA-C by Vpu variants from most primary HIV-1 isolates but not lab-adapted strains[188]. For non classical HLA-I molecules, it has been shown that HLA-E is downregulated by Nef in certain primary HIV-1 strains[189], and it is suggested that HLA-G1 is downregulated in monocyte/macrophages via Vpu[190],

although further studies are needed. It has also been shown that HLA-F is upregulated on CD4 T cells[23]. This generalized downregulation of HLA-I impairs the ability of CD8 T cells to mediate cytotoxicity but increases susceptibility of infected CD4 T cells to NK cell-mediated killing[191,192].

During HIV-1 infection, NK cell activation is mediated via missing-self mechanisms, but their ability to kill infected cells is dictated by the strength of the remaining inhibitory KIR/HLA interaction, as shown by the example of HLA-C with KIR2DL[193,194]. As high polymorphism exists both in HLA class I and KIRs, their combination leads to a high variability in binding affinities, explaining why a majority of risk/protective alleles are encompassing HLA-I molecules, as evident in the example of *KIR3DS1/HLA-Bw4*^{80I} described earlier.

Although NK cells are capable of activation and viral inhibition, there are defective at controlling the virus. Dysregulation of NK cell subset distribution is shown as early as in the acute phase of infection: increase in absolute number, expansion of $CD56^{dim}$ $CD16^{pos}$ and depletion of $CD56^{bright}$ $CD16^{neg}$ is noted[195]. Interestingly, Alter *et al.*[196] showed that early infection leads to the expansion of KIR3DS1+ and KIR3DL1+ NK cells. They also showed that early infection is linked to the general upregulation on mRNA level of activating KIRs while chronic infection is linked to the upregulation of inhibitory KIRs.

Chronic HIV-1 infection leads to the expansion of a dysfunctional CD56-CD16+ subset. Viral replication in peripheral blood does not lead to a decline of circulating NK cells but in a redistribution of the subsets[195,197–200]. The dysfunctional CD56- subset is shown to fail at producing inflammatory cytokines (IFNγ and TNFα)[195,197,198]. Their increased counts are associated with weak ADCC response[197,201] and the functional cytotoxicity correlates inversely with the level of HIV-1 plasma viremia[202].

Regarding NK receptor expression, downregulation of all NCRs[203] but high levels of inhibitory receptors have been reported in chronic HIV-1 infection[202,204]. The only exception is NKG2A, which is downregulated in chronic viremic settings, leading to an inversion of the NKG2A/NKG2C ratio, more pronounced in the dysfunctional CD56- subset[205]. NKG2D-mediated killing is affected by high expression of matrix metalloproteinase in chronic infection, which leads to the cleavage of NKG2DL from the cell surface and accumulation of their soluble form[206,207].

To escape the NK cell KIR/HLA immune pressure, HIV-1 infection leads to the selection of sequence polymorphisms enhancing the binding of inhibitory KIRs to infected cells, thus showing that KIRs are contributing to the viral evolution[177].

In summary, NK cells are triggered by missing self and induced self responses upon HIV-1 infection, but both the ligands and the NK receptors are targeted by immune evasion mechanisms, showing the importance of NK cell immune responses in controlling HIV-1 infection. The lack of

so far discovered immune evasive pathways targeting HLA-F expression and KIR3DS1 indicate that this activating axis, coupled with a potential immunoregulatory function of NK cells towards activated CD4 T cells, may be of potential therapeutic interest in HIV-1 infection.

Chapter 2: HLA-F and autoimmunity in the context of the liver

4 HLA-F in autoimmune diseases

TNFα can trigger inflammation, apoptosis or cellular proliferation depending on the signaling pathway triggered. The downstream signaling of TNF receptors, when inappropriately or excessively triggered, is crucial in autoimmune diseases, leading to chronic inflammation and mediating key pathological mechanisms as reviewed in [208]. HLA-F is capable of triggering NK cell activation and cytotoxicity via binding to KIR3DS1, and HLA-F expression has been linked to TNFα [66], therefore studying HLA-F as a disease-modifying factor in autoimmunity is of relevance. As listed in **Table 4**, HLA-F has been linked to autoimmune diseases via its upregulation at the cell surface, its polymorphism, and, more surprisingly, via the upregulation of autoantibodies directed against it. Data showing only mRNA upregulation need further investigation, given the tightly restricted surface expression of HLA-F. Interestingly, studies including analysis of patients during flares or symptoms, showed increased surface expression of HLA-F or suggested it as the upregulation of autoantibodies post-flare indicates an HLA-F surface expression during the flare leading to its targeting by autoreactive B cells. A role for both HLA-F and TNFα has been independently reported in these disease contexts: systemic lupus erythematous (SLE) flare onset is associated with increased TNFα serum levels[209,210], as well as for ankylosing spondylitis[211] ,ALS[212], rheumatoid arthritis[213] and type 1 diabetes[214]. Several diseases associated with HLA-F upregulation or polymorphisms are also linking *KIR3DS1* to an increased disease susceptibility (type 1 diabetes[215], ankylosing spondylitis[216–218] and rheumatoid arthritis[219]). This indicates that despite the lack of functional studies directly linking HLA-F expression and KIR3DS1 in these diseases, there may be an important role of NK cells in tissue-mediated damage via aberrant cell activation.

Autoimmune disease	Observation	Reference
SLE	anti-HLA-F IgG are detected after flare episodes	220
Ankylosing spondylitis	*HLA-F*01:01:02* is protective and the genotype *HLA-F*01:01:01/01:03:01* is linked with increased susceptibility	221
Rheumatoid arthritis	Upregulated mRNA expression in patient samples	222,223
	anti-HLA-F IgM/IgG are present in most patients and could show disease progression when associated with auto Ig anti-β2m	224
ALS	Upregulation of HLA-F protein expression in motor neuron despite general MHC I downregulation	225
Type 1 diabetes	upregulated mRNA and protein surface expression during onset	226,227

Table 4 HLA-F in autoimmune diseases. SLE: systemic lupus erythematous, ALS: amyotrophic lateral sclerosis, Ig: immunoglobulin.

5 Immunological context of the liver

The liver is an immune privileged organ whose main functions are metabolic activities, nutrient storage, and detoxification via hepatocytes. The liver is composed of several specialized immune cells: liver sinusoidal endothelial cells (LSECs) that separate hepatocytes from the blood stream by forming the space of Dissé; hepatic stellate cells (HSC) that are located in the space of Dissé; and Kupffer cells (KC) that are resident macrophages located in the sinusoidal lumen. Liver-associated dendritic cells (DC), NK cells and lymphocytes are also scattered across the organ, with NK cells being highly enriched in the liver.

The liver is perfused by the portal vein, receiving blood from the intestine, bringing alongside nutrients, highly inflammatory bacterial degradation products such as lipopolysaccharide (LPS), toxic components, and antigens from aged and damaged cells. To avoid chronic inflammation, hepatocyte killing, and fibrosis induced by this highly hostile environment, the liver microenvironment evolved tolerance mechanisms. The main mechanisms are mediated by the liver phagocytic cells (LSECs, KC and DCs) and include production of anti-inflammatory cytokines IL-10, IL-27, TGFβ, expression of the immune checkpoint PDL1 and death receptor ligand FasL.

This setting induces T cell hyporesponsiveness, upregulates generation of IL-10 producing regulatory T cells (Treg), skew the CD4 response towards Th2, and decrease production of the pro-inflammatory cytokines TNFα, IL-6 and reactive oxygen species (ROS) by macrophages (reviewed in [228]).

The high local concentration of IL-10 produced by Treg, Th2 and KC leads to immature phenotypes in all antigen presenting cells (APC) in the liver. This immature phenotype of APCs corresponds to a downregulation of HLA-II and co-stimulatory molecules CD80/86 and leads to T cells hyporesponsiveness.

It is interesting to note that immature DC drive CD8 T cell anergy and deletion, which have been shown to be overcome in mouse models by priming memory T cells with cross-presentation via LSECs[229]. HSC are also driven towards tolerance by expression of PDL1, TRAIL and IL-2 dependent Treg proliferation[228]. Finally, hepatocyte tolerance mechanisms are expression of PDL1, as well as ectopic expression of autoantigens in mice[230,231].

Although tolerance mechanisms are present, hepatocytes can be considered immunological agents. Upon stimulation by the pro-inflammatory cytokine IL-6, the expression of acute-phase proteins has been shown to be hepatoprotective in mice during hepatitis induced by bacterial endotoxins[232]. IL-6 production is mainly exogenous, but hepatocytes are capable of secreting this cytokine in response to LPS and hepatocyte growth factor (HGF)[233].

Of note, hepatocytes express Toll-like receptor (TLR)3 and 4 which drive NFκB activation[234,235].

Nonetheless, despite the highly evolved hepatic tolerance mechanisms, there are autoimmune diseases that specifically target the liver and ultimately result in complications leading to hepatocellular- or cholangiocellular carcinoma, need of liver transplantation and/or death when left untreated[236]. To date, it is still insufficiently understood what is causing the initial breach in tolerance and the ongoing immune reaction against the liver.

6 Autoimmune hepatitis

The classical autoimmune liver diseases (AILDs) are primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH). While PBC and PSC are targeting cholangiocytes, AIH is targeting hepatocytes. As AIH is the only AILD showing an increased susceptibility to TNFα, selective expansion of TNF producing T cells and successful anti-TNF treatment, AIH was chosen as a model to investigate surface expression of HLA-F [237–240].

AIH occurs in all ethnicities, affects predominantly women, both children and adult and has a worldwide incidence of 1.37 in 100000 and prevalence of 17.44 in 100000 persons[241]. Two subtypes of the disease exist[238]:

AIH-1 is the most studied subtype as it is also the most common (90% of the cases[242]). It is adult-predominant, with characteristic anti-nuclear (ANA) and anti-smooth muscle (SMA) autoantibodies, increased immunoglobulin G (IgG) as well as normal or slightly elevated alkaline phosphatase (ALP) level. This subtype is associated with mild to moderate severity but is nonetheless linked to extrahepatic autoimmune disorders in 20% of cases.

AIH-2 is paediatric predominant, with characteristic anti-liver kidney microsomal type 1 (LKM1), anti-liver cytosol type 1 (LC1) and anti-LKM3 autoantibodies as well as increased IgG and same biochemical features as AIH-1. The severity is higher than in AIH-1 with possible acute-onset liver failure. Interestingly, the autoantibodies associated with both subtypes are also associated with viral infections (HBV and HCV for ANA, SMA and HCV only for anti-LKM1)[238].

Genetic factors associated with AIH are HLA-II alleles, the Src homology 2-B adaptor protein 3 (*SH2B3*), the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), the cytotoxic T lymphocyte associated antigen 4 (*CTLA-4*), *TNF*, the transforming growth factor-β1 (*TGF-β1*), the signal transducer and activator of transcription 4 (*STAT4*) and epigenetic alterations[239,243–248].

Tolerance breakdown is essential to develop AILD[249]. Although the exact etiology is not known, several mechanisms leading to it have been proposed: molecular mimicry of autoantigens with viral pathogens, altered antigen presentation and dysregulated immune responses against autoantigens. Although the exact trigger has not been identified, these mechanisms lead to autoantigen presentation by APCs, unsuccessful control of auto-reactive T cells and production of autoantibodies.

It is still unclear if the defective response of Treg is leading to tolerance breakdown or if it is a result of the disease, but it is an important aspect of the disease[250]. Numerical, functional and loss of expansion of Treg in AIH have been described[251–253]. It has been reported that Treg have lower responsiveness toward IL-2, which results in a lower IL-10 production[254]. Treg count is also inversely correlated to the titer of some autoantibodies, indicating that this loss increases the severity of the disease[251].

Tolerance breakdown lead to the expression of co-stimulatory receptors and auto-antigens presentation by APC to naïve CD4 and CD8 T cells. CD4 T cells mature in different subpopulations capable of inducing liver damage upon cytokine stimulation: of note, Th1 produce IL-2, IFNγ and TNFα and Th17 produce IL-17/22 and TNFα. The IL-2 production activates CD8 T cell that will produce IFNγ, TNFα and express FasL. TNFα production induces apoptosis and IL-6 production by hepatocytes, which enhance Th17 activation and acute protein response[255]. In AIH, TNFα level are increased both in peripheral and liver environment and CD4 T cells producing TNFα are

preferentially expanded, underlining the importance of this cytokine in the physiopathology of the disease[240,256,257].

Current treatment for the induction of remission are immunosuppressive corticosteroids targeting T cells (prednisone or prednisolone) followed by a purine analogue (azathioprine) for maintenance of remission[258,259]. As current treatment is linked to secondary effects and does not reflect the need for individualized therapies, therapeutic advances are needed. Of note, monoclonal antibodies targeting TNF[260]and CD20[261] have already shown promising results in patients refractory to the first line treatment, strongly indicating a link to TNF in disease progression. In animal models, recombinant immune checkpoint CTLA-4[262] fused to immunoglobulin as well as pharmaceutical agents targeting CCR2/CCR5[263], TGFβ[264] and inhibiting NFκB pathway[265] have shown promising results in reducing liver inflammation and fibrosis. In summary, there is an urgent need in AIH to understand the inflammatory changes in hepatocytes as well as the immune responses directed against the liver to guide the development of new treatment approaches.

HLA-F surface expression has been shown in human hepatoma cell lines as well as humanized chimeric mouse liver and human liver biopsies by Lunemann *et al.*[64]. Independently, Kumar *et al*. showed that HLA-F surface expression is upregulated upon TNFα stimulation[66]. AIH immunopathology is linked to TNFα, which displays an increased level at peripheral and hepatic sites[256,257]. Given the tolerance breakdown in AIH, studying the hepatic expression of HLA-F, which is capable of binding to the NK activating receptor KIR3DS1 as shown by Lunemann *et al*[64] and putatively trigger acute liver damage, is relevant to investigate a potential detrimental effect of HLA-F in the context of autoimmune disease.

7 Liver organoids

The liver displays a high capacity for regeneration, that becomes evident in cases of partial hepatectomy (up to 2/3 of its size) or after injuries[266]. Two responses inducing the regeneration are described:

(i) a cell cycle entry of mature hepatocytes without apparent de-differentiation in response to acute liver damage[267]

(ii) a proliferation of activated cholangiocytes, the epithelial cells of the bile duct, acting as stem cells capable of regenerating hepatocytes and cholangiocytes[268]; The hepatocyte driven regeneration being the most prevalent form of regeneration *in vivo*, is the basis of the protocol used to generate hepatocyte organoids[269].

Designated method of the year 2017 in Nature Methods[270], organoids are organ-like self-organized 3D tissues that allow the study of patient samples and recapitulate the complex structure and function of *in vivo* tissue. These cells are derived from fetal or adult stem cells, healthy or diseased tissue, and are embedded in a basement membrane extracellular matrix (ECM) containing ECM components as well as growth factors[271].

Hepatocyte organoids from healthy samples were first established by Hans Clever's lab in 2018[272]. Mature hepatocyte reprogramming into proliferative bipotent progenitor cells is widely reported during chronic liver injury *in vivo*, but with this approach 3D culture was initially a fail[273,274]. The optimization of the culture medium by supplementing with Wnt/b-catenin activators, growth factors, antioxidant and other small molecules led to the growth of 3D structures displaying hepatocyte morphology. These organoids express hepatocyte markers Albumin, HFN4A and CYP3A4 while lacking cholangiocyte features such as the active transport of Rhodamine123. These 3D cultures, if derived from human fetal samples are capable of long-term culture (several months of passaging), while organoids derived from primary human hepatocytes showed a more limited culture time. Further protocols, such as the usage of induced pluripotent stem cells as a basis[275], establishment of CRISPR-Cas9 knockin/knockout system in hepatocyte organoids[276] and high resolution 3D imaging of full organoids[277] were published soon after, showing the wide range of options while using this model. This model hugely advanced the experimental approaches to study patient-derived primary hepatocytes to understand the inflammatory changes leading to a breakdown in tolerance in autoimmune hepatitis. Therefore, in this thesis, hepatocyte organoids derived from healthy and AIH patients were used to study HLA-F expression in steady conditions and upon pro-inflammatory stimulation.

Results
Chapter 1: The role of HLA-F in HIV-1 infection

1 Characterization of samples from therapy-naïve HIV positive individuals

To study the surface expression of HLA-F and its highly polymorphic receptors KIR3DS1, KIR3DL2 and KIR3DL1 during HIV-1 infection, multiparametric flow cytometry (FC) staining of PBMCs from therapy-naïve HIV positive individuals recruited via the translational platform (TP HIV) of the German Center for Infection Research (*n*=30) was performed and compared to HIV negative individuals recruited via the protocol "Immunologische Normwerte – Hamburger Gesundkohorte" (HCHH) (*n*=44). To link the multiparameter flow cytometry data to the underlying genotype, genotyping for *HLA class I* and *KIR* was performed in collaboration with the Körner lab by DKMS Life Science Lab. Genotyping results of the *KIR3DS1/L1* locus are summarized in **Table 5**.

Genotyping data revealed that the frequency of *KIR3DS1* expressing individuals was roughly identical (43 % vs 45 %), and the frequency of *KIR3DS1* homozygotes individuals was low in both cohorts (7 % vs 5 %). The frequency of individuals expressing the *Bw4-80Ile* allele and the protective combination of *KIR3DS1+/Bw4-80Ile+* alleles was lower in HIV positive individuals compared to controls (*Bw4-80Ile*:50 % vs 59 %) (*KIR3DS1+/Bw4-80Ile+*:17 % vs 25 %). The frequency of individuals expressing the risk combination *KIR3DS1+/Bw4-80Ile* [102] was 27 % in HIV positive individuals and 20 % in controls. These data are consistent with previous reports on larger cohorts[278,279].

KIR3DS1/L1	**TP-HIV cohort** number (%)	**HCHH cohort** number (%)
*KIR3DL1*hom	17/30 (57%)	24/44 (55%)
*KIR3DS1/L1*het	11/30 (37%)	18/44 (41%)
*KIR3DS1*hom	2/30 (7%)	2/44 (5%)
Bw4-80Ile	**TP-HIV cohort** number (%)	**HCHH cohort** number (%)
neg/neg	15/30 (50%)	18/44 (41%)
heterozygous	13/30 (43%)	26/44 (59%)
homozygous	2/30 (7%)	0/44 (0%)
Combination	**TP-HIV cohort** number (%)	**HCHH cohort** number (%)
none	7/30 (23%)	9/44 (20%)
KIR3DS1+Bw4-80Ile	5/30 (17%)	11/44 (25%)
KIR3DS1 only	8/30 (27%)	9/44 (20%)
Bw4-80Ile only	10/30 (33%)	15/44 (34%)

Table 5 *KIR3DS1/L1* and *Bw4-80Ile* genotyping of investigated donors.

1.1 Analysis of CD4 and CD8 T cells

1.1.1 Design and gating strategy of T cell ligand panel

The aim of the T cell panel was to comprehensively assess the expression of activating NK-cell receptors ligands on the CD4 and CD8 T cells of therapy-naïve HIV positive individuals (**Fig 3**). To this end, the well-described T-cell activation markers CD38 and HLA-DR were stained next to HLA-F and NKG2D ligands, as CD38 and HLA-DR both have been linked to disease progression and are well established markers of ongoing immune activation. The following gating strategy was used: After cleaning samples using the FlowAI plugin in FlowJo, and excluding doublets via SSC-A/SSC-H, the lymphocytes were gated using SSC-A/FSC-A, then selected for their expression of CD3 and lack of amine-reactive viability marker as well as a negative signal for the monocyte (CD14) and B-cell lineage markers (CD19). T cells were discriminated by their CD4 and CD8 expression. CD4 T cells subpopulations were investigated using Dunham *et al*[280] strategy: bulk CD4 T cells were gated by their expression of CD25 (IL-2Rα) and CD127 (IL-7Rα). $CD127^{low}$ $CD25^{high}$ subset was identified as Treg, and the CD127+/- CD25 low/neg subset as the non-Treg subset, termed here as conventional T cells.

To study the potential activation/inhibition via the NK cell receptors KIR3DS1/KIR3DL1/KIR3DL2, LILRB1 and NKG2D, comprehensive assessment of HLA-F and NKG2D-ligands was done by staining HLA-F (clone 3D11, first described by the Geraghty laboratory[50]) and combining the antibodies for MICA/B, ULB1, ULBP2/5/6 and ULBP3 (termed NKG2DL). HLA-DR/CD38 gating strategy was based on the publication of Kestens et al.[281]. Gating strategy for HLA-F was set on the unstained control from each donor. In summary, this gating strategy allowed us to characterize T cells for their activation and HLA-F/NKG2DL surface expression.

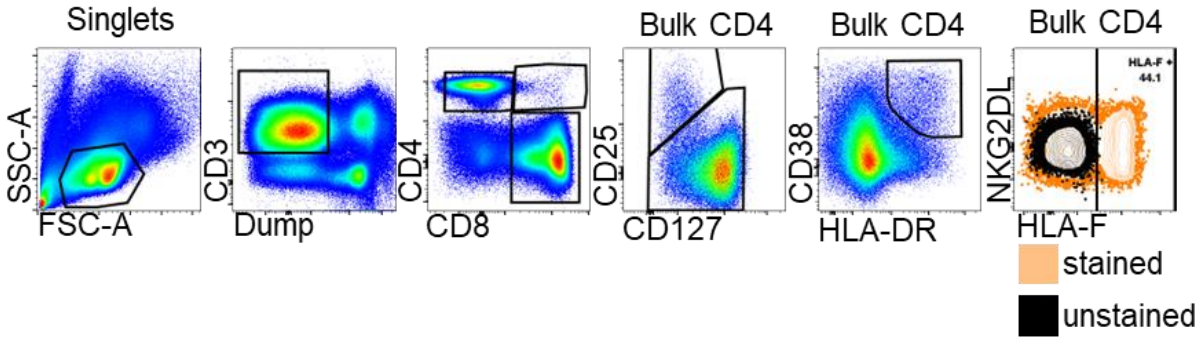


Figure 3 Gating strategy of the T cell-ligand panel. After cleaning samples using the FlowAI plugin in FLowJo and excluding doublets via SSC-A/SSC-H, the T cells were gated. Dump channel= viability marker+ CD14+CD19, orange=stained sample, black= unstained sample

1.1.2 Therapy-naïve HIV positive individuals display phenotypical features of HIV-1 infection

To investigate the impact of chronic untreated HIV-1 infection on the immune status, the CD4/CD8 ratio was assessed as a well-established clinical parameter of disease progression. The CD4/CD8 ratio was significantly reversed in HIV positive individuals (median: 0.85, interquartile range (IQR): 0.52-1.31) compared to HIV negative individuals (median: 1.99, IQR: 1.6-3.11, p< 0.0001) (**Fig 4, A**).

The relative proportion of CD4 and CD8 T cells double positive for the activation markers CD38 (early activation) and HLA-DR (late activation) were significantly higher in HIV positive (CD4 median: 2.27 %, IQR: 1.9 %-5.24 %) (CD8 median: 19.9 %, IQR: 13.6 %-31.2 %) compared to HIV negative individuals (CD4 median: 1.13 %, IQR: 0.81 %-1.54 %, p< 0.0001) (CD8 median: 3%, IQR: 2.13 %-5.57 %, p< 0.0001) (**Fig 4, B**). Interestingly, the difference of CD38+/HLA-DR+ CD8 T cells compared to CD4 T cells was 8.8-fold in HIV-positive individuals and 2.7-fold in HIV negative individuals. This result is in line with published literature on CD8 T cell activation due to ongoing virus-mediated activation of antiviral CD8 T cell responses[201].

The relative proportion of Treg remained stable in HIV positive (median: 3.66 %, IQR: 2.6 %-4.97 %) compared to HIV negative individuals (median: 3.24 %, IQR: 2.35 %-4.84 %, ns). (**Fig 4, C**). Additionally, the relative proportion of double positive CD4+/CD8+ T cells was similar in HIV positive (median: 0.46 %, IQR:0.25 %-0.97 %) and HIV negative individuals (median: 0.46 %, IQR:0.35 %-0.86 %, ns) (**Fig 4, D**).

In summary, our cohort showed the expected reversed CD4/CD8 ratio and an increased expression of activation markers on CD4 and CD8 T cells consistent with previous reports. However an increased proportion of double positive CD4+/CD8+ T cells and relative proportion of Treg among CD4 T cells as described by previous reports was not observed in our data set [282–286].

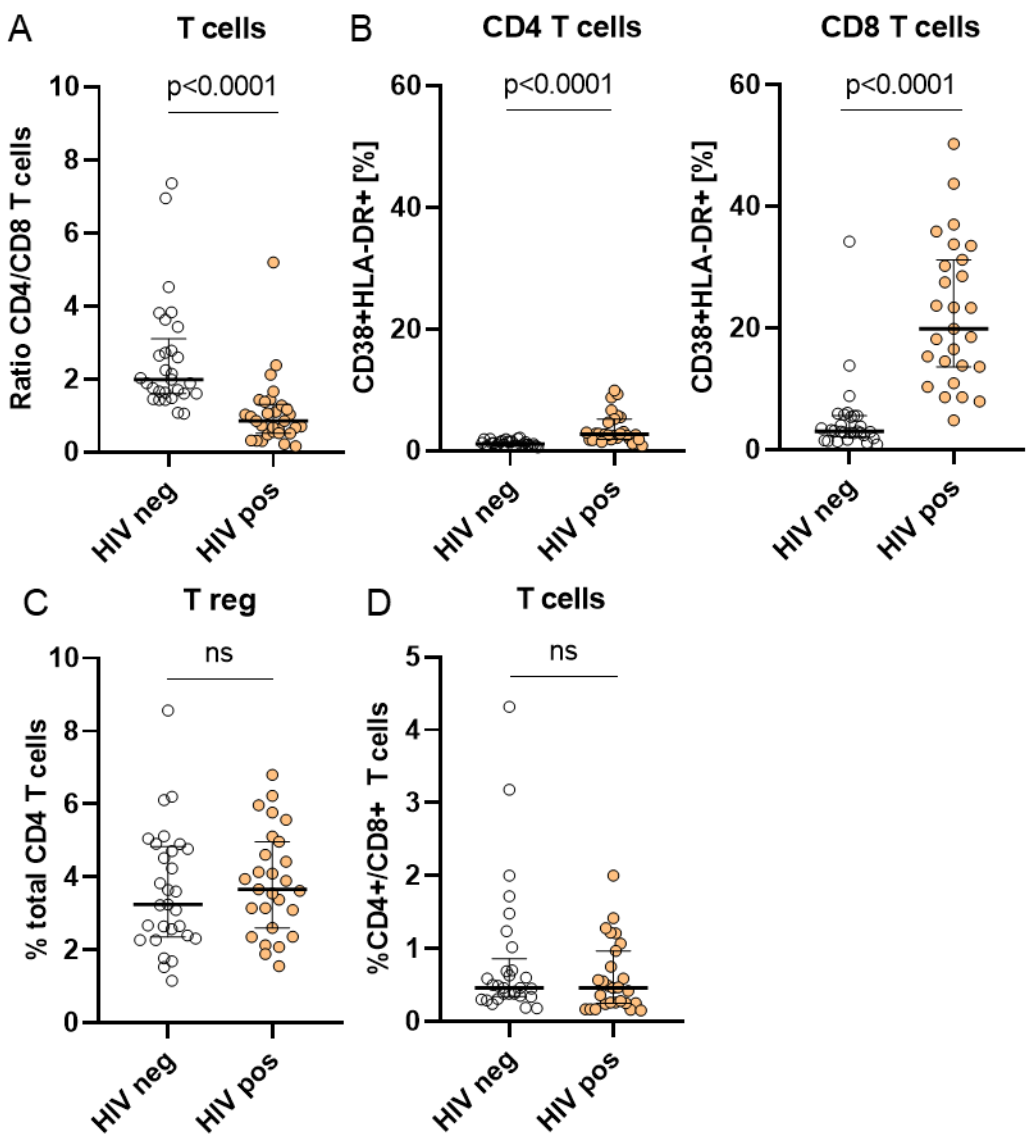


Figure 4 Therapy-naïve HIV positive individuals display phenotypical features of HIV-1 infection. Ratio CD4/CD8 T cells (A), percentage of CD4 (left) and CD8 (right) T cells expressing CD38+/HLA-DR+ (B), percentage of Treg among total CD4 T cells (C) and percentage of double positive CD4+/CD8+ among T cells (D). Statistical analyses were done using the Mann-Whitney test. Median±95%CI.

1.1.3 HLA-F and NKG2DL surface levels are upregulated on CD4 T cells in untreated HIV-1 infection

To investigate the potential engagement of activating and inhibitory NK receptors in chronic HIV-1 infection, HLA-F and NKG2DL surface expression were assessed.

As expected, HLA-F was barely detectable at the cell surface of CD4 T cells from HIV-1 negative individuals (**Fig 5, A**). Strikingly, chronic HIV-1 infection led to a substantial increase of HLA-F on CD4 T cells. The highest HLA-F surface expression on CD4 T cells was measured in HIV positive

individuals, with a 3.3-fold change in median fluorescence index (MdFI) compared to HIV negative individuals (MdFI: 1680 vs 516) (**Fig 5, A**).

Additionally, the percentage of CD4 T cells positive for surface expression of HLA-F was significantly increased in HIV positive individuals (median: 7.16 %, IQR: 3.1 %-15.3 %) compared to HIV negative individuals (median: 1.33%, IQR: 0.99%-1.9%, p<0.0001). The same observation was made for CD8 T cells (HIV positive median: 3.8 %, IQR: 2 %-6.53 %) (HIV negative median: 1.42 %, IQR: 1.01 %-2.11 %, *p*=0.0001) (**Fig 5, B**). Interestingly, the increase in HLA-F positive cells was higher on CD4 than CD8 T cells. This observation contrast with the higher increase of immune activation markers CD38 and HLA-DR on CD8 described earlier.

In line with this result, HLA-F MdFI on CD4 T cells was significantly increased in HIV positive (median: 579, IQR: 448-732) compared to HIV negative individuals (median: 305, IQR: 268-351, p<0.0001). The same observation –albeit to a lower extent– was made for CD8 T cells (HIV positive median: 532, IQR: 461-685) (HIV negative median: 343, IQR: 315-382, p<0.0001) (**Fig 5, C**). Of note, our panel was not designed to detect productive infection of CD4 T cells, estimated at 100 p24+ cells/10^6 CD4 T cells in therapy-naïve HIV positive individuals[287]. These results are in line with ongoing activation, possibly infection, and cellular stress of CD4 T cells during HIV-1 infection.

As Treg have been described to not upregulate HLA-F *in vitro* [18], we next investigated whether HLA-F surface expression could be detected on Treg of HIV-1positive individuals *ex vivo*. Indeed, the relative proportion of CD4 Treg expressing HLA-F was significantly higher in HIV positive (median: 5.86 %, IQR: 3.07 %- 8.77 %) compared to HIV negative individuals (median: 2.15 %, IQR: 1.57 %- 3.07 %, p< 0.0001) (**Fig 5, D**). HLA-F surface level on Treg of HIV positive individuals correlated with the expression on bulk CD4 T cells (r= 0.9095, p< 0.0001).

NKG2DL MdFI on CD4 T cells was slightly increased in HIV positive (median: 69.2, IQR: 35-102) compared to HIV negative individuals (median: 34.1, IQR: 23.2-65.2, *p*=0.0153) (**Fig5, E**). The same observation was made on CD8 T cells to a higher extent (HIV positive median: 117, IQR: 55.1-241) (HIV negative median: 52.5, IQR: 25.35-85.05, *p*=0.0104). It is worth noting that the MdFI observed are substantially lower compared to CD4 T cells stimulated with sodium propionate (NaP) to upregulate NKG2DL (median: 522.5, range: 337-708), as described by Nielsen *et al*[135].

In summary, our cohort showed an upregulation of HLA-F and NKG2DL on CD4 and CD8 T cells during chronic HIV-1 induced inflammation. Staining HLA-F in the larger number of individuals reported to date, our HIV negative control cohort showed no HLA-F detection and our HIV positive cohort revealed higher HLA-F surface levels on CD4 than CD8 T cells, contrasting with activation markers CD38 and HLA-DR, which were higher on CD8 T cells. These results support HLA-F surface expression as a marker of ongoing immune activation on CD4 T cells in HIV-1 infection.

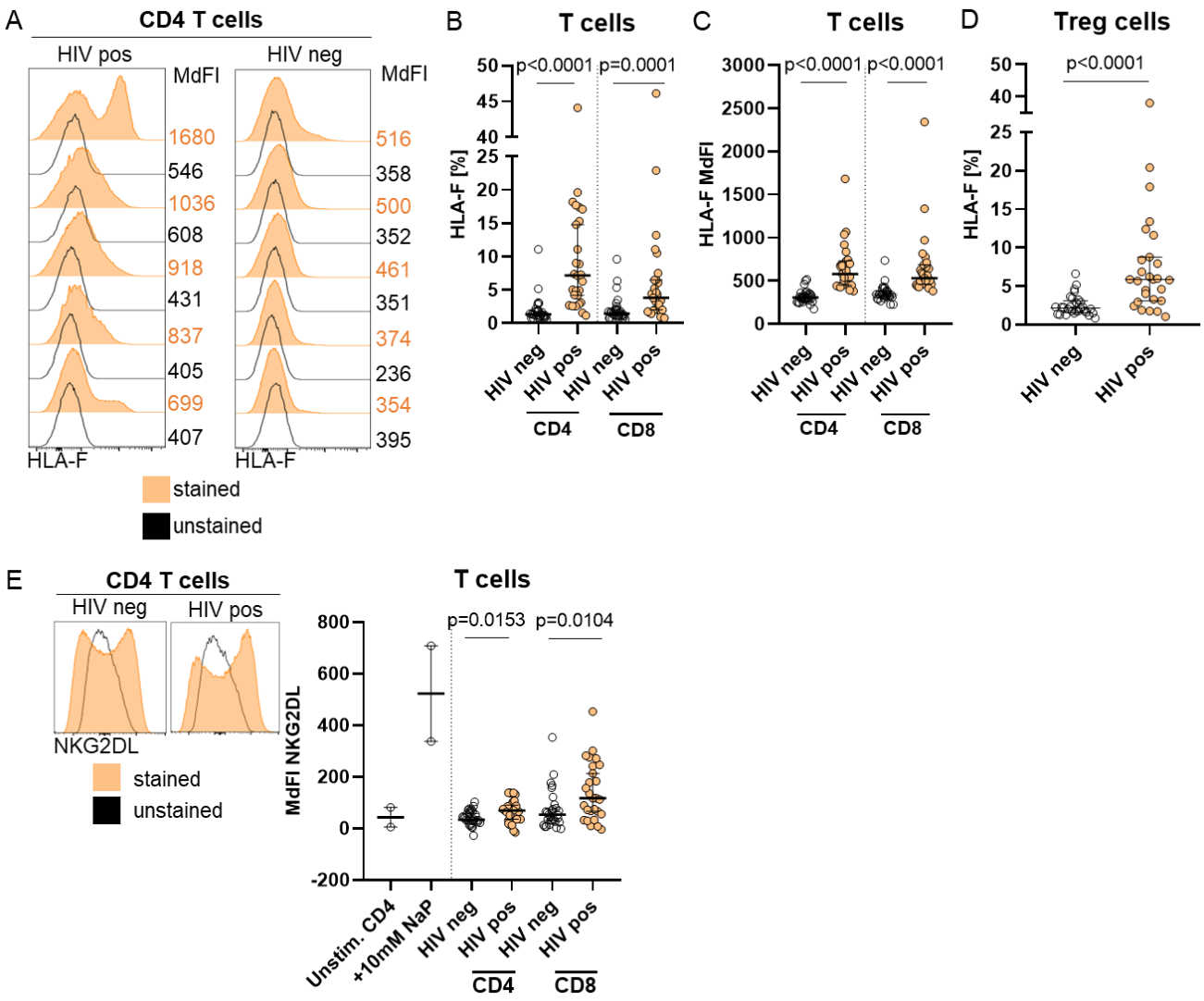


Figure 5 HLA-F and NKG2DL surface levels are upregulated on T cells in untreated HIV-1 infection. Histograms of HLA-F staining; the five donors with highest HLA-F MdFI from each cohort are displayed (A), percentage of CD4 and CD8 T cells expressing HLA-F (B), MdFI HLA-F on CD4 and CD8 T cells (C), percentage of Treg expressing HLA-F (D), histograms (left) and MdFI (right) NKG2DL on CD4 and CD8 T cells (E). Statistical analyses were done using the Mann-Whitney test. Median±95%CI

1.1.4 Autoantibodies against HLA-F but not against HLA-E are detected in HIV-1 positive individuals

To understand whether the increased surface expression of HLA-F in a setting of chronic HIV-1 induced inflammation is triggering an autoantibody response (as was described for autoimmune diseases such as SLE[220]), we established a protocol to assess the presence of anti-HLA-F IgM and IgG autoantibodies. HLA-E autoantibodies were used as a comparison given the ubiquitous expression of HLA-E.

To this end, we coated streptavidin coated beads with biotinylated HLA-F or HLA-E proteins and incubated them with plasma samples from HIV-positive (*n*=92) and HIV negative (*n*=50) individuals. Next, autoantibodies were detected via flow cytometry using anti-human IgG and anti-human IgM antibodies (**Fig 6, A**). To control for successful coating of the beads with HLA-F or HLA-E protein, we stained coated beads with commercial antibodies against HLA-F and -E. As control for coated and uncoated beads, we used biotin-coated and protein-coated beads stained for human IgG and IgM. To assess donor background staining, biotin coated beads were incubated with plasma from each donor (donor background) and stained for human IgG and IgM. The standard cut-off for autoantibody positivity was set at MdFI=500 for anti-HLA-F and anti-HLA-E IgG detection and MdFI=1000 for anti-HLA-F and anti-HLA-E IgM detection.

Anti-HLA-F IgG antibodies were detected in 11 out of 92 HIV positive and 3 out of 50 HIV negative individuals (**Fig6, B**) (Fisher's exact test, $p = 0.3788$). Anti-HLA-F IgM were neither detected in HIV positive nor HIV negative individuals.

Anti-HLA-E IgG antibodies were detected in 1 out of 92 HIV positive and not in HIV negative individuals (Fisher's exact test, $p > 0.99$). Anti-HLA-E IgM were not detected in HIV positive or HIV negative individuals.

In summary, our cohort showed an increased number of donors with detectable anti-HLA-F IgG compared to the control, however, the difference between the groups was not significant, likely due to differences in sample sizes. Anti-HLA-F IgM autoantibodies as well as anti-HLA-E IgM and IgG were not detected.

To sum up the analysis of CD4 and CD8 T cells, our flow cytometry panel focusing on the expression of NK receptors ligands on T cells of therapy-naïve HIV positive individuals revealed that our cohort displayed phenotypical features of HIV-1 infection. HLA-F and NKG2DL were upregulated on CD4 and CD8 T cells in comparison to HIV-1 negative individuals, where no HLA-F was detected. Anti-HLA-F IgG but not IgM were detected in therapy-naïve HIV positive individuals. Taken together, these results highlight the potential of HLA-F as a marker of CD4 T cell inflammation in HIV-1 infection.

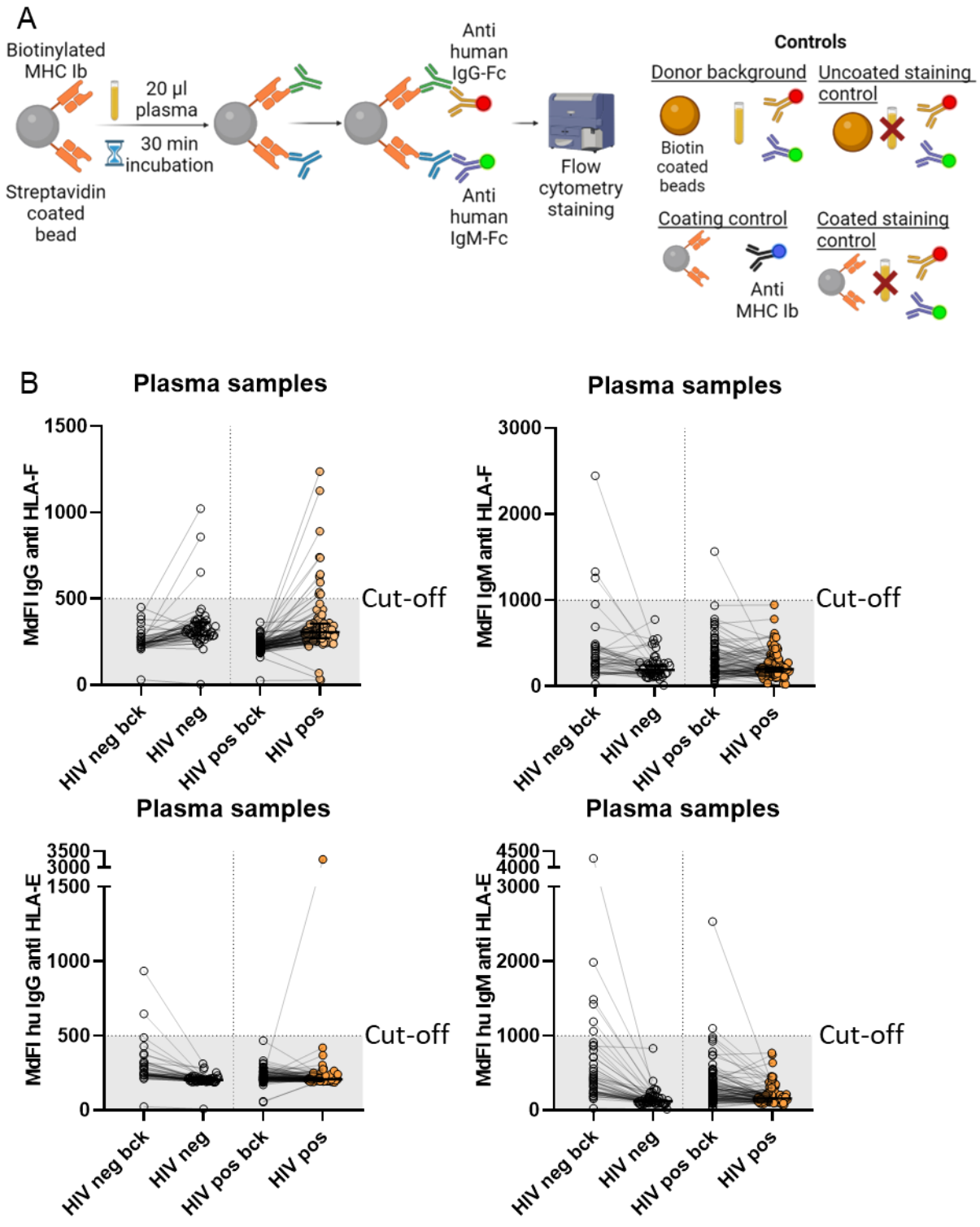


Figure 6 Autoantibodies against HLA-F but not against HLA-E are detected in HIV-1 positive individuals. Protocol of autoantibodies staining, *created with BioRender.com* (A), MdFI anti-HLA-F IgG (top left) and IgM (top right), anti-HLA-E IgG (bottom left) and IgM (bottom right), bck= donor background, grey line represent the standard cut-off for antibody positivity and was set at MdFI=500 for IgG detection and MdFI=1000 for IgM detection (B). Statistical analyses were done using the Fisher's exact test. Median±95%CI.

1.2 Analysis of NK cells

1.2.1 Design and gating strategy of the NK cell receptor panel

The aim of the NK-cell receptor panel was to comprehensively assess the expression of NK receptors binding to HLA-F and NKG2DL in therapy-naïve HIV positive individuals. To this end, KIR receptors KIR3DS1/KIR3DL1/KIR3DL2, NKG2D and LILRB1 were stained.

The following gating strategy was used: samples were cleaned using the FlowAI plugin in FlowJo, doublets were excluded, lymphocytes were gated using SSC-A/FSC-A, then discriminated by their lack of CD3 expression and lack of amine-reactive viability marker/CD14/CD19. NK cells were identified by their expression of CD56 and CD16 (**Fig 7, A**).

To study the potential binding of HLA-F and NKG2DL on CD4 T cells, comprehensive assessment of KIR3DS1/KIR3DL1/KIR3DL2, LILRB1 and NKG2D on bulk NK cells was performed by staining the designated markers. KIR3DS1 and KIR3DL1 gating in bulk NK cells was performed as described by Alter *et al.*, given the lack of commercially available antibodies specific to KIR3DS1 [176]: KIR3DS1L1+ KIR3DL1- cells were labeled as KIR3DS1+ subset and KIR3DS1L1+KIR3DL1+ were labeled as KIR3DL1+ subset (**Fig 7, B**). The discrimination between subsets was performed by analyzing donors with KIR3DS1^{hom}, KIR3DL1^{hom} and KIR3DS1L1^{het}. KIR3DL2, LILRB1 and NKG2D were gated on bulk NK cells using FMO controls.

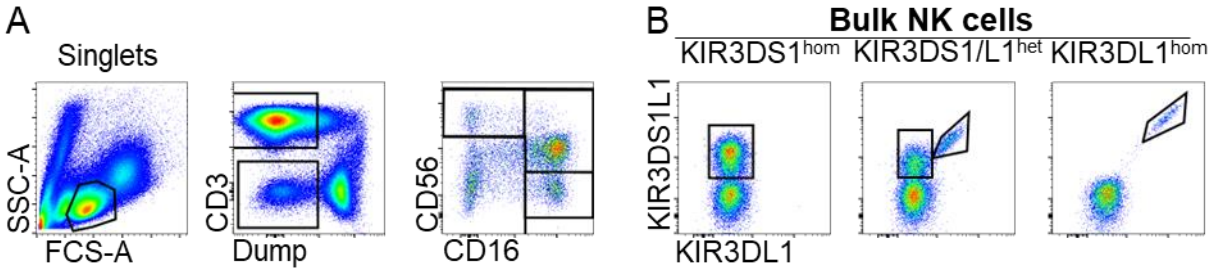


Figure 7 Gating strategy of the NK-cell receptor panel. After cleaning samples using the FlowAI plugin in FlowJo and excluding doublets via SSC-A/SSC-H, the NK cells were gated. Dump channel= viability marker+ CD14+CD19

1.2.2 NK subsets are dysbalanced during HIV-1 infection

To assess the impact of chronic untreated HIV-1 infection on NK cells, the distribution of the CD16-CD56^{bright}, CD16+CD56^{dim} and CD16+CD56- subsets was studied **(Fig 8, A**). The latter has been described to be functionally defective (poor cytotoxic function, reduced cytokine production) and highly expanded in HIV-1 infected individuals[197,198].

The relative proportion of CD16-CD56^{bright} NK cells was significantly decreased in HIV positive (median: 3.5 %, IQR: 2.38 %-6.09 %) compared to HIV negative individuals (median: 5.78 %, IQR: 3.95 %-10.14 %, p=0.0055). The relative proportion of CD16+CD56^{dim} was significantly decreased

in HIV positive (median: 48.3%, IQR: 36.3%-62.8%) compared to HIV negative individuals (median: 64.95%, IQR: 57.9%-74.7%, *p*=0.0007). This was likely caused by a significant increase in the relative proportion of CD16+CD56- in HIV positive (median: 8.06%, IQR: 4.21%-16.1%) compared to HIV negative individuals (median: 5.18%, IQR: 2.2%-7.32%, *p*=0.0143).

Thus, in line with previous findings, our cohort showed that NK subsets are dysbalanced with an increase in the percentage of the dysfunctional subset CD16+CD56- in HIV positive compared to HIV negative individuals.

1.2.3 NKG2D is upregulated in chronic untreated HIV-1 infection

To assess whether NKG2DL upregulation in HIV positive individuals may potentially activate NKG2D and to confirm reports showing decreased NKG2D expression in therapy naïve HIV positive individuals[206,207], NKG2D expression was studied given that NKG2D triggering leads to its internalization[128]. To our surprise, the NKG2D MdFI on NK cells was significantly higher in HIV positive (median: 367, IQR: 304-550) compared to HIV negative individuals (median: 304, IQR: 181-411, *p*=0.0294) (**Fig 8, B**). In summary, the decrease in NKG2D expression in therapy naïve HIV positive individuals described in previous reports was not observed in our cohort, which showed an increased NKG2D expression on NK cells.

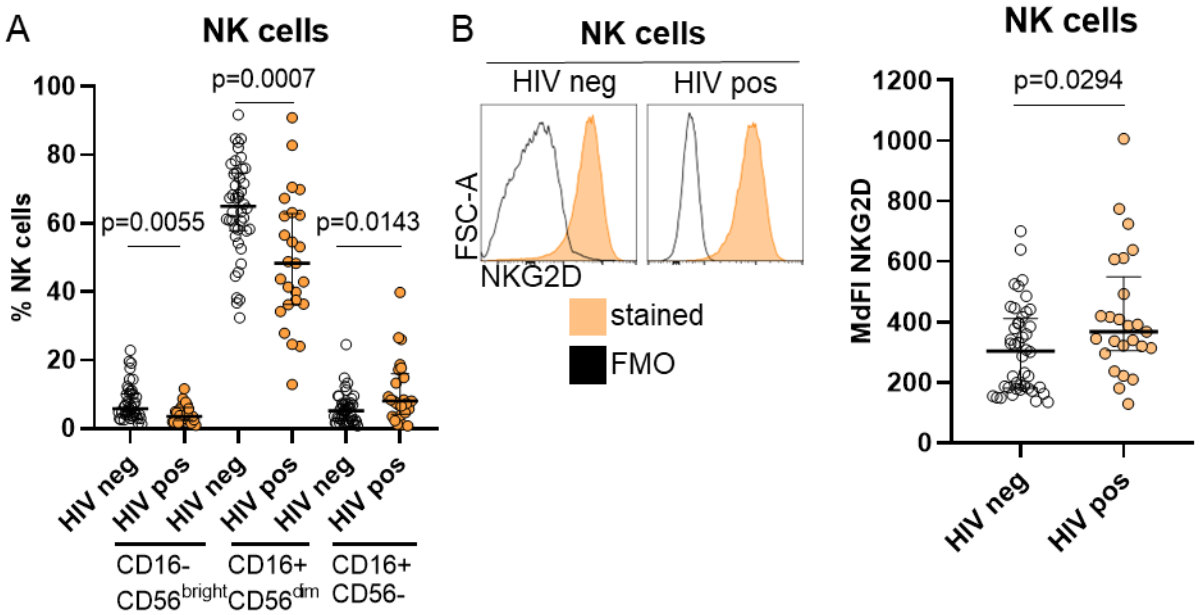


Figure 8 NK subsets are dysbalanced during HIV-1 infection. NK subsets distribution (A), NKG2D expression among NK cells, FMO= fluorescence minus one (B). Statistical analyses were done using the Mann-Whitney test. Median±95%CI.

1.2.4 KIR3DL2 and LILRB1 but not KIR3DS1 or KIR3DL1 are upregulated on NK cells in chronic untreated HIV-1 infection

To assess whether HLA-F upregulation in HIV positive individuals can potentially trigger the activating NK receptor KIR3DS1 and the inhibitory receptors KIR3DL1, KIR3DL2 and LILRB1, their surface expression on NK cells was assessed in therapy naïve HIV positive individuals.

Of note, we cannot draw definitive conclusions from KIR3DS1 data given the small proportion of donors expressing KIR3DS1 in both cohorts (HIV positive:13/30, 43%) (HIV negative:20/44, 45%) (**Fig 9, A**). However, higher frequencies of KIR3DS1+ NK cells were observed in HIV positive individuals expressing *KIR3DS1* (median:27.5 %, IQR:17.1 %-51.3 %), compared to HIV negative individuals expressing *KIR3DS1* (median:30.05 %, IQR:20.15 %-37 %, p=0.8771). Higher frequencies of KIR3DL1+ NK cells were observed in HIV negative individuals expressing KIR3DL1 (median:15 %, IQR:7.15 %-25 %), compared to HIV positive individuals expressing KIR3DL1 (median:12.95 %, IQR:6.4 %-17.93 %, p=0.2012).

Interestingly, the percentage of KIR3DL2+ NK cells was significantly higher in HIV positive (median: 24.2 %, IQR: 14.2 %-35.2 %) compared to HIV negative individuals (median: 14.4 %, IQR: 10.13 %-26.28 %, p=0.0262) (**Fig 9, B**). Given the gradual shifts in LILRB1 surface expression, we decided to use MdFI here as a read out (similar to NKG2D). LILRB1 MdFI on NK cells was significantly increased in HIV positive (median: 475, IQR: 396-587) compared to HIV negative individuals (median: 400, IQR: 343-440, p=0.0015) (**Fig 9, C**).

In summary, our cohort showed an upregulation of the inhibitory receptors KIR3DL2 and LILRB1, as well as a slight decrease and the inhibitory receptor KIR3DL1 on NK cells. Nevertheless, no conclusions can be drawn for the activating receptor KIR3DS1 given the low proportion of donor expression in both cohorts.

To sum up the analysis of NK cells, our flow cytometry panel focusing on the expression of NK receptors binding to HLA-F and NKG2DL in therapy-naïve HIV positive individuals revealed that NK subsets are dysbalanced with a decrease in the classical subsets and an increase of the dysfunctional subset CD16+CD56- compared to HIV-1 negative individuals.

Analysis of NK receptors revealed an upregulation of activating receptor NKG2D and inhibitory receptors KIR3DL2 and LILRB1. No definitive conclusions can be drawn for the activating receptor KIR3DS1 given the low proportion of donor expression in both cohorts, nonetheless, highest frequencies of KIR3DS1+ NK cells were detected in HIV-1 positive donors.

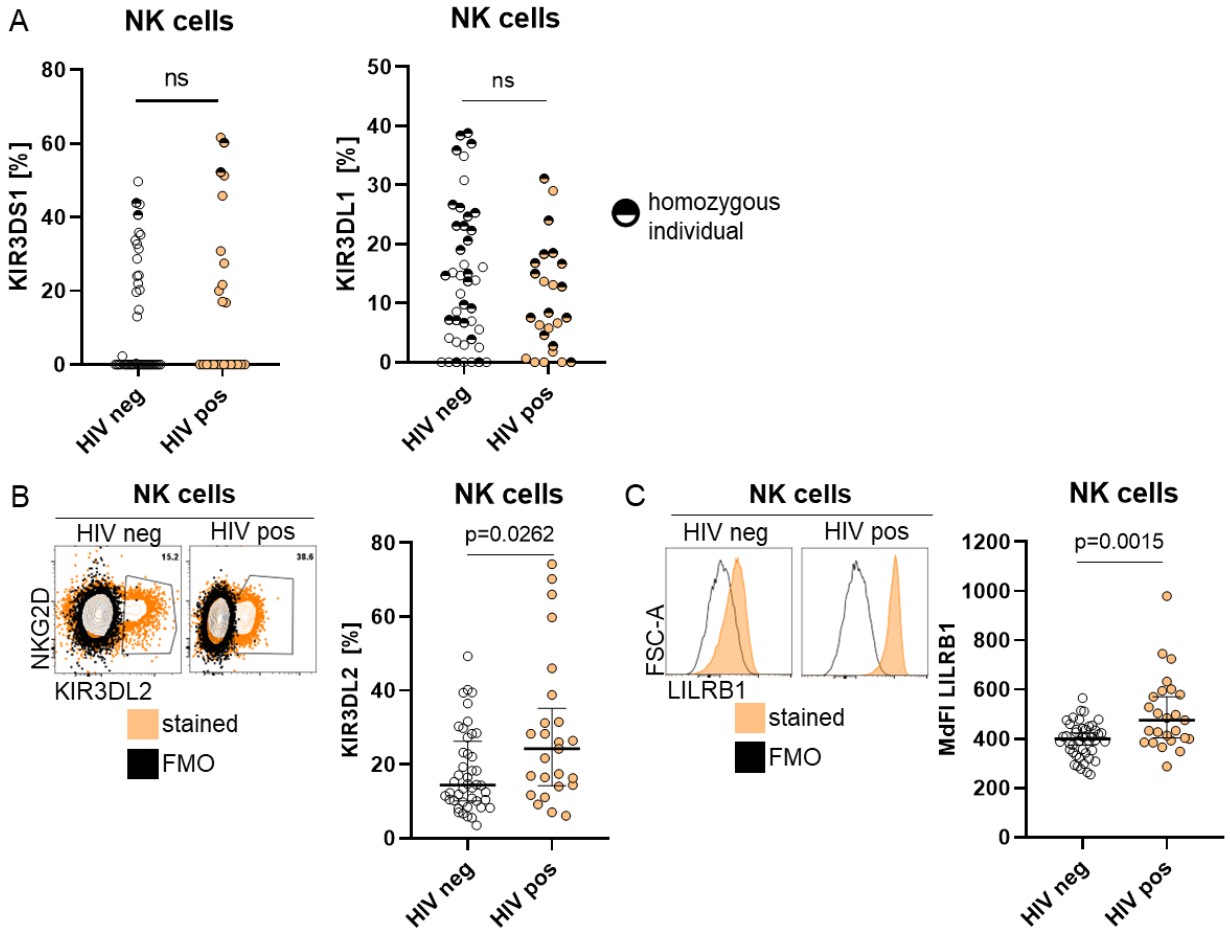


Figure 9 KIR3DL2 and LILRB1 are upregulated on NK cells in chronic untreated HIV-1 infection. Percentage of NK cells expressing KIR3DS1 (left) and KIR3DL1 (right) (A), KIR3DS1 homozygous and KIR3DL1 homozygous individuals are plotted as half-full circles in their respective graphs. histograms (left) and percentage (right) of NK cells expressing KIR3DL2 (B), histograms (left) and MdFI (right) LILRB1 expression on NK cells (C). Statistical analyses were done using the Mann-Whitney test. Median±95%CI.

2 Assessment of upregulation of HLA-F surface expression on stimulated CD4 T cells

Given the variable surface levels of HLA-F on CD4 T cells observed in therapy naïve HIV-1 positive individuals, we next investigated via *in vitro* stimulation the dynamic and donor variability of HLA-F upregulation on primary activated CD4 T cells from HIV-1 negative donors. To this end, PBMCs from the healthy control cohort Hamburg (*n*=22) were enriched for CD4 T cells and stimulated with CD2/CD3/CD28 tetramers and IL-2 (**Fig 10, A**).

2.1 HLA-F surface expression increases with longer stimulation time.

We first performed a time course experiment of isolated CD4 T cells (*n*=3) to optimize the time point for HLA-F staining following CD2/CD3/CD28 tetramer stimulation.

In line with published data, resting CD4 T cells do not express HLA-F at the cell surface, or express it at a very low level, while stimulation leads to upregulation at the cell surface[18].

A time course experiment of stimulated CD4 T cell showed that the highest HLA-F surface levels were reached after 7 days of stimulation (from 4.5-fold to 12.3-fold increase in MdFI compared to resting) (**Fig 10, B**).

All donors showed a similar pattern of expression, but maximum HLA-F surface levels differed substantially between donors (donor1 D7 MdFI: 526) (donor2 D7 MdFI: 977) (donor3 D7: 1563)

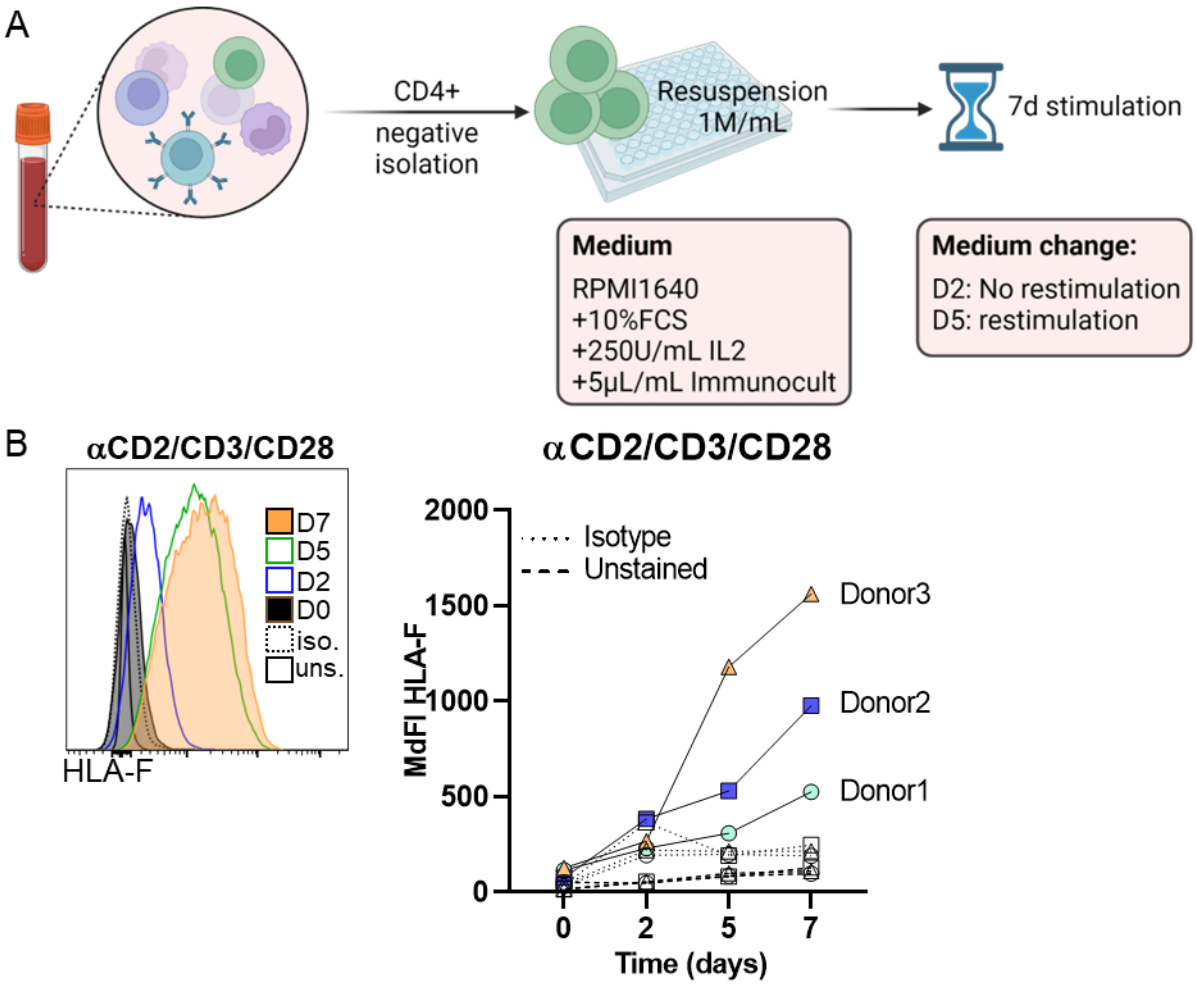


Figure 10 Assessment of upregulation of HLA-F surface expression on stimulated primary CD4 T cells. Protocol of CD4 stimulation, *created with BioRender.com* (A), time course expression of HLA-F on stimulated CD4 T cells, histogram (left) and MdFI (right), uns.=unstained, iso.= isotype (B).

Interestingly, the differences in HLA-F surface expression among donors became more apparent after five days of stimulation. Of note, re-stimulation with CD2/CD3/CD28 tetramers occurred on the second medium change (D5). This experiment set the protocol for upscaling of such stimulation to more donors to investigate the donor variability in HLA-F surface expression upon T cell activation.

2.2 HLA-F surface expression in stimulated CD4 T cells is not proliferation dependent

Given that other MHC-like stress ligands such as MIC-A and –B depend on cell cycle[141], we next wanted to investigate whether the same regulation pattern occurs for HLA-F surface expression.

To study proliferation dependence of HLA-F surface expression, cell trace violet (CTV), a proliferation staining binding to free amines on the surface and inside of cells was used. The signal is divided upon each cell division, displaying highly proliferated cells at low CTV MdFIs and low proliferated cells with a high CTV MdFI. After 7 day-stimulation of CD4 T cells, activation markers, to control for activation levels, and HLA-F were plotted against CTV (**Fig 11, A**). The variability in maximum HLA-F surface level between donors was not affected by the expression of activation markers.

Proliferation data of stimulated CD4 T cells from 21 healthy donors were compiled and analyzed for CD38, HLA-DR and HLA-F expression per CTV peak (peak1= highest possible CTV signal, peak8= lowest possible CTV signal) (**Fig 11, B**).

CD38 MdFI showed a 5.5-fold increase to unstimulated on the unproliferated CD4 T cell population (unstimulated median:164, IQR:92.5-194) (peak1 median:910, IQR:560-1192.5) and a 19-fold increase on the late CTV peaks (peak8 median:3120, IQR:1932.75-3912.5). These results are in line with the upregulation of CD38 expression of stimulated CD4 T cells[288].

HLA-DR MdFI showed a 3.68-fold increase to unstimulated on the unproliferated CD4 T cell population (unstimulated median:111, IQR:103-117) (peak1 median:409, IQR:375-459) and a 15.82-fold increase on the late CTV peaks (peak8 median:1756, IQR:1160.75-2017). These results are in line with late expression of HLA-DR on stimulated CD4 T cells[289].

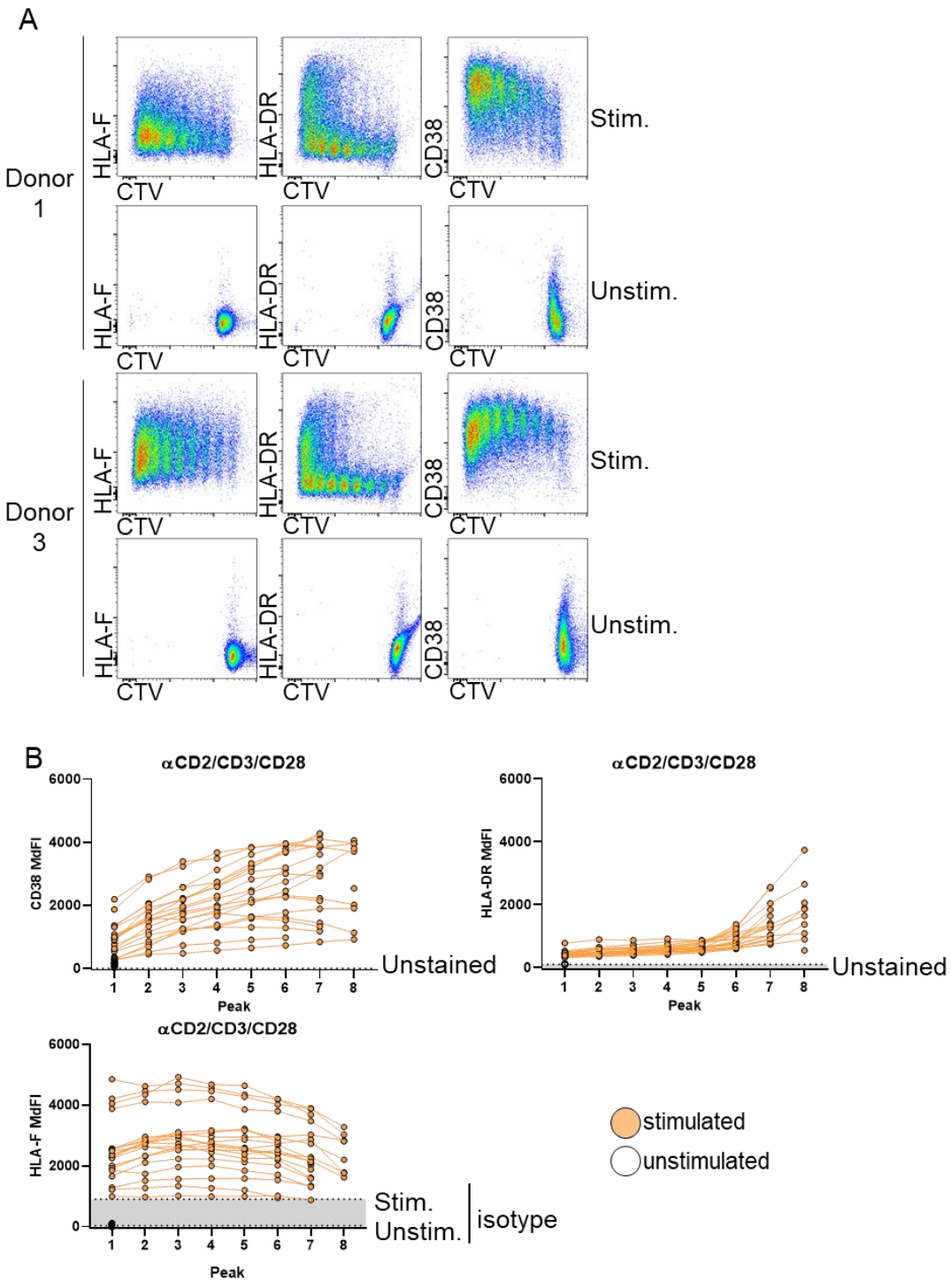


Figure 11 HLA-F surface expression on proliferating T cells. HLA-F/HLA-DR/CD38 expression against CTV of two donors (A), analysis of HLA-F (bottom left)/HLA-DR (top right)/CD38 (top left) expression from 21 donors (B). Stim.= mean isotype staining from stimulated samples, Unstim.= mean isotype staining from unstimulated samples

HLA-F MdFI showed a 50.97-fold increase to unstimulated on the unproliferated CD4 T cell population (unstimulated median:46.2, IQR: -3.85-83.5) (peak1 median:2355, IQR:1884.5-2558.5) and a 54.18-fold increase on the late CTV peaks (peak8 median:2503, IQR:1776-2881). In summary, our cohort showed that HLA-F upregulation during stimulation of CD4 T cells is upregulated independently of number of cell division.

2.3 HLA-F is heterogeneously expressed on stimulated CD4 T cells

To investigate whether HLA-F upregulation upon stimulation of CD4 T cells is a conserved feature between individuals, isolated CD4 T cells from 22 healthy donors were stimulated according to the setup details above. Stimulations using the same donor were replicated in a separate experiment to analyze the robustness of the HLA- F signal (**Fig 12**).

HLA-F MdFI was significantly upregulated in the first replicate upon stimulation (median:2877, IQR: 2288-3454) compared to unstimulated cells (median:62.9, IQR: 3.85-102, $p< 0.0001$), as well as in the second replicate (stimulated median:2660, IQR: 1720-3569) (unstimulated median:107, IQR: 80.3-136, $p< 0.0001$).

The relative proportion of CD4 T cells expressing CD38 was significantly upregulated in the first replicate upon stimulation (median:72.3 %, IQR: 58.7 %-85.5 %) compared to unstimulated cells (median:11.1 %, IQR: 8.77 %-14.7 %, $p<0.0001$), as well as in the second replicate (stimulated median:66.9 %, IQR :51.6% -71.3 %) (unstimulated median:9.48 %, IQR: 7.8 %-11.7 %, $p< 0.0001$). Likewise, the relative proportion of CD4 T cells expressing HLA-DR was significantly upregulated in the first replicate upon stimulation (median:30.9 %, IQR: 21.6 %-36.8 %) compared to unstimulated cells (median:4.39 %, IQR: 2.65 %-6.04 %, $p< 0.0001$), as well as in the second replicate (stimulated median:16.1 %, IQR: 12.9 %-20.5 %) (unstimulated median:4.06 %, IQR: 3.14 %-7.89 %, $p<0.0001$).

Interestingly, although substantial inter-donor variability was found for HLA-F as well as CD38 and HLA-DR expression, no correlation was detected between HLA-F surface expression and CD38 (r=-0.126, p=0.586) or HLA-DR (r=0.237, p=0.302). In summary, our cohort showed that all donors tested upregulate HLA-F on CD4 T cells upon stimulation, albeit to various degrees.

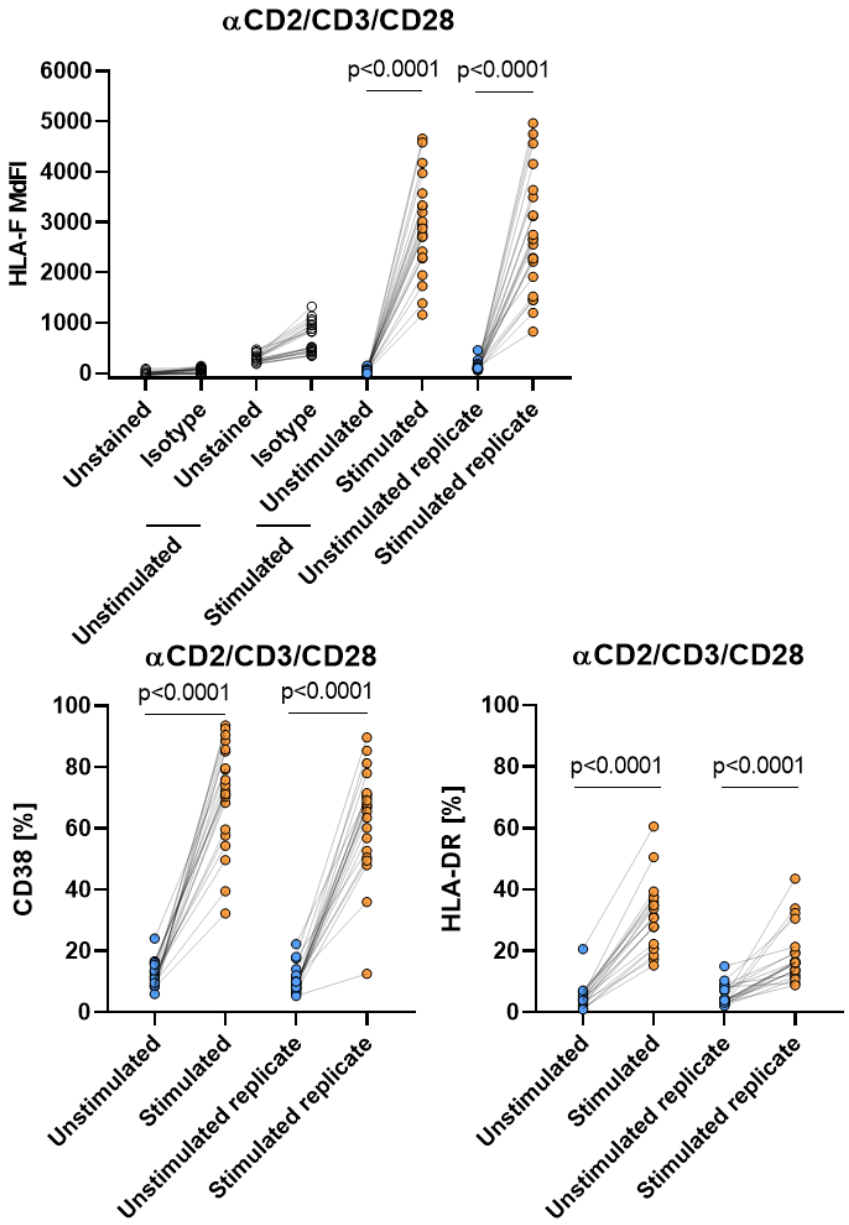


Figure 12 HLA-F is heterogeneously expressed on stimulated CD4 T cells. HLA-F (top), CD38 (bottom left) and HLA-DR (bottom right) expression from 22 donors. Statistical analyses were done using the Wilcoxon test. Median±95%CI.

2.4 HLA-F on stimulated T cell is present in a conformation allowing KIR3DS1-Fc binding

To investigate whether HLA-F upregulation on stimulated CD4 T cells induces surface expression of a conformation of HLA-F capable of binding to KIR3DS1, stimulated CD4 T cells from 17 healthy donors were stained with KIR3DS1 extracellular domain fused with the FC fragment of human IgG1 (KIR3DS1-Fc) and with a secondary antibody against human IgG. As a background control,

secondary antibody only was used (**Fig 13**). Control staining of stimulated CD4 T cells showed the expected HLA-F surface expression upregulation (median:1439, IQR: 1098-1841) compared to unstimulated CD4 T cells (median:8.98, IQR: -11.6-34, p<0.0001).

KIR3DS1-Fc binding was increased on stimulated CD4 T cells (median:9929, IQR: 8741-11089) compared to unstimulated CD4 T cells (median:2076, IQR: 1631-2338, p<0.0001). KIR3DS1-Fc binding to unstimulated CD4 T cells to a low level is in line with data published by Garcia-Beltran on binding of heparan sulfate to KIR3DS1[96]. Interestingly, the binding of KIR3DS1-Fc did not correlate with HLA-F surface expression (*r*=-0.222, *p*=0.405).

In summary, our cohort showed that stimulated CD4 T cells from all donors tested upregulate a conformation of HLA-F allowing for KIR3DS1-Fc binding.

Altogether, HLA-F surface expression on stimulated CD4 T cells was detected independent of cell division and already at early time points after stimulation. All tested donors upregulate HLA-F surface expression upon stimulation of CD4 T cells but to various degree. All donors express a conformation of HLA-F on stimulated CD4 T cells that is detected by KIR3DS1-Fc.

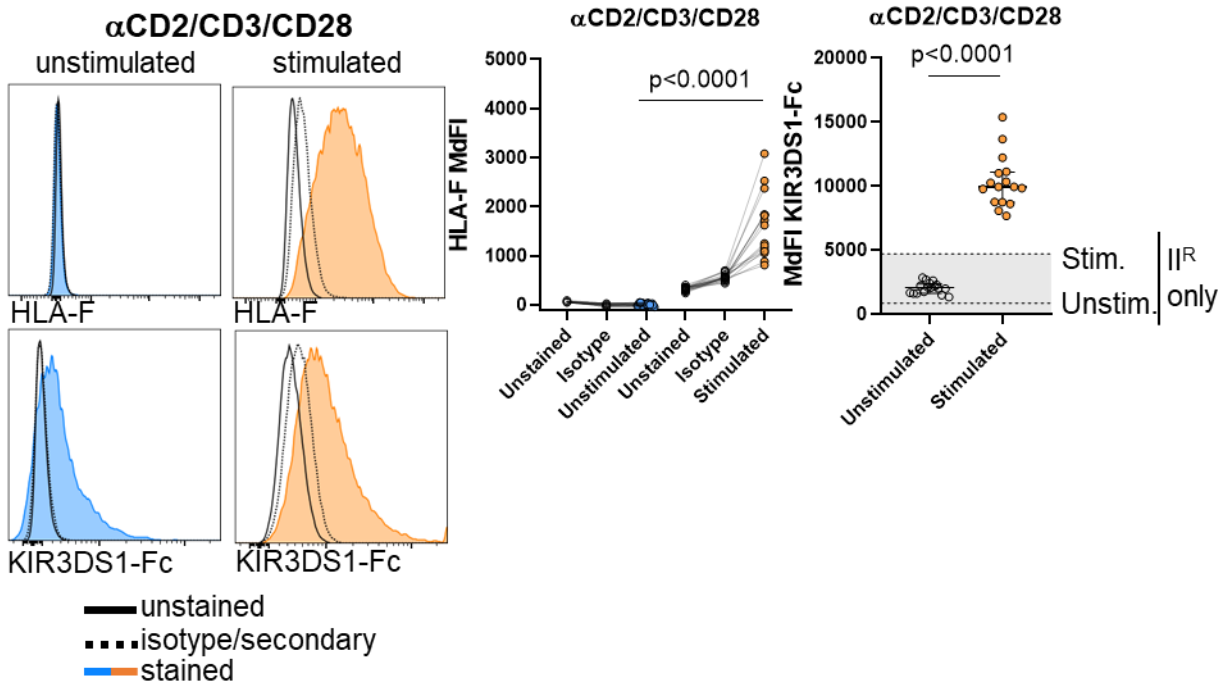


Figure 13 HLA-F on stimulated T cell is present in a conformation allowing KIR3DS1-Fc binding. HLA-F histograms (top left) and MdFI (middle), KIR3DS1-FC staining histograms (bottom left) and MdFI (top right) of stimulated CD4 T cells from 17 donors. Statistical analyses were done using the Wilcoxon test. Median±95%CI. IIR only= secondary antibody anti human Fc only, Stim= mean of secondary only staining of stimulated samples, Unstim= mean of secondary only staining of unstimulated samples.

3 Bystander killing of activated CD4 T cells by NK cells

To investigate the implications of HLA-F surface expression in the killing of uninfected but activated CD4 T cells in HIV-1 infection (referred to as bystander cells), co-culture experiments of activated CD4 T cells and NK cells were performed.

To this end, CD4 T cells were isolated and stimulated 7 days with CD2/CD3/CD28 tetramers and IL-2. NK cells were sorted for KIR3DS1+ and KIR3DS1- subsets using a KIR3DL1^{hom} donor to set the KIR3DS1+ gate and stimulated with NK medium (NKM_V1) containing IL-2 and human serum (**Fig 14, A**). Activated CD4 T cells and NK cells were co-cultured for 16 hours and stained to calculate target lysis and assess surface levels of markers of interest (**Fig 14, B**). K562 cells, a myelogenous leukemia cell line devoid of HLA-I and highly sensitive to NK killing, were used as control target.

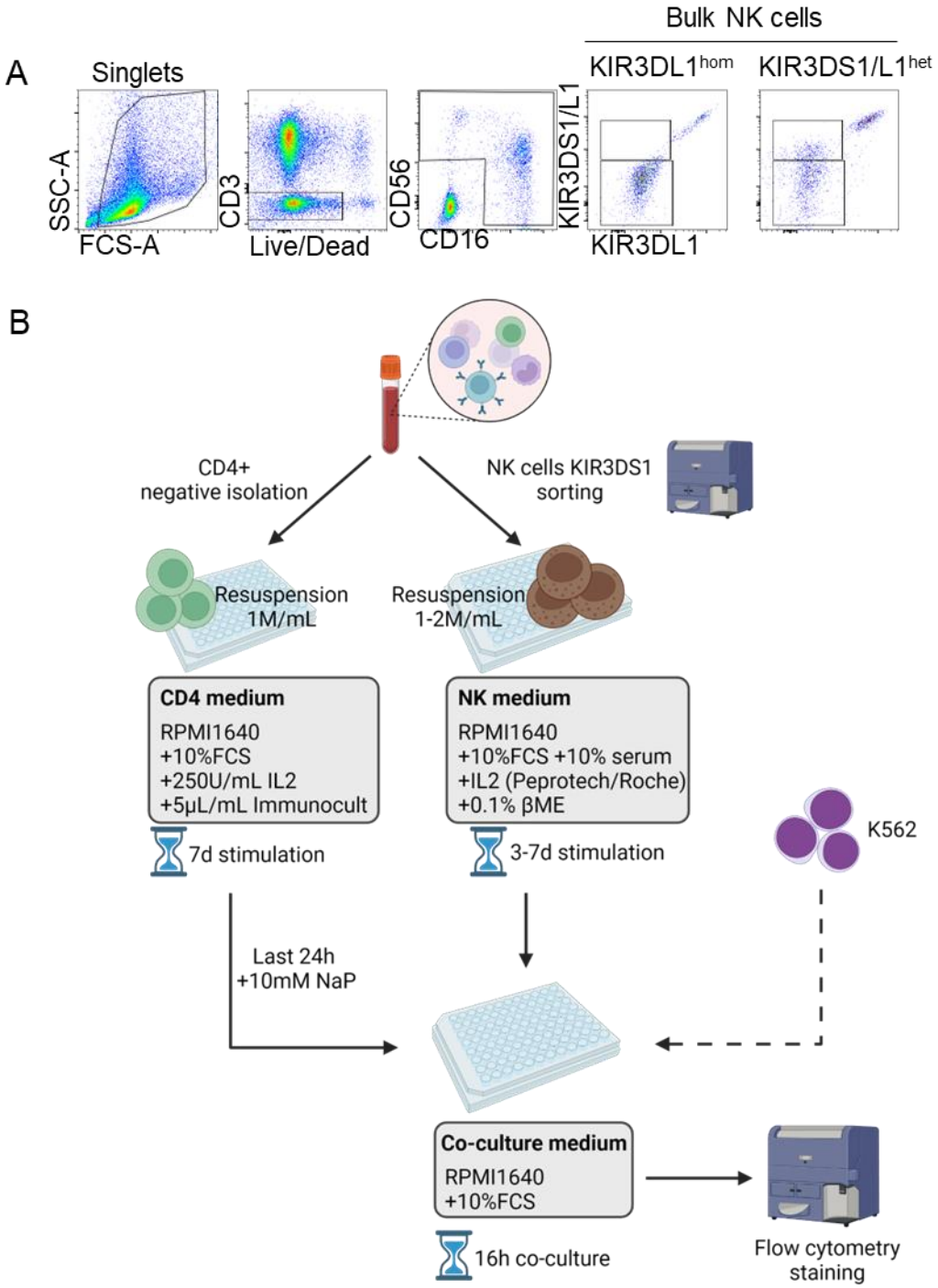


Figure 14 Gating strategy for sorting and protocol of bystander killing assay. Samples were cleaned using the FlowAI plugin in FlowJo and excluding doublets via SSC-A/SSC-H. Cells were then discriminated by their lack of CD3 expression and lack of amine-reactive viability marker. NK cells were identified by their expression of CD56 and CD16. The KIR3DS1L1+/KIR3DL1- gate was set by staining NK cells from a KIR3DL1 homozygous donor. KIR3DS1L1+/KIR3DL1- NK cells were sorted as KIR3DS1+ cells and KIR3DS1L1-/KIR3DL1- NK cells were sorted as KIR3DS1- cells (A), protocol of bystander killing assay, *created with BioRender.com* (B).

3.1 Sorted NK cells retain phenotypic features after stimulation

To assess if KIR3DS1 sorted NK cells retain the same phenotypical features as isolated NK cells after culture, sorted KIR3DS1+ and KIR3DS1- NK cells from a KIR3DS1^{hom} and a KIR3DS1L1^{het} donors were cultured and compared to isolated bulk NK cells from the same donors to assess their phenotypic features after 3 days of stimulation.

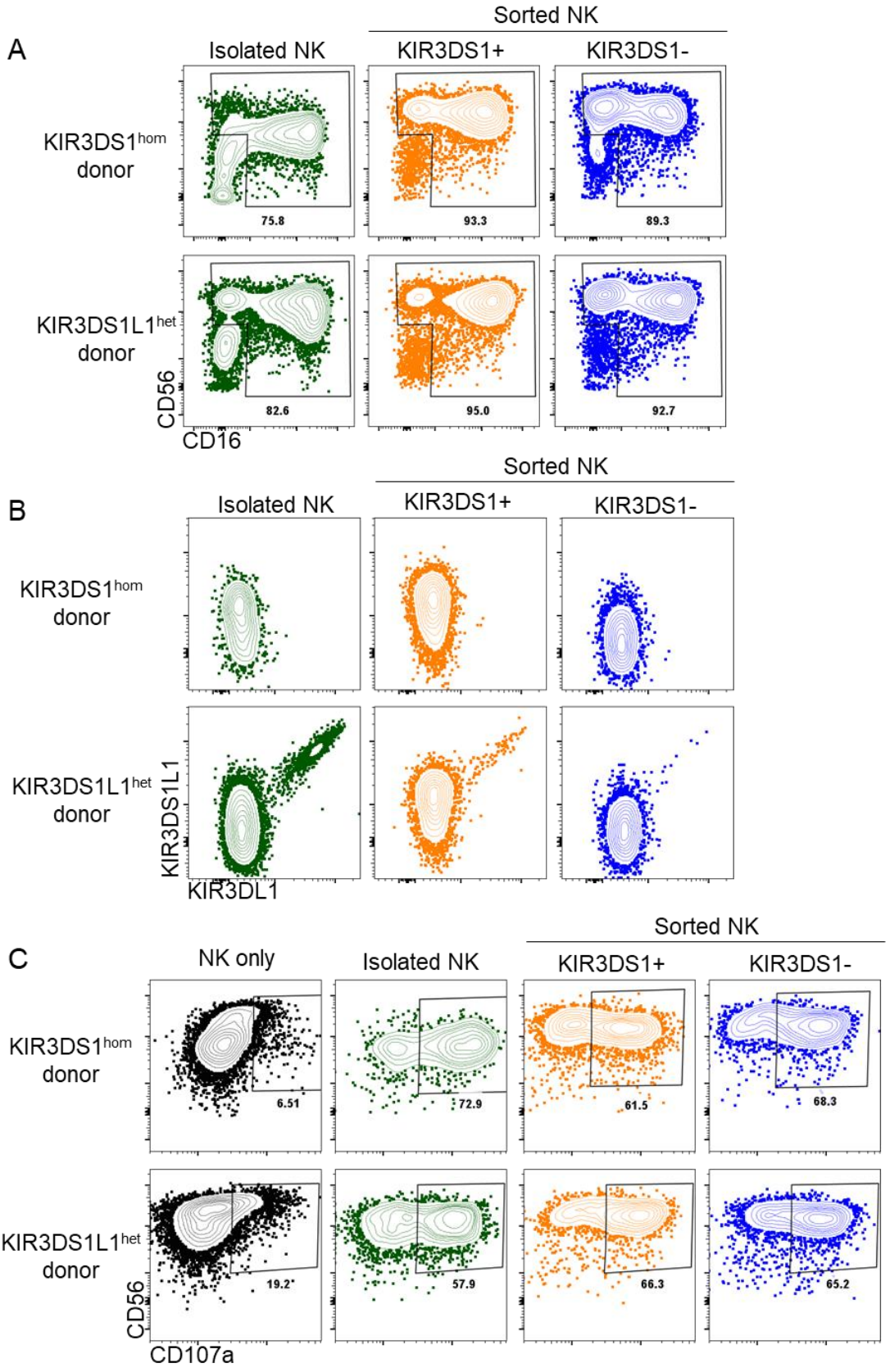
NK cell subset distribution was similar in both donors for all conditions, with the CD56^{dim}CD16+ subset showing increased CD56 expression by the stimulation, as expected. The relative proportion of NK cells among live CD3- cells was slightly higher in sorted (KIR3DS1^{hom} donor: 93.3 %/89.3 %) (KIR3DS1L1^{het} donor: 95 %/92.7 %) than isolated NK cells (KIR3DS1^{hom} donor: 75,8 %) (KIR3DS1L1^{het} donor: 82.6 %), which is due to the higher specificity of the sorting method compared to magnetic bead enrichment of NK cells (**Fig 15, A**).

KIR3DS1/L1 staining on NK cells (gated as described in **Fig 7, B**) confirmed that KIR3DS1 MdFI was increased in KIR3DS1+ sorted (KIR3DS1^{hom} donor MdFI:1474) (KIR3DS1L1^{het} donor MdFI:1172) compared to the KIR3DS1- sorted (KIR3DS1^{hom} donor MdFI:296) (KIR3DS1L1^{het} donor MdFI:271) and isolated cells for both donors (KIR3DS1^{hom} donor MdFI:897) (KIR3DS1L1^{het} donor MdFI:408) (**Fig 15, B**). Interestingly, despite no sorting of KIR3DL1+ NK cells, both sorted conditions from the KIR3DS1L1^{het} donor showed re-expression of KIR3DL1 on NK cells (isolated NK:8.55 %, KIR3DS1+ sorted:2.03 %, KIR3DS1- sorted:0.39 %, N=1).

To determine if NK cytotoxicity varied depending on the isolation or sorting condition, NK cell degranulation was investigated by staining CD107a during co-culture and compared to NK cells without target cells (**Fig 15, C**).CD107a staining of NK cells co-cultured for 16 h with K562 cells showed no remarkable difference between sorted conditions (KIR3DS1^{hom} donor: 61.5 %/68.3 %) (KIR3DS1L1^{het} donor: 66.3 %/65.2 %) and isolated NK cells for both donors (KIR3DS1^{hom} donor: 72.9 %) (KIR3DS1L1^{het} donor: 57.9 %).

Next, we assessed target cell lysis (calculation described in material and methods). In line with the degranulation data, the cytotoxic capacity of NK cells against K562 cells after 16 h co-culture at varying effector target ratios (E:T 1:1 and 2:1) was similar between sorted conditions (KIR3DS1^{hom} donor KIR3DS1+ sorted:96.89 %/96.55 %) (KIR3DS1^{hom} donor KIR3DS1- sorted:96.41 %/95.01 %) (KIR3DS1L1^{het} donor KIR3DS1+ sorted:95.03 %/93.94 %) (KIR3DS1L1^{het} donor KIR3DS1- sorted:92.22 %/92.41 %) and isolated NK cells for both donors (KIR3DS1^{hom} donor:79.35 %/96.84 %) (KIR3DS1L1^{het} donor:92.67 %/95.64 %) (**Fig 15, D**).

In summary, this experiment showed that KIR3DS1+ and KIR3DS1- sorted NK cells retain the same subset distribution and do not differ in their capacity to kill the control target cell line K562. Additionally, they are as capable of degranulation and cytotoxicity as isolated bulk NK cells, showing no functional impairment by FACS-sorting.


A
Sorted NK
Isolated NK
KIR3DS1+
KIR3DS1-
KIR3DS1hom donor
KIR3DS1L1het donor
75.8
93.3
89.3
82.6
95.0
92.7
CD56
CD16
B
Sorted NK
Isolated NK
KIR3DS1+
KIR3DS1-
KIR3DS1hom donor
KIR3DS1L1het donor
KIR3DS1L1
KIR3DL1
C
Sorted NK
NK only
Isolated NK
KIR3DS1+
KIR3DS1-
KIR3DS1hom donor
KIR3DS1L1het donor
6.51
72.9
61.5
68.3
19.2
57.9
66.3
65.2
CD56
CD107a

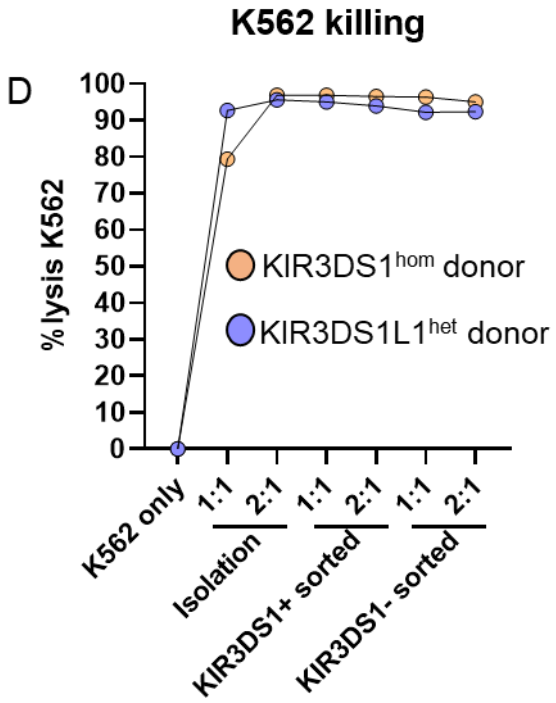


Figure 15 Sorted NK cells retain phenotypic features after stimulation. NK subset distribution (A), NK KIR3DS1 (KIR3DS1L1+/KIR3DL1-) and KIR3DL1 (KIR3DS1L1+/KIR3DL1+) expression (B) and degranulation assay (C) of isolated and KIR3DS1+/KIR3DS1- sorted NK cells from two donors. Killing assay of K562 cells by isolated and KIR3DS1+/KIR3DS1- sorted NK cells (D).

3.2 HLA-F is not the only trigger of CD4 bystander killing

To assess the impact of HLA-F upregulation on bystander killing by stimulated NK cells, NK cells from 3 donors (Donor A: KIR3DS1^{hom}; Donor B and C: KIR3DS1L1^{het}) were sorted as in the established set up, cultured for 4 days in NK-cell medium (NKM_V1) and co-cultured for 16 h with 7 day-CD2/CD3/CD28 tetramer stimulated CD4 T cells (**Fig 16**).

HLA-F MdFI (**Fig16, left histograms**) was increased in the CD4 T cells of all donors after stimulation (Donor A MdFI:2357) (Donor B MdFI:1433) (Donor C MdFI:1559) compared to unstimulated cells (Donor A MdFI:916) (Donor B MdFI:398) (Donor C MdFI:583).

Next, we investigated the killing of activated CD4 T cells by KIR3DS1+ (**Fig16, middle graphs**) and KIR3DS1- (**Fig16, right graphs**) sorted NK cells. E:T ratios of 1:1 and maximal amount of effectors:1, as determined by NK cell counts after culture. CD4 lysis data showed that, on all donors and conditions, the stimulated CD4 T cells were more killed than unstimulated cells, and increasing killing was observed with increase in E:T ratios.

Notably, the impact of KIR3DS1 varied substantially between donors. Donor A (**Fig16, A**) showed a lower bystander killing of activated CD4 T cells by KIR3DS1+ NK cells (8.55 %/22.53 %)

compared to KIR3DS1- NK cells (33.36 %/46.57 %). Donor B (**Fig16, B**) showed an approximate same level of bystander killing of activated CD4 T cells by KIR3DS1+ (2.87 %/18.69 %) and KIR3DS1- NK cells (10.88 %/18.07 %). Donor C (**Fig16, C**) showed a higher bystander killing of activated CD4 T cells by KIR3DS1+ sorted (34.81 %/52.56 %) compared to KIR3DS1- NK cells (-6 %/27.58 %). In summary, this experiment demonstrates that HLA-F is not the only trigger for KIR3DS1+ NK cells, and that other axes are additionally involved in the NK cell killing of activated CD4 T cells.

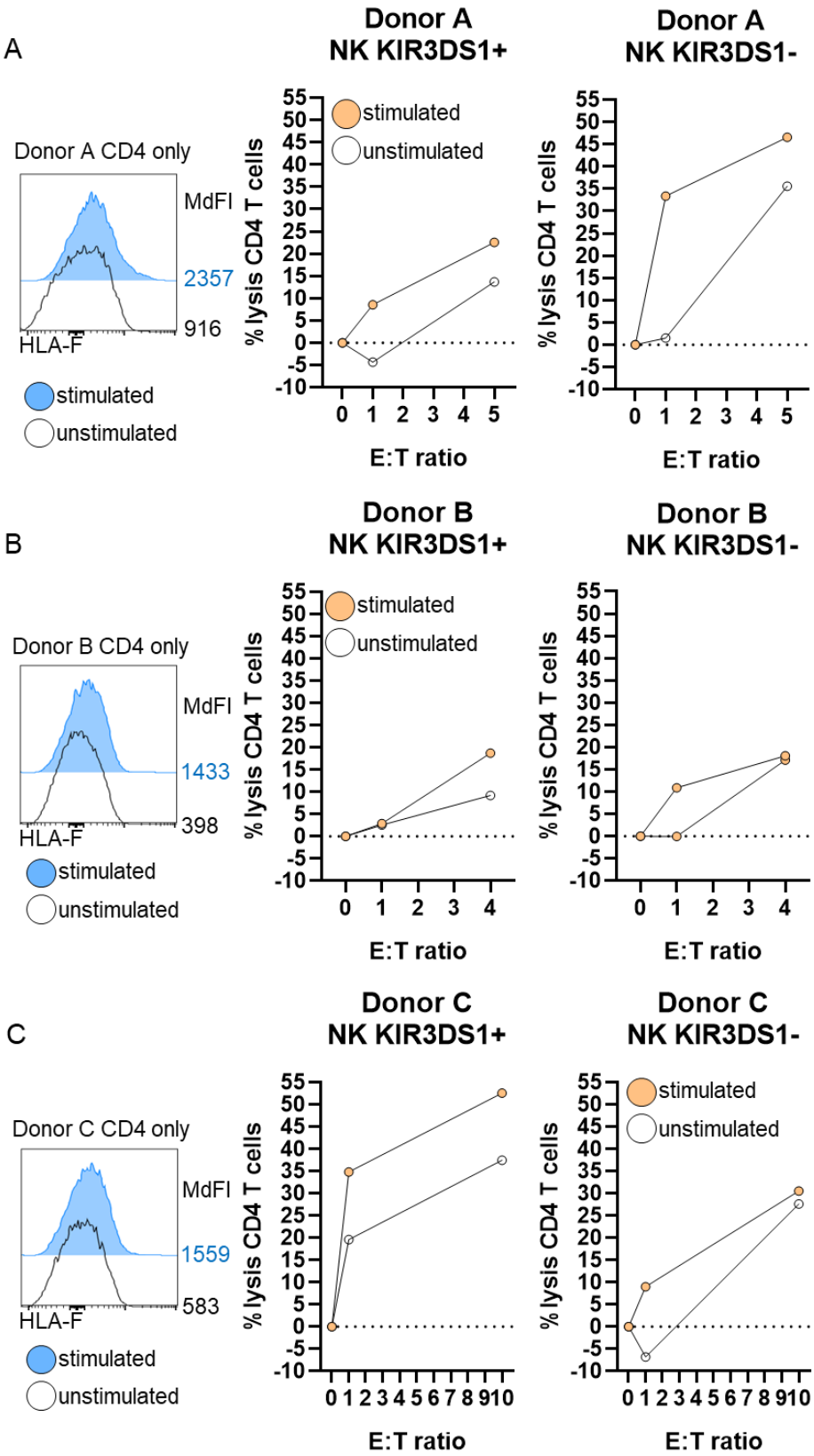


Figure 16 HLA-F is not the only trigger of CD4 bystander killing. HLA-F expression and lysis of stimulated and unstimulated CD4 T cells after co-culture with NK cells from 3 different donors (A, B, C). HLA-F histograms (left), lysis of CD4 T cells after KIR3DS1+ NK cells co-culture (middle) and lysis of CD4 T cells after KIR3DS1- NK cells co-culture (right)

3.3 NKG2D:NKG2DL axis is involved in CD4 bystander killing

Given our previous data indicating that HLA-F is not the only axis of CD4 bystander killing by NK cells, the NKG2D:NKG2DL axis was investigated, supported by previous reports[135,139–141].

As NKG2D is downregulated upon triggering, the aim of this experiment was to study NKG2D downregulation after co-culture. Upon culture optimization to ensure maximal viability and count of NK cells and CD4 T cells, KIR3DS1L1^{het} NK cells were sorted for KIR3DS1+ and KIR3DS1- and cultured for 7days in NKM_V2. Autologous isolated CD4 T cells were stimulated with CD2/CD3/CD28 tetramers for 7 days and resuspended at 1×10^6 cells/ mL every second or third day.

On the last 24 h before co-culture, stimulated CD4 T cells were additionally stimulated with sodium propionate (NaP),a short fatty acid chain that upregulates NKG2DL expression on stimulated CD4 T cells[135,290]. NK cells and stimulated CD4 T clls were co-cultured at E:T 1:1 for 16h before surface staining.

HLA-F MdFI was increased on CD4 T cells after NaP stimulation (MdFI:445) compared to unstimulated cells (MdFI: -187) (**Fig 17, A**). HLA-F MdFI on CD4 T cells decreased when co-cultured with KIR3DS1+ (MdFI:400), but not with KIR3DS1- NK cells (MdFI:527). This hints at potential killing via the KIR3DS1:HLA-F axis, with HLA-F+ CD4 T cells being targeted by KIR3DS1+ but not KIR3DS1- NK cells.

As expected, MICA/B MdFI was increased on CD4 T cells after NaP stimulation (MdFI:1081) compared to unstimulated cells (MdFI:-30). MICA/B MdFI on CD4 T cells decreased when co-cultured with KIR3DS1+ (MdFI:586) or KIR3DS1- NK cells (MdFI:633) (**Fig 17, B**). This hints at potential killing of MICA/B+ CD4 T cells by NK cells irrespective of KIR3DS1 via the NKG2D:NKG2DL axis. In line with this, the MdFI of NKG2D, which gets internalized after ligand binding, was decreased on KIR3DS1+ (MdFI: -34) and KIR3DS1- (MdFI: -114) NK cells co-cultured with K562 compared to their NK only control, as expected (KIR3DS1+ MdFI:1225) (KIR3DS1- MdFI:869) (**Fig 17, C**).

In addition, NKG2D MdFI on KIR3DS1+ NK cells was decreased when co-cultured with NaP stimulated (MdFI:616) compared to unstimulated CD4 T cells (MdFI:1105). The same observation was made on KIR3DS1- NK cells when co-cultured with NaP stimulated (MdFI:790) compared to unstimulated CD4 T cells (MdFI:841).

In summary, this experiment showed that HLA-F and NKG2DL expression on NaP stimulated CD4 T cells were decreased upon co-culture with stimulated NK cells, and simultaneously, NKG2D expression was decreased on NK cells when co-cultured with NaP stimulated CD4 T cells. These data indicate that NKG2DL:NKG2D and HLA-F:KIR3DS1 axis could synergize during bystander

killing, calling for follow up studies using blocking experiments on both ligands and receptors of these axes.

To sum up the NK cell bystander killing of activated CD4 T cells, our experiments showed that KIR3DS1+ and KIR3DS1- NK cells are as capable of degranulation and cytotoxic effect as isolated bulk NK cells. KIR3DS1+ NK cells did not uniformly kill activated CD4 T cells better than their KIR3DS1- counterparts, indicating that other activating receptor:ligand interactions play a role next to KIR3DS1 and HLA-F .Our data hints at the NKG2D:NKG2DL axis being in the bystander killing activity of NK cells, possibly synergizing with the HLA-F:KIR3DS1 axis.

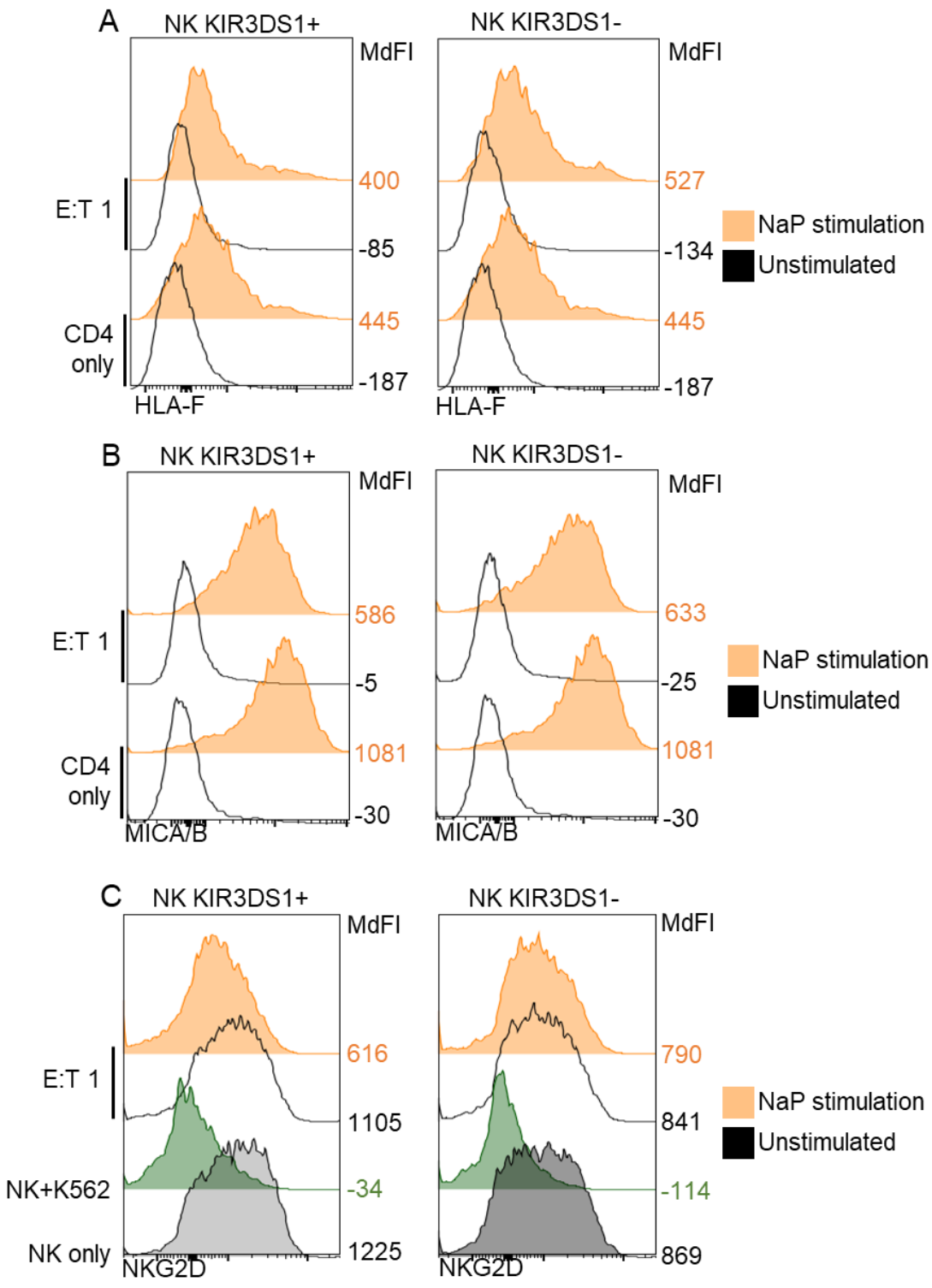


Figure 17 NKG2D:NKG2DL axis may be involved in CD4 bystander killing. HLA-F, MICA/B and NKG2D expression on respectively CD4 and NK cells after co-culture.

Chapter 2: HLA-F and auto-immunity in the context of the liver

1 Characterization of human hepatocyte organoids

As a cell culture system to study HLA-F upregulation and auto-immunity in the context of the liver, we used human hepatocyte organoids that were previously established and characterized in collaboration with the laboratory of Prof.Altfeld and Prof.Bunders based on the founding paper from the laboratory of Hans Clevers[272]. Hepatocyte organoids were generated from healthy tissue derived tumor resection samples performed at the Asklepios Klinik Barmbek and AIH patient samples from the Universitätsklinikum Hamburg-Eppendorf.

When observed under the light microscope, the culture appeared to be partially contaminated by cholangiocyte organoids. Hepatocyte organoids are easily distinguishable by their dark color, blobby aspect, and the presence of folds at the surface (**Fig 18, A**), while contaminating cholangiocyte organoids display a round shapes with a lack of coloration. Both organoids subsets

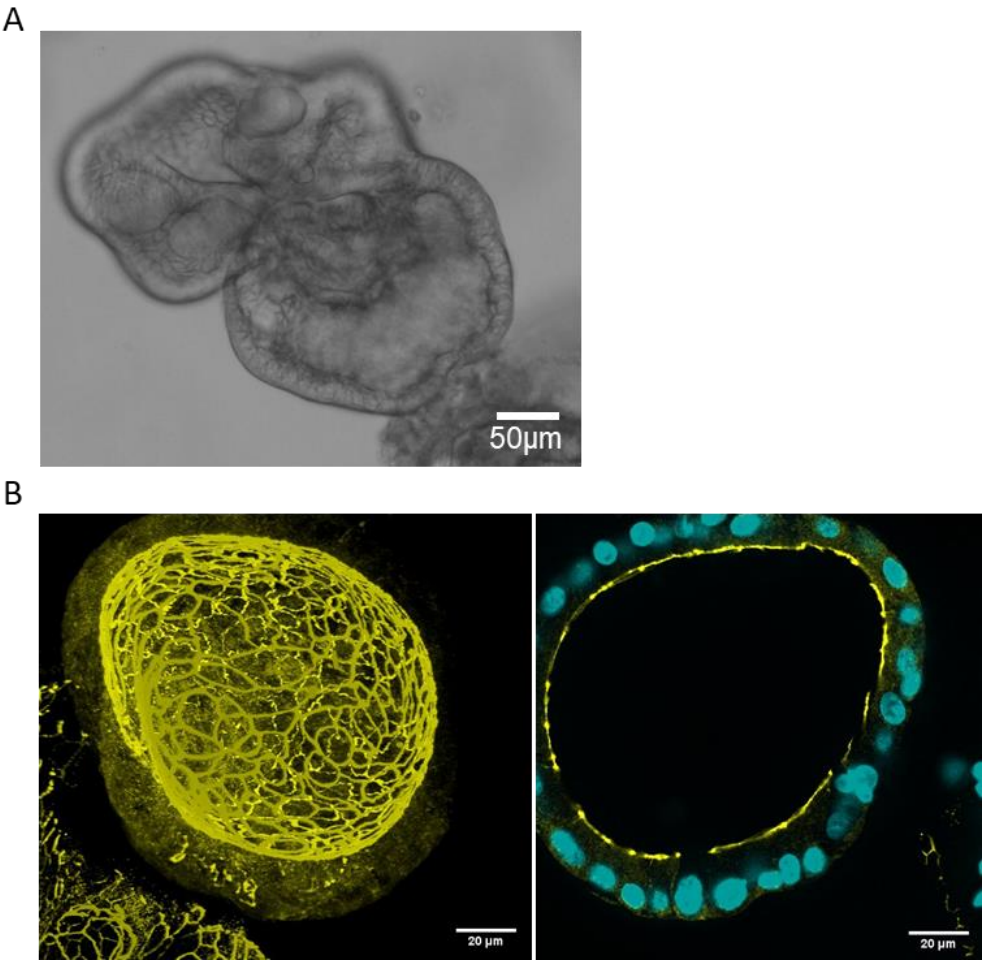


Figure 18 Characterization of human hepatocyte organoids. Light microscopy picture of human hepatocyte organoid, scale bar=50µm (A), Immunofluorescence of human hepatocyte organoids, yellow= ZO-1 blue= Hoechst, scale bar=20µm (B)

are hollow, suggesting a basolateral cellular organization. After immunohistochemistry staining, hepatocyte organoids appear to express Zonula occludens 1 (ZO-1), an apical sided tight junction marker, on the inside face, which means the cellular organization is basal out/apical in (**Fig 18, B**). This observation is interesting as the same feature was previously observed in human intestinal organoids[65].

Hepatocyte organoids cultured between passage 1 (P1) and P5 were used for experiments, as their viability, growth rate, size, and morphology are optimal to maximize the obtaining of single cells.

2 HLA-F is not surface expressed on hepatoma cell lines and healthy human hepatocyte organoids at baseline

To assess the surface expression of HLA-F at baseline level on hepatocyte, the hepatoma cell lines Huh7 and HepG2 and healthy hepatocyte organoids (AKB) (*n*=3) were stained. Isotype staining and unstained samples were used as controls (**Fig 19**).

HLA-F was neither detected on Huh7, HepG2 nor on the healthy hepatocyte organoids.

In summary, this experiment showed that HLA-F is not expressed at baseline level at the cell surface of hepatoma cell line and healthy hepatocyte organoids.

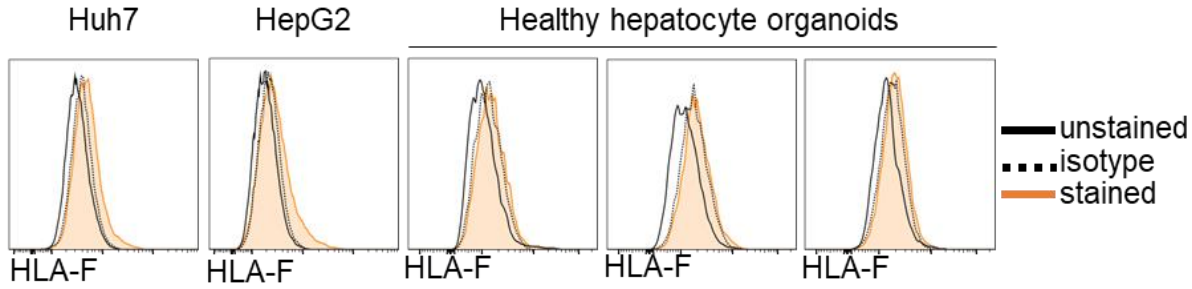


Figure 19 HLA-F is not surface expressed on hepatoma cell lines and healthy human hepatocyte organoids at baseline. HLA-F surface expression on resting hepatoma cell lines and hepatocyte organoids generated from three different donors

3 HLA-F is surface expressed on hepatoma cell lines upon stimulation with type I IFN and TNFα

To understand whether pro-inflammatory stimulation leads to HLA-F surface expression on hepatoma cell lines, Huh7 and HepG2 cells were stimulated with various pro-inflammatory cytokines and a TLR 4 stimulant according to **Table 9**. Staining with IgG1 isotype for HLA-F and unstained samples were used as controls (**Fig 20**).

The combination of IFNα and IFNβ induced HLA-F surface expression on Huh7 (MdFI isotype:671, MdFI stained:977) and HepG2 cells (MdFI isotype:561, MdFI stained:914). TNFα induced HLA-F

surface expression on Huh7 (MdFI isotype:806, MdFI stained:2152) and HepG2 cells (MdFI isotype:615, MdFI stained:900).On the contrary, neither IFNγ did increase HLA-F surface expression on Huh7 (MdFI isotype:565, MdFI stained:710) and HepG2 cells (MdFI isotype:305, MdFI stained:420), nor did LPS did increase HLA-F surface expression on Huh7 (MdFI isotype:403, MdFI stained:488) and HepG2 cells (MdFI isotype:212, MdFI stained:262).

In summary, this experiment showed that the pro-inflammatory cytokines IFNα+IFNβ and TNFα but not IFNγ and LPS were inducing HLA-F surface expression on hepatoma cell lines.

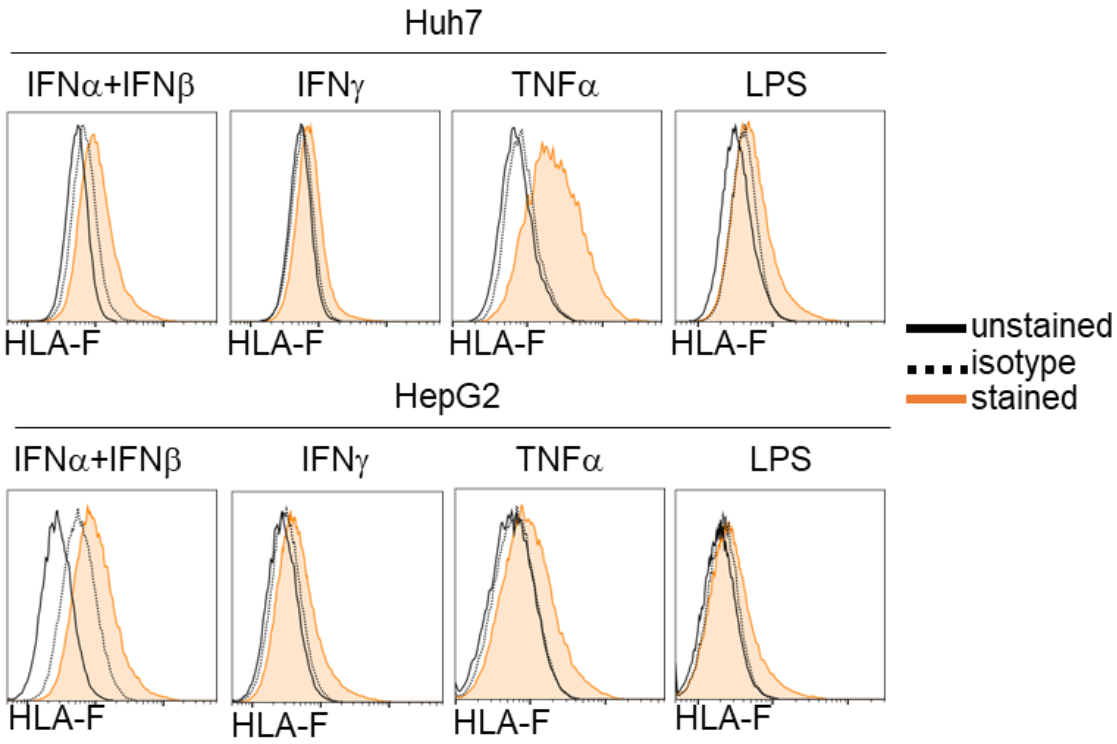


Figure 20 HLA-F is surface expressed on hepatoma cell lines upon stimulation with type I IFN and TNFα. HLA-F surface expression on stimulated hepatoma cell lines.

4 HLA-F is not surface expressed on healthy hepatocyte organoids upon stimulation

Following the induction of HLA-F surface expression on hepatoma cell lines after pro-inflammatory stimulations, healthy hepatocyte organoids (*n*=3, except for LPS stimulation *n*=2) were stimulated with various pro-inflammatory cytokines and TLR 3/4 stimulants according to **Table 9**. As above, IgG1 isotype staining and unstained samples were used as controls (**Fig 21**).

None of the proinflammatory cytokines (IFNα and IFNβ, TNFα, IFNγ) did increase HLA-F surface expression in hepatocyte organoids. In addition, neither TLR4-stimulation with LPS nor TLR3-stimulation with Poly I:C did result in HLA-F upregulation.

In summary, no stimulus was identified that upregulated HLA-F on the surface of hepatocyte organoids derived from healthy donors.

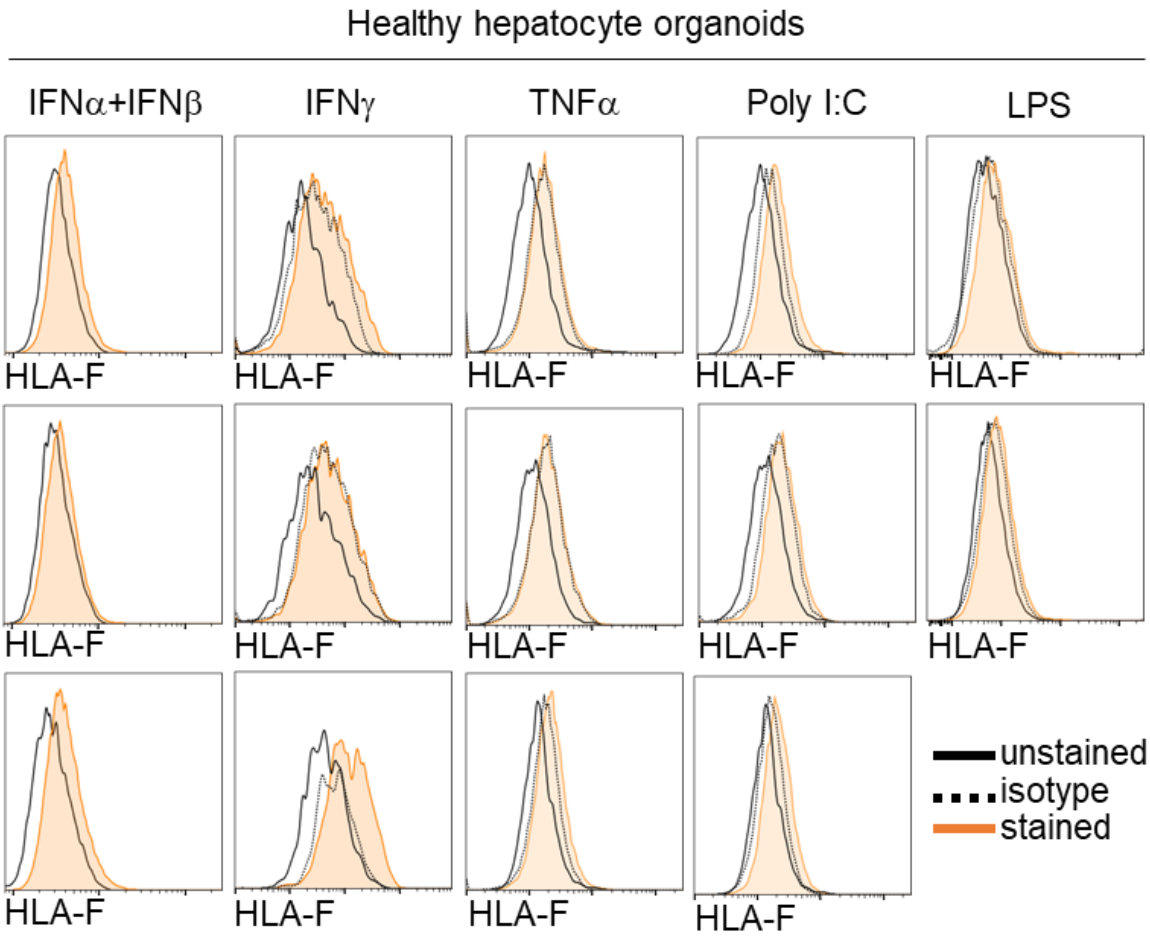


Figure 21 HLA-F is not surface expressed on healthy hepatocyte organoids upon stimulation. HLA-F surface expression on hepatocyte organoids generated from two to three different donors.

5 HLA-F surface expression is upregulated on AIH but the effect of pro-inflammatory stimulation remains unclear

To assess if hepatocyte organoids generated from AIH patient samples display a different HLA-F surface expression pattern than healthy hepatocyte organoids, AIH hepatocyte organoids (*n*=3, duplicated for baseline) were stained without stimulation and after pro-inflammatory cytokine TNFα and TLR3 stimulation. Healthy hepatocyte organoids (*n*=6 for baseline, *n*=3 for stimulations), isotype staining and unstained samples were used as controls.

At baseline, HLA-F was slightly expressed on AIH (median MdFI:1487, IQR:909-2916) but not on healthy hepatocyte organoids (median:1277, IQR:1012-1920, ns) (**Fig 22, A**).

After TNFα stimulation, HLA-F MdFI was increased on AIH (median MdFI:4125) compared to healthy hepatocyte organoids (median MdFI:1892) but a substantial isotype background was detected on AIH hepatocyte organoids (median MdFI:4711) (**Fig22, B**). After poly I:C stimulation, HLA-F MdFI was increased on AIH (median MdFI: 3847) compared to healthy hepatocyte organoids (median MdFI: 2010) but also here background staining with the IgG1 isotype was increasing, albeit to a lower degree (median MdFI:2888). Thus, further blocking conditions and modifications to the staining protocol are needed to reduce the unspecific binding of the isotype to the stimulated hepatocytes and tease out the effect of TNFα on HLA-F surface levels. Optimally, staining with an antibody of the same Ig subclass as the HLA-F clone but specific for an epitope not found on hepatocytes should be compared next.

In summary, this experiment showed that AIH hepatocyte organoids express HLA-F at low level at baseline but the effect of pro-inflammatory stimulation remains unclear given the high level of unspecific staining detected after TNFα stimulation. Thus, any observed increase in HLA-F signal should be followed up by Western Blot assessment of HLA-F protein levels for further validation.

To sum up the study of HLA-F surface expression in the context of the liver and AIH, HLA-F is not expressed at baseline on hepatoma cell lines and healthy hepatocyte organoids but is detectable at low level on AIH hepatocyte organoids to varying degrees. Despite IFNα+IFNβ and TNFα stimulations being effective to induce HLA-F surface expression on hepatoma cell lines, no effect on healthy hepatocyte organoids was detected, and the impact of TNFα on HLA-F surface levels of AIH hepatocyte organoids needs to be further studied.

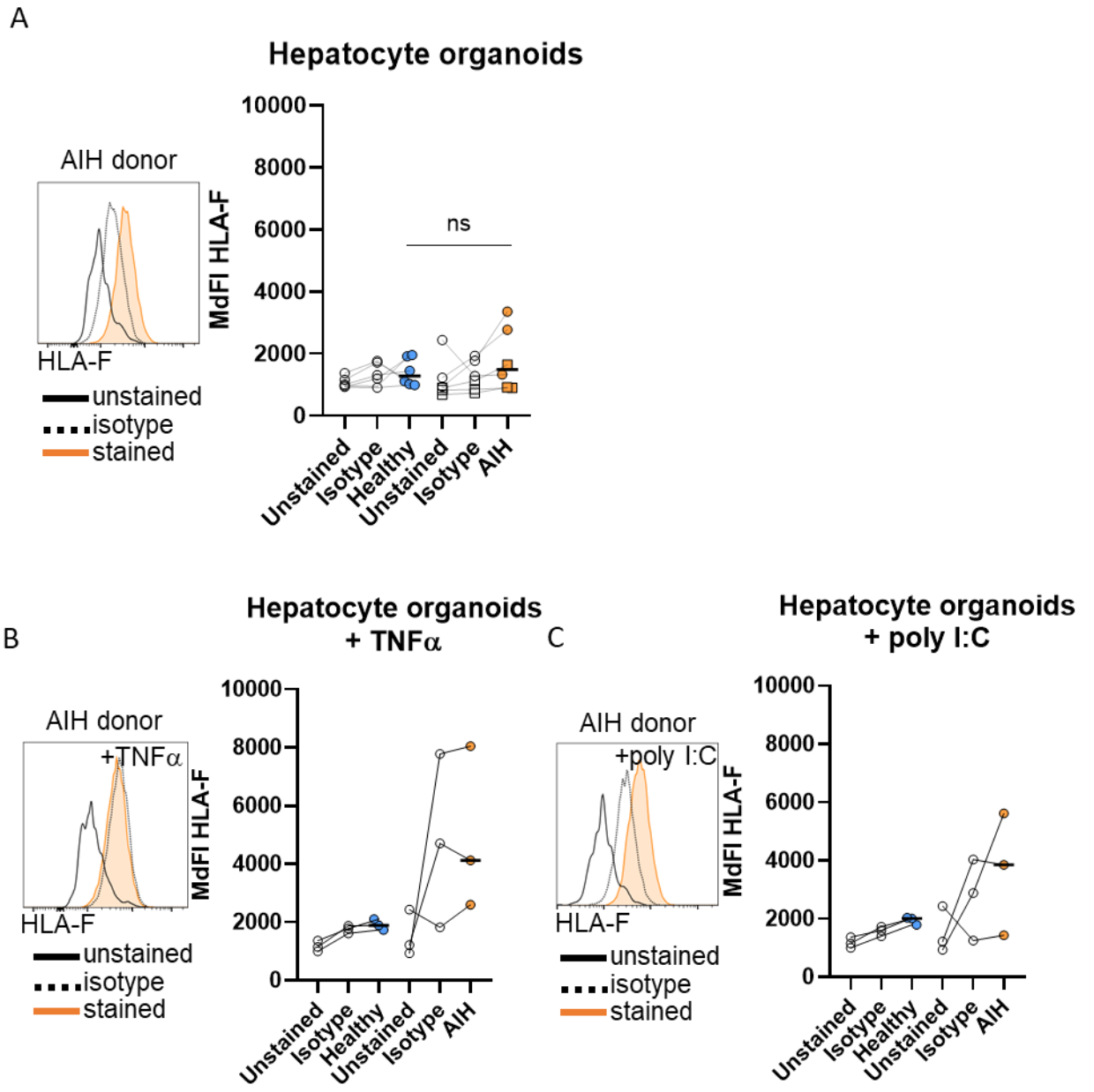


Figure 22 HLA-F surface expression is upregulated on AIH but the effect of pro-inflammatory stimulation remains unclear. HLA-F surface expression on rested hepatocyte organoids generated from six healthy and three AIH donors with technical replicate (A), HLA-F surface expression on TNFα (B) and poly I:C (C) stimulated hepatocyte organoids generated from three healthy and AIH donors.

Discussion

This thesis focused on investigating the role of HLA-F in human diseases, focusing on two types of pathologies: viral infections and autoimmune diseases. HLA-F surface expression was increased on CD4 T cells and anti-HLA-F autoantibodies were detected in the serum of therapy-naïve HIV-1 positive individuals. Subsequent analysis of HLA-F surface expression on stimulated CD4 T cells from HIV-1 negative donors showed that all donors upregulated HLA-F surface levels but to highly varying degrees. Increased KIR3DS1-Fc staining of activated CD4 T cells compared to unstimulated cells was detected among all donors. Co-culture experiments revealed that NK cells were capable of bystander killing of uninfected stimulated CD4 T cells. Evidence points towards an involvement of the NKG2D:NKG2DL axis in the bystander killing activity of NK cells, possibly synergizing with the HLA-F:KIR3DS1 axis. In the liver, low levels of surface HLA-F were detected on hepatocyte organoids derived from AIH patients. Cytokine stimulation of hepatoma cell line revealed that HLA-F surface expression can be upregulated by pro-inflammatory cytokines; however, further studies are needed to fully understand HLA-F expression on hepatocytes.

1 The role of HLA-F as a marker of ongoing immune activation in HIV-1 infection

The results of this thesis show that chronic HIV-1 infection induces an immune activation and inflammation. This was observed with the increase of HLA-DR and CD38 on CD4 and CD8 T cells of HIV-1 positive individuals, a phenomenon previously described in the literature[281]. Results of this thesis demonstrated that in therapy naïve HIV-1 positive individuals HLA-F surface expression was increased on CD4 and CD8 T cells[23]. Prior to this, Garcia Beltran *et al.* had described increased HLA-F mRNA in *in vitro* HIV-1 infected CD4 T cells[23]. For the first time, the results provide evidence of *ex vivo* HLA-F surface expression induced by the immune activation mediated by HIV-1 infection. A limitation from our cohort was the lack of time course samples to investigate the role of HLA-F in acute versus chronic infection, especially on uninfected CD4 T cells. In addition, we cannot differentiate between potentially infected CD4 T cells, estimated at 100 p24+ cells/10^6 CD4 T cells in therapy-naïve HIV positive individuals[287] and uninfected T cells in our HIV-1 samples.

Results of this thesis also demonstrate increased surface expression of ligands for the NKG2D receptor on primary T cells in HIV-1 infection. Previous *in vitro* experiments focusing on the late expressed viral protein Vpr showed a NKG2DL increase on primary CD4 T cells, while experiments focusing on the viral protein Nef showed a NKG2DL decrease. However these

studies only focused on infected CD4 T cells[179,183]. On the contrary, our results were obtained by investigating all CD4 and CD8 T cells in the context of immune activation induced by the HIV-1 infection, thus not focusing only on infected CD4 T cells. In HIV-1 infection NKG2DL are cleaved from the cell surface in a mechanism known as shedding, leading to their extracellular release in a soluble form. Our results support the idea that both infected and uninfected T cells can be a source of soluble NKG2DL[206,207]. NKG2DL shedding is an important immune evasion mechanism as soluble NKG2DL can binds to the NKG2D receptor of circulating NK cells, impairing their cytotoxicity[206].

Given the similar expression pattern between HLA-F and NKG2D on activated CD4 T cells and in ongoing immune activation[18,141], soluble HLA-F (sHLA-F) in chronic HIV-1 infection will be interesting to investigate, especially to study the potential blockade of the activating NK receptor KIR3DS1, as described with NKG2DL shedding in HIV-1 infection.

sHLA-F detection has been described in cancer but it has not been particularly studied in the context of viral infections and autoimmune diseases[291,292]. Additionally, two previous reports described the upregulation of anti-HLA-F autoantibodies in other pathologies different than HIV-1 infection: Noguchi *et al*. performed a wide screening in the sera of patients with various cancers using a Western-Blot detection and they showed an upregulation of anti-HLA-F autoantibodies [293]. Jucaud *et al.* described in SLE the upregulation of anti-HLA-F IgG, but not IgM, especially after disease flare, using coated microbeads[220]. The results of this thesis hint at a potential upregulation of anti-HLA-F IgG but not IgM in therapy-naïve HIV-1 positive individuals (**Fig 6, B**), similar to what has been observed in SLE. The class specificity of detected autoantibodies is a good indicator that the production of anti-HLA-F autoantibodies is induced during the chronic phase of the pathology and that a memory response is generated against HLA-F. Analyses of autoantibodies present in acute infection could potentially lead to the detection of anti-HLA-F IgM but this approach has never been tested. Longitudinal plasma samples would also help to elucidate sHLA-F levels and their potential correlation with viral immune evasion in early or late HIV-1 infection. Last, study of conformation-dependent epitopes in anti-HLA-F autoantibodies via ELISA could be of interest to decipher the importance of the conformation of HLA-F in the generation of an autoantibody response.

Taken together, these data indicated that HLA-F surface expression and the subsequent upregulation of anti-HLA-F IgG in the context of chronic HIV-1 infection are potential markers of the ongoing immune activation.

Frequencies of Tregs among CD4 T cells, as crucial immunosuppressive cells, did not differ between HIV positive and negative individuals in our study, confirming the data previously published by Dunham *et al*[280]. Given the correlation between CD4 T cell activation and Treg depletion in chronic HIV-1 infection[294,295], Tregs have been extensively studied[296] with contradictory descriptions of phenotypical and functional dysfunctions[297–302]. Interestingly, data from this thesis show increased surface expression of HLA-F *ex vivo* on Tregs of therapy naïve HIV positive individuals, hence supporting the expression of HLA-F as a marker of ongoing immune activation, even on T cell subsets such as Tregs where *in vitro* activation showed no surface expression of HLA-F[18].

Double positive CD4+/CD8+ T cells are characterized by highly proliferative and effector capacities in HIV-1 infection[303,304]. Double positive CD4+/CD8+ T cells showed no increase in frequencies in HIV positive individuals (**Fig 4, D**), contrary to what has been described in treated HIV positive individuals[282,305]. This difference might be due to the advanced state of disease in our cohort of therapy naïve individuals, since it has been described that double positive CD4+/CD8+ T cells are upregulated in acute infection. Given that they show cytotoxic effects, an early exhaustion and activation-induced cell death of this cell subset might occur in HIV-1 infection.

HLA-F surface upregulation raises the question of which conformation of HLA-F is expressed at the cell surface, as only HLA-F OC is binding to the activating receptor KIR3DS1, thus capable of triggering cytotoxic functions on NK cells[23]. This question is technically difficult to answer as no HLA-F OC antibody is commercially available. Nonetheless, it can be partially assessed by KIR3DS1-Fc staining. As the regulation of HLA-F surface expression remains vastly unknown, the ratio of HLA-F OC vs HLA-F:β2m conformer expression on the cell surface is not fully understood. However, some studies support the notion that HLA-F is mainly expressed as HLA-F OC[16,306]. The results of this thesis demonstrated that stimulated CD4 T cells from all donors upregulated a conformation of HLA-F allowing KIR3DS1-Fc binding (**Fig 13**). Therefore, it can be hypothesized that activated lymphocytes express a combination of different HLA-F conformers on the cell surface. It has also been speculated by Dulberger *et al* that the expression of HLA-F OCs on the cell surface is mediated via either direct expression or conformer switch from HLA-F:β2m[11]. This HLA-F OC vs HLA-F:β2m conformer ratio is dynamic and could be influenced by *HLA-F* polymorphism as well as presented peptides, given its unusual presentation of N terminal unanchored peptides of unusual length. One could also speculate that the stimulation and/or infection of CD4 T cells leads to the expression of both conformers. HLA-F OCs may be preferentially expressed in HIV-1 infection as they potentially bypass the folding and loading

mechanisms of classical HLA-I, contrary to the HLA-F:β2m conformer which could be targeted by HIV-1 evasion mechanisms focusing on the PLC.

2 Phenotypic alterations of NK cells in HIV-1 infection

NK cells are altered during HIV-1 infection. The results of this thesis demonstrate that NK cell subsets are dysbalanced in chronic HIV-1 positive individuals with a decreased relative proportion of the conventional subsets and an increase of the CD16+CD56- dysfunctional subset, confirming previously published data[197,200].

NKG2D was upregulated on NK cells and CD8 T cells in therapy naïve HIV positive individuals, contrary to previous published data on chronic untreated donors [198,207]. While Mavilio *et al.* showed only minor changes of NKG2D on NK cells in chronic untreated individuals[198], Nolting *et al.* showed a significant decrease in NKG2D+ NK cells in the same type of cohort[207]. Disparities between these results might be linked to either different disease stages among donors included in the cohort or accumulation of soluble NKG2D ligands, as the alteration of NKG2D expression in HIV-1 infection has been proposed to be mediated by the shedding and accumulation of soluble NKG2D ligands. Using K562 cells with increasing concentrations of soluble MICA[207], Nolting *et al*. suggested that the accumulation of soluble NKG2D ligands reduces the capacity of NK cells to downregulate NKG2D. Therefore, the higher expression of NKG2D shown in this thesis might be due to a mix of activating signaling on NK cells as well as the presence of soluble NKG2D ligands, although this needs further confirmation.

The results from this thesis demonstrate an increase of NK cells expressing the inhibitory receptors KIR3DL2 (**Fig 9, B**) and LILRB1 (**Fig 9, C**), according to what has been described for inhibitory receptors in the literature[202,204]. Studies of KIR3DL2 expression in HIV-1 infected individuals are scarce, and they show similar expression frequencies in PBMCs between healthy and HIV positive individuals. However, these studies did not investigate specifically the frequency of KIR3DL2+ NK cells[307,308]. Nonetheless, a previous study has linked increased mRNA expression of inhibitory receptors to chronic HIV-1 infection[196]. The functional impact of KIR3DL2 upregulation in chronic therapy naïve HIV-1 infection remains to be further investigated.

Here, we observed no significant expansion in KIR3DS1+ or KIR3DL1+ NK cells contrary to data published by Alter *et al.* (**Fig 9, A**)[196], which is potentially due to the low number of donors in the HIV positive (*n*=30) and HIV negative (*n*=44) cohort. Alter *et al.* described a preferential expansion of KIR3DS1+ (and to a lower extent of KIR3DL1+) NK cells in acute infection. This expansion was maintained to a slightly lower extent in chronically infected donors harboring the $Bw4^{80I}$ motif. Our data indicate that in certain individuals KIR3DS1+ NK cells did expand to high levels in chronic

HIV-1 infection. Upregulation of activating NK receptors has also been described with the expansion of NKG2C+ NK cells in HCMV co-infected individuals[205].

The potential expansion of KIR3DS1+ NK cells might be due to the inflammatory environment that occurs during HIV-1 infection. This speculation is in line with data from Horowitz *et al.*, that described that genetics largely determine inhibitory KIR expression, whereas activating KIR expression was heavily environmentally influenced[91]. Among KIRs, the *KIR3DS1/KIR3DL1* locus is unique as it encodes functionally divergent alleles[309]. The frequency of *KIR3DS1* carriers varies between 20-60% across different ethnicities and so far, KIR3DS1 has not been shown to be targeted by HIV-1 immune escape mechanisms[279]. KIR/HLA interactions have been extensively studied as shown in the KIR and diseases database (KDDB)[310]. *KIR3DS1/HLA-Bw4* association has been particularly studied (41 studies report this association on KDDB, as of May 2023) as a largely protective association in viral infection, cancer, and autoimmune diseases. Nevertheless, a direct interaction of KIR3DS1 and HLA-Bw4 is still unclear and likely dependent on rare peptides[23]. Hölzemer *et al*. described several models as potential mechanisms to explain a protective effect of KIR3DS1/HLA-Bw4 in HIV-1 infection by linking it to HLA-F[24]. One of these is that the upregulation of HLA-F might occur preferentially as heterodimer together with the HLA-Bw4^{I80} heavy chain[306]. Whether this heterodimer is a conformation of HLA-F which would allow for KIR3DS1-binding is unclear to date. One could speculate that given the high expression of *Bw4^{I80}* in HIV positive (50%) and HIV negative (59%) individuals in our cohort, HLA-F: HLA-Bw4^{I80} heterodimers could lead to more effective clearing of infected cells.

3 Role and mechanism of NK cell bystander killing

The results of this thesis demonstrate that HLA-F upregulation on activated CD4 T cells is associated with binding to KIR3DS1-Fc, indicating that the HLA-F OC is upregulated during activation. Activated but uninfected CD4 T cells (referred as bystander cells) are more sensitive to NK cell killing than unstimulated CD4 T cells. HLA-F expression on CD4 T cells may trigger KIR3DS1+ NK cells, however, to conclusively demonstrate this, further blocking experiments are needed. Nonetheless, the results of this thesis indicate that HLA-F likely is not the only trigger of bystander T-cell killing, and that other axes, in particular NKG2D, are involved, as previously described [135,139,141]. It is known that TRAIL[135,137] and NKp46[140] are also involved in bystander killing of activated CD4 T cells by NK cells.

In the context of HIV-1 infection, bystander killing of activated CD4 T cells by NK cells may have opposing consequences for the immune control of HIV-1 infection. On one hand, this mechanism can lead to a rapid control of immune inflammation, on the other hand it can also trigger an excessive killing of uninfected CD4 T cells. Rapid control of immune inflammation is associated

with slower disease progression[311]. Excessive killing of CD4 T cells might lower the nadir in the early phase of infection and aggravate the CD4 count loss in chronic infection. Waggonner et al. described in mice during LCMV infection[136] the effect of excessive killing of activated CD4 T cells by NK cells. In that study, mice depleted on NK cells were compared to control mice with intact immunity. When NK-depleted mice were infected with a high dose of LCMV, they showed lower survival compared to immunologically intact control mice. However, NK-depleted mice infected with a lower dose of virus depicted an increased survival compared to immunologically intact control mice. The reason behind this effect was shown to be a disproportionate killing of activated CD4 T cells by NK cells hampering the adaptive immune response. Thus, whether early killing of activated CD4 T cells by NK cells in HIV-1 infection is detrimental or beneficial needs to be further determined.

To further understand the exact contribution of receptor: ligand pairs in bystander killing of activated CD4 T cells by NK cells, experiments blocking NK cell receptors and their targets on CD4 T cells are needed. Understanding whether bystander killing can be triggered by one or more of the different axes above described is of high interest, especially in the context of HIV-1 infection, where activating receptors are also targeted by viral proteins to evade immune responses.

4 HLA-F regulation is associated with pro-inflammatory pathways in viral infection and autoimmune diseases

The regulation of HLA-F surface expression remains largely unknown. Gobin *et al*. first studied the transcriptional regulation of non classical HLA-I molecules in 2000[21]. This study showed that the upstream promoter region of *HLA-F, -E* and *-G* possesses regulatory elements containing binding sites for NFκB, IRF1 and CIITA, but that only HLA-F transcription is induced by all three pathways, indicating the importance of proinflammatory cytokines in the transcriptional regulation of *HLA-F*.

NFκB is a heterodimer of p50 and p65 subunits. NFκB is translocated in the nucleus by the phosphorylation of Iκ B. Iκ B is phosphorylated by IKKβ, which is activated via phosphorylation by TAK1. Recruitment of TAK1 can be mediated by several activation pathways such as TCR/CD3 signaling, TLR signalling or engagement of TNFR1 by TNF trimers[312].

IRF1 is induced by the triggering of the IFNAR receptor by type I interferon (IFNα and IFNβ) [313].

The class II transactivator (CIITA) regulates IFNγ-activated transcription of HLA-I and -II molecules by acting as transcriptional activator and as a general transcription factor[314].

Transcriptional upregulation of *HLA-F* via NFκB was corroborated by Kumar *et al*., who demonstrated that upregulation of HLA-F surface expression during JEV infection was mediated

by TNFα-dependent NFκB induction[66]. However, no data showing HLA-F surface expression mediated by type 1 IFN triggering of IRF1 or IFNγ-triggering of CIITA have been published so far. The results of this thesis demonstrate that HLA-F is not present at the cell surface under resting conditions but it is increased on CD4 T cells upon *in vitro* activation after CD3/CD28-stimulation (**Fig 12**), a mechanism potentially implicating NFκB[315]. Our data show that HLA-F surface expression is also upregulated *ex vivo* on CD4 and CD8 T cells during chronic HIV-1 infection (**Fig 5, B**). In HIV-1 infection, TNFR and downstream NFκB pathways are targeted by Vpr, Tat, Nef and Env to increase HIV-1 replication in infected cells[316], leading therefore to an increased activation of NFκB and production of TNFα by infected T cells. TNF produced by infected cells is shown to activate NFκB in latently infected CD4 T cells, facilitating viral reactivation from HIV-1 reservoirs[317]. Plasma and tissue levels of TNF are increased at all stages of the disease, even during ART with undetectable viremia[318,319]. Usage of anti-TNF treatments such as antibodies targeting TNF (infliximab, adalimumab, golimumab, certolizumab pegol), TNF receptor fusion protein (etanercept) and TNF inhibitors (thalidomide, LMP-420) have been shown to improve HIV-1 symptoms in treated patients without increasing the mortality rate, as described by Pasquereau *et al*.[316]. Given the link of TNF and HLA-F in HIV-1 infection based on our data and the literature, future studies investigating the impact of TNF and NFκB in inducing HLA-F surface expression, used as a biomarker for inflammation in HIV-1 infection, is of interest.

Finally, HLA-F expression was studied in the context of the liver using hepatocyte organoids, which express mRNA for p50, p65, IRF1 and CIITA (the Human Protein Atlas, as of May 2023[15,320]). HLA-F surface expression was not expressed at baseline on hepatoma cell lines and healthy hepatocyte organoids (**Fig 19**), but it was detected at low and varying levels on AIH hepatocyte organoids (**Fig 22**). Moreover, IFNα+IFNβ and TNFα stimulation increased HLA-F surface expression on hepatoma cell lines (**Fig 20**) but not on healthy hepatocyte organoids (**Fig 21**). In AIH, the importance of TNFα and NFκB in the pathophysiology of the disease is highlighted by the selective expansion of TNF-producing CD4 T helper 1 (Th1) in the liver infiltrate of patients, as described by Bovensiepen *et al*.[240]. Moreover, therapeutic targeting of NFκB pathway and TNF has shown good results[260]. Our results on HLA-F surface upregulation of TNFα-stimulated AIH-derived organoids were potentially interesting, but need and warrant further study, in particular given the strong clinical link between TNFα and AIH.

Taken together, these results suggest a two-step model for HLA-F surface expression on lymphocytes and hepatocytes. First, *HLA-F* mRNA expression is upregulated upon activation or pro-inflammatory cytokine stimulation via NFκB, IRF1 (or CIITA)[21]. *HLA-F* mRNA is then translated

but the protein potentially remains trapped in the ER[16,22] . Upon infection, cellular transformation or chronic inflammation, ER retention is lifted, leading to HLA-F surface expression.
However, the mechanism resulting in egress of HLA-F protein remains to be investigated. Infection, cellular transformation and chronic inflammation are known to trigger the unfolded protein response (UPR), which is characterized by chaperone recruitment and ER-associated degradation (ERAD), two mechanisms that do not lead to ER release[321]. One could speculate that an alternate mechanism allows selective HLA-F egress from the ER despite the UPR.

Given the similarities in expression pattern between HLA-F and NKG2D ligands, it is tempting to look for potential common ways of surface regulation. Gowen *et al*. described novel regulators of ULBP1 in a tumor derived near-haploid cell line by using a retroviral gene-trap mutagenesis screening. The hits were confirmed by CRISPR/Cas9 mutation, gene restoration and establishment of double/triple mutant to assess independence or common pathways for the hits[322]. This publication described that ATF4, a stress induced transcription factor, supports ULBP1 transcriptional and surface expression in the HAP-1, K562 and Jurkat cell lines in steady state and in response to amino acid starvation and UPR. The strategy of this paper is of high interest for the study of HLA-F transcriptional and potentially surface expression regulation as it could help to identify potential overlapping regulators with NKG2DL and to better understand HLA-F ER retention. Nonetheless, mutagenesis screening via gene trap or CRISPR-Cas9 library as used by Klein *et al*. [96] requires a cell culture model with high and stable surface expression of the protein of interest, which remains challenging for HLA-F.

Tumor	mRNA/protein expression	Observation	Reference
HBV-related HCC	mRNA expression	lower overall survival	[323]
HCC	intracellular+ surface expression	lower overall survival	[324]
Brain glioma and GBM	mRNA+ intracellular/surface expression	lower overall survival	[325]
	mRNA expression	tendency to higher overall survival	[326]
NPC	intracellular+ surface expression	lower overall survival	[292]
ESCC	intracellular+ surface expression	lower overall survival	[327]
NSCLC	intracellular+ surface expression	lower overall survival	[328]
breast cancer	intracellular+ surface expression	lower overall survival	[329]
gastric cancer	intracellular+ surface expression	lower overall survival	[330]

Table 6 HLA-F expression in cancer. HCC: hepatocellular carcinoma, GBM: glioblastoma, NPC: nasopharyngeal carcinoma, ESCC: esophageal squamous-cell carcinoma, NSCLC: non small cell lung cancer.

In this thesis, I also addressed the question of HLA-F tissue regulation. No tissue has been described to express high HLA-F on the cell surface at baseline. Nonetheless, viral infections (**Table 1**), autoimmune diseases (**Table 4**), and various cancers (**Table 6**) have been described to upregulate HLA-F surface expression. The combination of data from this thesis looking at HLA-F on liver tissue as well as published literature supports the theory that despite absence of HLA-F under steady-state conditions, a two-step model of regulation of HLA-F surface expression could explain the wide tissue expression of HLA-F during inflammatory diseases.

5 HLA-F in HIV-1 and AIH: Working model

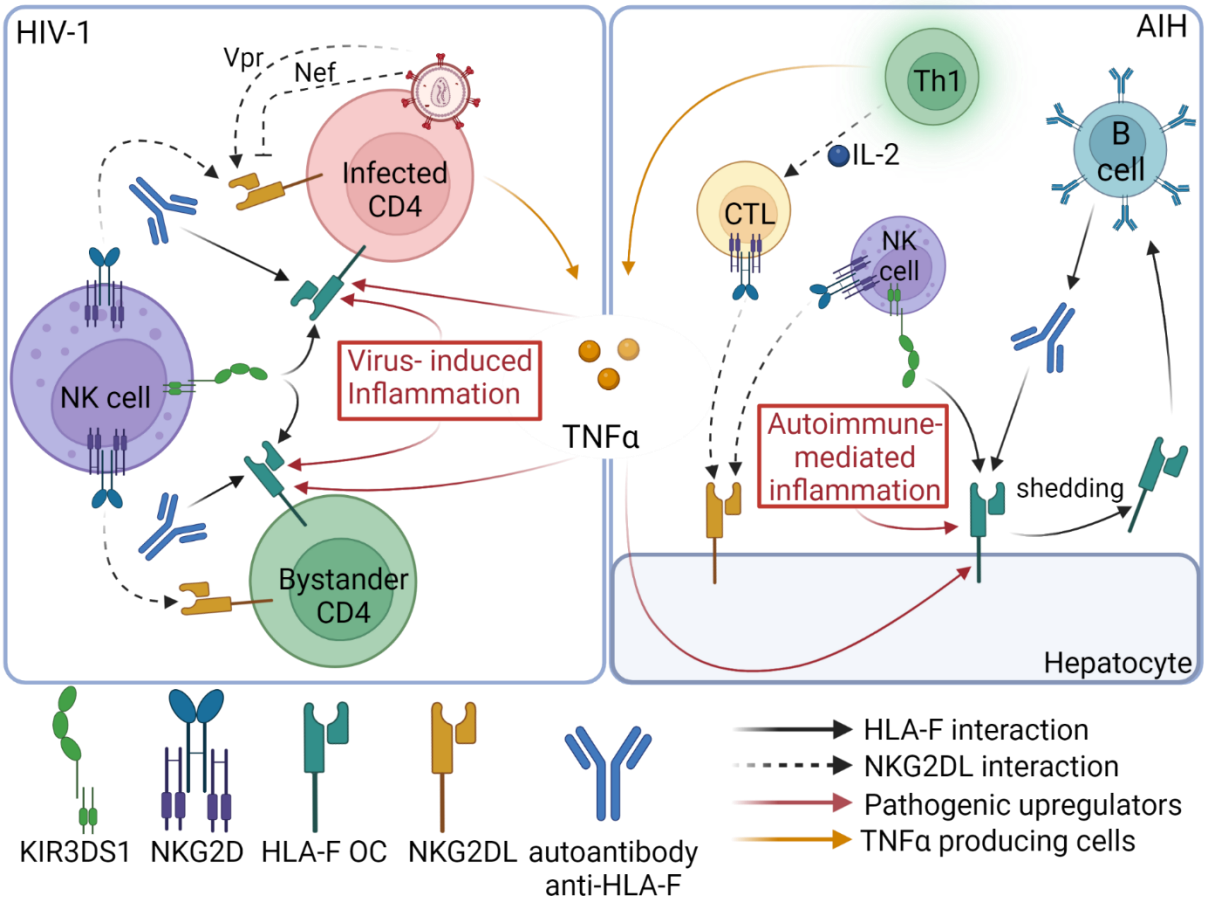


Figure 23 HLA-F in HIV-1 and AIH: working model. CTL: cytotoxic lymphocyte, Th1: T helper 1 cell, *created with BioRender.com*

In summary, the following working model is proposed (**Fig 23**):

In HIV-1 infection, HLA-F surface expression is induced via the ongoing immune activation, along with the stress ligands NKG2DL. This immune activation is fueled by the pro-inflammatory environment, especially the elevated levels of TNFα, produced by infected CD4 T cells. Such immune activation upregulates HLA-F surface expression in infected and uninfected activated

(bystander) CD4 T cells. NK cells expressing the activating receptor KIR3DS1 and NKG2D are then capable of killing activated (and/or infected) T cells by targeting HLA-F and NKG2DL-expressing CD4 T cells. Anti-HLA-F autoantibodies can potentially assist this phenomenon by promoting ADCC against HLA-F-expressing CD4 T cells.

In AIH, the tolerance breakdown and expansion of TNF-producing Th1 cells leads to a chronic inflammation and damage of the liver. Such inflammation and cellular stress drives HLA-F surface expression on hepatocytes via our proposed two-step model: mRNA expression is upregulated by the pro-inflammatory stimulation (TNFα) and surface expression is induced by lifting ER retention due to the ongoing cellular stress. The ongoing inflammation also upregulates NKG2DL, leading to potential hepatocyte killing by cytotoxic lymphocytes (CTL) via the NKG2DL:NKG2D axis and by NK cells via the combination of HLA-F:KIR3DS1 and NKG2DL:NKG2D axes.

Hepatocyte lysis and potential HLA-F shedding could lead to the production of autoantibodies against HLA-F. Anti-HLA-F autoantibodies could lead to ADCC against HLA-F expressing hepatocytes, further aggravating the chronic inflammation. Altogether, in this model, viral and autoimmune diseases lead to HLA-F surface expression because of the chronic pro-inflammatory environment induced by these pathologies.

6 Conclusions

This thesis work characterized HLA-F is a potential marker of persisting immune activation in HIV-1 infection and in AIH. Given the conserved expression of *HLA-F,* it is likely relevant in other inflammatory pathologies or cancers as well. Potential applications of HLA-F as a marker could entail the tracking of disease progression in HIV-1, and intensity of onset/flares in autoimmune disease, or as an indicator of treatment efficiency. Additionally, culture and stimulation of hepatocyte organoids revealed that this 3D-culture model offers the potential to study HLA-F in liver disease. In summary, HLA-F remains an enigmatic stress ligand that can trigger NK cells through KIR3DS1 and potentially through ADCC. Future studies will need to further elucidate to what extent this interaction shapes the course of HIV-1 and AIH disease progression.

Materials and methods

1 Materials

1.1 Antibodies

Marker	Fluoro-phore	Clone	Manufacturer	Cat#	Dilution	Application
CD107a	PE-Cy7	H4A3	Biolegend	328618	1:100	Fig 16 Fig 17
CD107a	BV510	H4A3	Biolegend	328632	1:100	Fig 15
CD127	KIRAVIA FITC	A019D5	Biolegend	351360	1:25	Fig 3-6
CD14	APC-Cy7	HCD14	Biolegend	325620	1:100	Fig 7-9
CD14	APC-Cy7	63D3	Biolegend	367108	1:100	Fig 3-6
CD155 (PVR)	PE-Cy7	SKII.4	Biolegend	337613	1:50	Fig 3-6
CD16	BV785	3G8	Biolegend	302046	1:100	Fig 7-9 Fig 14 Fig 15
CD19	APC-Cy7	HIB19	Biolegend	302218	1:100	Fig 3-6 Fig 7-9
CD25	BV650	BC96	Biolegend	302634	1:100	Fig 3-6
CD3	AF700	UCHT-1	Biolegend	300424	1:100	Fig 7-9
CD3	BUV395	UCHT-1	Becton Dickin-son	563546	1:100	Fig 3-6 Fig 12 Fig 13 Fig 14-17
CD3	BUV737	UCHT1	Biolegend	564307	1:100	Fig 10-13
CD38	BV711	HIT2	Biolegend	303528	1:200	Fig 12
CD38	BV785	HIT2	Biolegend	303530	1:200	Fig 10-13
CD38	BV785	HIT2	Biolegend	303530	1:100	Fig 3-6
CD4	AF700	RPA-T4	Biolegend	300526	1:100	Fig 12 Fig 13
CD4	BV711	RPA-T4	Biolegend	300558	1:100	Fig 16 Fig 17
CD4	PerCP-Cy5.5	OKT4	Biolegend	317432	1:200	Fig 3-6
CD45RA	BV711	HI100	Biolegend	304138	1:500	Fig 3-6
CD56	BV510	HCD56	Biolegend	318339	1:100	Fig 7-9
CD56	BUV737	NCAM16.2	Becton Dickin-son	612766	1:100	Fig 3-6 Fig 14-17
CD8	FITC	RPA-T8	Biolegend	301006	1:100	Fig 12
CD8	PE-Cy7	SK1	Biolegend	344712	1:200	Fig 7-9
CD8	AF700	SK1	Biolegend	344724	1:300	Fig 3-6
HLA-DR	BV510	L243	Biolegend	307645	1:50	Fig 3-6
HLA-DR	BV510	L243	Biolegend	307646	1:100	Fig 11 Fig 16 Fig 17
HLA-DR	FITC	L243	Becton Dickin-son	347363	1:50	Fig 21

HLA-DR	FITC	G46-6	Becton Dickinson	555811	1:100	Fig 16 Fig 17
HLA-DR	APC	G46-6	Becton Dickinson	560896	1:100	Fig 22
HLA-E	APC	3D12	Biolegend	342606	1:50	Fig 6 Fig 21
HLA-E	APC	3D12	Biolegend	342606	1:25	Fig 12 Fig 13
HLA-E	BV421	3D12	Biolegend	342612	1:25	Fig 3-6 Fig 16-17 Fig 19-22
HLA-F	PE	3D11	Biolegend	373204	1:50	Fig 6
HLA-F	PE	3D11	Biolegend	373204	1:25	Fig 10-13
HLA-F	PE	3D11	Biolegend	373204	1:20	Fig 3-6
HLA-F	PE	3D11:HLA-F	Biolegend	373204	1:25	Fig 19-22
HLA-F	APC	3D11	Biolegend	373208	1:25	Fig 16 Fig 17
HLA-F (mouse IgG)	purified	3D11:HLA-F	Biolegend	373202	1:50	Fig 18
Hoechst			Invitrogen	C10083	1:2000	Fig 18
human IgG	PE-cy7	G18-145	Becton Dickinson	561298	1:50	Fig 6
human IgM	APC	MHM-88	Biolegend	314510	1:100	Fig 6
Iso IgG1κ C	PE	MOPC-21	Biolegend	400114	1:40	Fig 3-6 Fig 19-22
Iso IgG2a, κ C	APC	MOPC-173	Biolegend	400221	1:20	Fig 3-6
KIR3DL1	FITC	DX9	Biolegend	312706	1:100	Fig 7-9 Fig 14 Fig 15
KIR3DL2	PE	539304	R&D	FAB2878P	1:50	Fig 7-9
KIR3DS/L1	APC	REA168	Miltenyi	130-104-485	1:50	Fig 7-9 Fig 15
KIR3DS1-Fc			R&D systems	4136-KR	25μg/ml	Fig 13
KIR3DS1L1	PE	Z27.3.7	Beckman coulter	IM3392	1:50	Fig 14
LILRB1	PerCP-Cy5.5	GHI/75	Biolegend	333714	1:100	Fig 7-9
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit	APC-Cy7		Life Technologies	L34976	1:1000	All flow cytometry stainings
MICA/B	PE	6D4	Biolegend	320906	1:100	Fig 17

MICA/B	APC	6D4	Biolegend	320908	1:20	Fig 3-6
Mouse IgG	AF647	polyclonal	Invitrogen	A32787	1:500	Fig 18
NKG2D	BV421	1D11	Biolegend	320822	1:100	Fig 7-9 Fig 15
NKG2D	BV605	1D11	Biolegend	320832	1:100	Fig 17
PDL1	BV785	29E.2A3	Biolegend	329736	1:50	Fig 22
PDL1	BV785	29E.2A3	Biolegend	329735	1:25	Fig 21
Rabbit IgG	AF546	polyclonal	Invitrogen	A10040	1:500	Fig 18
ULBP1	APC	BaF3	Bio-Techne	FAB1517 A	1:50	Fig 3-6
ULBP3	APC	BaF3	Bio-Techne	FAB1517 A	1:50	Fig 3-6
ULPB2/ 5/6	APC	BaF3	Bio-Techne	FAB1298 A	1:50	Fig 3-6
ZO-1 (Rabbit IgG)	purified	polyclonal	Invitrogen	40-2200	1:100	Fig 18

Table 7 List of antibodies

1.2 Reagents

1.2.1 Cell culture reagents

Reagent	Manufacturer	Cat#	Application
A83-01	Tocris	2939	HOM preparation
Advanced DMEM/F-12 (high glucose, GlutaMAX, pyruvate)	Gibco	12634-010	AD+++/ Wash medium preparation
B27 w/o vitamin A	Gibco	12587-010	HOM preparation
β-mercaptoethanol	Sigma Aldrich	M3148-25ml	NKM preparation
BME2Basement membrane extract, type 2 (BME2)	R&D	3532-010-02	hepatocyte organoids culture
Cell Trace Violet	Invitrogen	C34557A	CD4 proliferation tracking
CHIR99021	Tocris	4423	HOM preparation
Collagenase D	Sigma Aldrich	C9407-100mg	Liver digestion solution preparation
DMEM high glucose	Sigma Aldrich	D6429-500ml	adherent cell culture medium
DMSO	Sigma Aldrich	D5879-100ml	Freezing medium preparation
DNase I	Stemcell	07470	Liver digestion solution preparation
EasySep human CD4+ T cell enrichment kit	Stemcell	19052	CD4 enrichment
EasySep human NK cell enrichment kit	Stemcell	19055	NK enrichment
EBSS	Gibco	24010-043	Liver digestion solution preparation
EDTA	Promega	V4231	Isolation buffer preparation
EGF	Peprotech	AF-100-15-500UG	HOM preparation

Fetal bovine serum (FBS)	Capricorn scientific	FBS-11A	Supplement cell culture
FGF-10	Peprotech	AF-100-26-500UG	HOM preparation
FGF-7	Peprotech	100-19-50UG	HOM preparation
Gastrin	Sigma Aldrich	G9145-.5MG	HOM preparation
GlutaMax	Gibco	35050061	Supplement cell culture
HEPES	Gibco	15630-056	AD+++ preparation
HGF	Peprotech	100-39H-100UG	HOM preparation
n-acetyl-L-cysteine	Merck	A9165-25g	HOM preparation
Nicotinamide	Sigma Aldrich	N0636-100G	HOM preparation
Penicillin-Streptomycin	Sigma Aldrich	P4333-100ml	AD+++/ Wash medium preparation
recovery freezing medium	Gibco	12648010	Freezing medium hepatocyte organoids
RPMI-1640	Gibco	21875-034-500ml	non adherent cell culture medium
Rspondin conditioned medium	produced in-house	produced in-house	HOM preparation
TGF-α	Peprotech	100-16A-100UG	HOM preparation
TrypLE Express	Gibco	12605-028	Hepatoma cell line/ hepatocyte organoids trypsinization
Y-27632	Stemcell	72308	HOM preparation

Table 8 List of cell culture reagents

1.2.2 Cell culture stimulants

Stimulant	Concentration	Duration	Manufacturer	Cat#	Application
IFNα2	100 U/mL	5d	Invivogen	rcyc-hifna2b	Fig 20 Fig 21
IFNβ	100 U/mL	5d	Peprotech	300-02BC-20CG	Fig 20 Fig 21
IFNγ	500U/mL	5d	Peprotech	300-02-100UG	Fig 20 Fig 21
IL-15	1ng/ml	16h	Peprotech	200-15 500UG	Fig 17
IL-2	250 U/ml	7d	Peprotech	200-02 500UG	Fig 10-13 Fig 15 Fig 16
IL-2	1000U/ml	7d	Roche	11147528001	Fig 17
ImmunoCult Human CD3/CD28/CD2 T Cell Activator	5µl/10^6 cells	7d	Stemcell	10970	Fig 10-13
LPS	1µg/ml	2d cell line 5d heporgs	Sigma Aldrich	4391	Fig 20 Fig 21
NaP	10mM	24h	Sigma Aldrich	P5436-100G	Fig 17
Poly I:C	5µg/ml	5d	Invivogen	trlr-pic	Fig 21 Fig 22

TNFα	10ng/ml	5d	Peprotech	300-01A-50UG	Fig 20-22

Table 9 List of cell culture stimulants

1.2.3 Other reagents

Reagent	Manufacturer	Cat#	Application
1,5 mL Low protein binding tubes	Sarstedt	72.706.600	Fig 6
ACK lysing buffer	Gibco	A1049201	PBMC isolation
Benzonase Nuclease	Millipore	70664-3	Fig 3-6 Fig 7-9
Biotin-HLA-E	Fred Hutchinson cancer research center		Fig 6
Biotin-HLA-F	Fred Hutchinson cancer research center		Fig 6
BV stain buffer	Becton Dickinson	566349	Fig 3-6 Fig 7-9
DNeasy Blood and Tissue Kit	QIAGEN	69504	genomic DNA isolation
Hank's buffer	Capricorn scientific	HBSS-2A	PBMC isolation
Lymphocyte separation media	Capricorn scientific	LSM-A	PBMC isolation
PBS	Sigma Aldrich	D8537-500ml	various
Streptavidin Dynabeads	Thermo Fischer Scientific	11205D	Fig 6

Table 10 List of other reagents

1.3 Cell lines

Cell line	Source	Identifier
HEK293T	ATCC	CRL-3216
HepG2	ATCC	HB-8065
Huh7	ATCC	CVCL-0336
K562	ATCC	CCL-243

Table 11 List of cell lines

1.4 Device and software

1.4.1 Device

Device	Manufacturer	Application
BD LSR Fortessa	Becton Dickinson	Fig 3-6 Fig 10-13 Fig 14-17 Fig 19-22
BD FACS Aria	Becton Dickinson	Fig 14
BD Aria Fusion	Becton Dickinson	Fig 7-9
Eclipse Ti2	Nikon	Fig 18

Table 12 List of devices

1.4.2 Software

Software	Source	Version
Excel	Microsoft Corporation	2302
Fiji	[331]	2.11.0
Flowjo	Becton Dickinson	10.8.2
Prism	GraphPad software	9.5.1
Word	Microsoft Corporation	2302

Table 13 List of softwares

1.5 Buffers and solutions

Solution	Substance	Concentration
AD+++	Advanced DMEM/F-12 (high glucose, GlutaMAX, pyruvate)	97 % (v/v)
	GlutaMAX	1 % (v/v)
	HEPES	10 mM
	Penicillin/Streptomycin	1 % (v/v)
D10	DMEM high glucose	90% (v/v)
	FBS	10% (v/v)
freezing medium	FBS	90% (v/v)
	DMSO	10% (v/v)
HOM	AD+++	81 % (v/v)
	B27 w/o vitamin A	2 % (v/v)
	n-Acetyl-L-Cysteine	1,25 mM
	EGF	50 ng/ml
	Rspondin conditioned medium	15 % (v/v)
	FGF-7	100 ng/ml
	FGF-10	100 ng/ml
	HGF	50 ng/ml
	TGF-α	20 ng/ml
	Gastrin	10 nM
	CHIR99021	3 µM
	A83-01	2 µM
	Nicotinamide	10 mM
	Y-27632	10 µM
Isolation buffer	PBS	98% (v/v)
	EDTA	1mM
	FBS	2% (v/v)
NKM_V1	R10	90% (v/v)
	IL-2 (peprotech)	500U/ml
	Serum (from donor)	10% (v/v)
	55 mM beta-mercaptoethanol in PBS	0,1% (v/v)
NKM_V2	R10	90% (v/v)
	IL-2 (roche)	200U/ml
	Serum (from donor)	10% (v/v)
	55 mM beta-mercaptoethanol in PBS	0,1% (v/v)
R2	RPMI-1640	98% (v/v)
	FBS	2% (v/v)
R10	RPMI-1640	90% (v/v)
	FBS	10% (v/v)
Staining buffer	PBS	99,5% (v/v)
	FBS	0,5% (v/v)

Table 14 List of buffers and solutions

2 Methods

2.1 Cell culture and freezing

All cells were maintained at 37°C/5% CO_2. K562 were cultured at 3 × 10^5 cells/ mL in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (R10) and 1% Glutamax and were split 1:3 every third day.

HepG2, Huh7, and other cell lines were cultured at 2 × 10^5 cells/ mL in DMEM high glucose supplemented with 10% fetal bovine serum (D10) and split 1:4 using TrypLE Express every third day. Hepatoma cell lines were stimulated with IFNα+β, IFNγ, TNFα, Poly I:C and LPS according to **Table 9**.

For freezing, cells were harvested (trypsinized for adherent cells) and centrifuged 5 min at 500 × *g*. The pellet was then resuspended in 1 mL of freezing medium (see **Table 14**) per 1-50 times 10^6 cells and the solution distributed into cryotubes. Cryotubes were transferred into a freezing container filled with isopropanol and placed in a −80°C freezer to cool down at a rate of 1°C/min. On the next day, cryo-preserved cells were transferred to a liquid nitrogen tank for long-term storage.

Cryopreserved 1 mL-tubes containing cells in freezing medium were thawed in a 37°C water bath under gentle agitation for 30-45 sec. Cells were then immediately resuspended in 9 mL warm corresponding medium and centrifuged 5 min at 500 × *g*. The supernatant was aspirated and the pellet resuspended in warm medium for culture.

2.2 Peripheral blood mononuclear cell and serum isolation

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood from healthy blood donors by density gradient centrifugation. Fresh blood was resuspended in Hank's buffer to reach 40 mL per tube, and gently layered on top of 15 mL Lymphocyte separation media. The gradient was then centrifuged for 20 min at 950 × *g* with a slow start and no brakes (Acc1/Dec0). The interphase, composed of PBMC and platelets, was harvested using a 5 mL pipette, washed with up to 50 mL of Hank's buffer and centrifuged 5 min at 500 × *g*. The pellet was resuspended in 3 mL ACK lysing buffer and incubated for exactly 3 min to lyse residual erythrocytes. Following this incubation, the cells were washed in 40 mL Hank's buffer and centrifuged 5 min at 500 × *g*. To pellet only PBMC but not platelets, a final centrifugation of 13 min at 3000 × *g* was performed. PBMC were frozen according to the procedure described above.

Serum was isolated by centrifugation of whole blood 10 min at 200 × *g* and then collection of the supernatant was done. Following centrifugation for 15 min at 1000 × *g*, the supernatant was

collected, heat inactivated 30 min at 56°C in a water bath and sterile filtered. Serum samples were aliquoted in PCR tubes and conserved at – 20°C.

2.3 Antibody staining and flow cytometry

Samples were washed in PBS, transferred in a 96-well plate at a range of 2×10^4 to 2×10^5 cells/well and centrifuged 5 min at 500 × *g*. The antibody master mix was prepared in staining buffer (see **Table 14**) and added at 50 µL/well. All antibodies used in this thesis and corresponding dilutions are listed in **Table 7**. Cells were incubated for 30 min at 4°C in the dark, washed in staining buffer and centrifuged 5 min at 500 × g. Except for cell sorting samples and staining using the KIR3DS1/L1 antibody (Z27.3.7 clone), stained cells were fixed in 4% paraformaldehyde for 20 min at room temperature in the dark. Fixed cells were washed with staining buffer, centrifuged 5 min at 500 × *g* and resuspended in staining buffer at 200 µL/well before acquisition.

2.4 Staining of human PBMC samples

Cryopreserved PBMC from therapy-naïve HIV-1-positive individuals (*n* = 30) from the Translational Platform HIV (TP-HIV) Cohort by the German Center for Infection Research (DZIF) and cryopreserved PBMC from healthy individuals (*n* = 44) from the Healthy cohort Hansestadt Hamburg (HCHH) cohort were thawed as described above, after keeping 20% of frozen samples for genomic DNA isolation. Genomic DNA was isolated with the DNeasy Blood and Tissue Kit by collaborators from Körner lab. *HLA class I* and *KIR* genotyping was performed by the DKMS Life Science Lab, Dresden, Germany as described in[332,333]. Thawed cells were resuspended in 10 mL R10+ 25 U/ml Benzonase Nuclease and centrifuged for 10 min at 200 × *g*. The pellet was resuspended in 9 mL R10 and split into 3 equal samples for staining the two panels described in this thesis and an additional panel described in [61].

Samples used for the ligand panel were rested prior staining in R10+100 U/ml IL-2 (Peprotech) at 5 M/mL for 3 h at 37°C/5% CO_2 to minimize the surface expression of stress ligands due to the thawing. The first 15 healthy donors were excluded due to recording issue.

The TP-HIV ligand panel was a master mix in BV Stain Buffer containing a dump channel (viability, CD14, CD19), the lineage markers CD3, CD4, CD8, CD56, the CD4 subpopulation markers CD45RA, CD25, CD127, the activation markers CD38 and HLA-DR and the markers of interest HLA-F, HLA-E, CD155, the NKG2DL (MICA/B, ULBP 1, 2/5/6/, 3). Isotype, fluorescent minus one (FMO) staining for HLA-F and the NKG2DL and unstained samples were used as control.

The TP-HIV receptor panel was a master mix in BV Stain Buffer containing a dump channel (viability, CD14, CD19), the lineage markers CD3, CD8, CD16, CD56 and the markers of interest

NKG2D, LILRB1, KIR3DS1/L1, KIR3DL1 and KIR3DL2. FMO staining for NKG2D, LILRB1 and KIR3DL2 and unstained samples were used as control.

2.5 Autoantibodies detection assay

For coating magnetic beads with HLA-F/HLA-E, Streptavidin Dynabeads were vortexed for >30sec and 30 µL of the 6,5 × 10^5 beads/µL stock was transferred in a 1,5 mL low protein binding tube. Dynabeads were washed 3 times by adding 1 mL PBS, placing the 1,5-mL tube on a Dynabeads magnet for 30 sec and taking out the PBS.

Biotin control beads were coated by resuspending washed beads in 150 µL PBS and add 50 µL Biotin solution (10 mg/50 mL stock).

Biotinylated HLA-F and HLA-E proteins were obtained from the Fred Hutchinson Cancer Research Center (Seattle, USA) and coated by resuspending washed beads in 150 mL PBS and add 40 µL of the protein of interest diluted at 0,25 µg/µL.

Beads and their coating agent were incubated at 4°C on orbital shaker for 4 h to overnight (ON), washed 3 times as described above and resuspended in 300µl PBS to reach a final concentration of 6,5 × 10^4 coated beads/µL.

Plasma samples from TP-HIV DZIF cohort (*n* = 92) and healthy donors from HCHH cohort (*n*= 50) were stained for anti-HLA-F and -HLA-E autoantibodies. In a 96-well plate V-bottom, 10^5 beads were transferred. For each donor, one well with protein coated beads with 25 µL of plasma (stained well) and one well with biotin coated beads with 25 µL of plasma (donor background) were prepared. Per batch, one well with biotin coated beads with 25 µL of staining buffer (uncoated staining control), and two well with protein coated beads with 25 µL of staining buffer (coating control and coated staining control) were prepared.

All samples were incubated for 30 min on orbital shaker at room temperature and washed 3 times with 200 µL of staining buffer after centrifuging 3 min at 1000 × g. Stained, donor background, coated and uncoated staining controls were stained in a master mix in staining buffer for human IgG and IgM. The coating control well was stained in staining buffer for HLA-F or HLA-E. Stained wells were incubated 30 min on orbital shaker at room temperature and washed 3 times with 200 µL of staining buffer after centrifugation 3 min at 1000 × g. Samples were fixed with 2% paraformaldehyde, incubated 20min at room temperature, centrifuged 3 min at 1000 × g, and resuspended in 200 µL staining buffer before acquisition.

2.6 Magnetic enrichment of primary immune cells and fluorescence-activated cell sorting

Primary CD4 T and NK cells were negatively enriched from fresh or cryopreserved PBMCs using respectively the EasySep human CD4+ T cell enrichment kit and EasySep human NK cell enrichment kit. PBMC were resuspended at 5 M/mL in isolation buffer (see **Table 14**) according to manufacturer's recommendation, using half of recommended antibody mix and magnetic beads.

Cell sorting of NK cells was performed by staining 1×10^8 PBMCs for flow cytometry in 1000 µL following the procedure detailed above. Stained cells were resuspended in 2 mL R10 (see **Table 14**) and sorted by gating singlets, viable CD3-, CD16/56 NK cells, and setting the sorting gates on KIR3DS1L1+/KIR3DL1- and KIR3DS1L1-/KIR3DL1- (**Fig 14, A**).

2.7 *In vitro* stimulation, cell tracing and staining of CD4 T cells

PBMC from healthy donors (*n* = 22) were obtained from the HCHH cohort and were enriched for CD4 T cells as described earlier. CD4 T cells were cultured at 1×10^6 cells/mL in R10 supplemented with 250 U/mL IL-2 (Peprotech) at 37°C/5% CO_2 in 96 well plate V-bottom. CD4 stimulation was performed by adding 5 µL /10^6 cells ImmunoCult Human CD3/CD28/CD2 T Cell Activator. Half of the medium was exchanged every second or third day, with restimulation every second medium exchange.

Prior to seeding for culture, CD4 T cells were stained with cell trace violet (CTV). The cells were washed with PBS, centrifuged 5 min at 500 × g and resuspended at 2×10^6 cells/mL in warm PBS prior staining. 0,5 µL CTV/10^6 CD4 T cells were added and incubated for exactly 8 min at room temperature. To stop the staining, 1 volume of FBS was added to the staining mix and incubated for 1 min at room temperature. 5 volumes of R10 were then added to the mix and incubated for 5 min at room temperature, before filling the tube up with R10 and centrifugating 5 min at 500 × g. The washed cells were then resuspended in the corresponding medium and cultured as described above.

For the optimization experiment, enriched CD4 T cells (*n*=3) were cultured for 0, 2, 5, and 7 days as described above and stained as described above for viability, the proliferation marker CTV, the lineage markers CD3, CD4 and CD8, the activation markers CD38 and HLA-DR and the marker of interest HLA-F. Isotype staining for HLA-F and unstained samples were used as controls.

For the replicated staining, enriched CD4 T cells (*n*=22) were cultured for 7 d as described above and stained as described above with the addition of HLA-E.

For the KIR3DS1-Fc staining, enriched CD4 T cells (*n*=17) were cultured for 7 d as for the replicated staining. CD4 T cells were resuspended in staining buffer at 1 × 10^5 per condition, resuspended with 25 µg/mL KIR3DS1-FC and incubated 45 min at 4°C on orbital shaker. Stained cells were washed with cold staining buffer, centrifuged 5 min at 500 × g and stained at 50 µL /well with a master mix in staining buffer containing viability marker, anti-human IgG-Fc, CD3 and CD4. The cells were incubated 30 min at 4°C on orbital shaker, washed with staining buffer and centrifugated 5 min at 500 × g before fixation in 2% paraformaldehyde as detailed above. Secondary only and unstained samples were used as control.

2.8 NK cell co-incubation with stimulated autologous CD4 T cells and CD107a assay

PBMC and serum from healthy donors (*n*=4) were obtained from the HCHH cohort. CD4 T cells and NK cells were enriched as described earlier.

For the optimization experiment (**Fig 15**), K562 cells were cultured as described earlier. PBMCs were cultured at 1 × 10^6 cells/ mL for 4 days in NKM_V1 media (see **Table 14**) at 37 °C/5 % CO_2 prior to NK sorting or isolation. Sorted and isolated NK cells were cultured at 1 × 10^6 cells/ mL for 3d in NKM_V1 at 37 °C/5 % CO_2 prior co-culture. For co-culture, 5 × 10^4 K562 cells were seeded and NK cells were added to reach an E:T ratio of 1:1 and 2:1 in a final volume of 200 µL in R10. Co-culture conditions were incubated 16 h at 37 °C/5 % CO_2 with CD107a antibody. NK only and K562 only conditions were used as controls. After co-incubation, cells were washed once and stained for flow cytometry as described earlier.

For the HLA-F bystander killing experiment (**Fig 16**), CD4 T cells were cultured and stimulated as described earlier. K562 cells were cultured as described earlier. PBMCs were cultured at 1 × 10^6 cells /mL for 3 days in NKM_V1 (see **Table 14**) at 37 °C/5 % CO_2 prior NK sorting. Sorted NK cells were cultured at 1 × 10^6 cells /mL for 4 days in NKM_V1 at 37 °C/5 % CO_2 prior co-culture. For co-culture, 5 × 10^4 K562 or CD4 T cells were seeded and NK cells were added to reach an E:T ratio of 1:1 and maximum effector cells:1 in a final volume of 200 µL in R10. Co-cultures were incubated for 16 h at 37 °C/5 % CO_2 with CD107a antibody. NK only and CD4 only conditions were used as controls. After co-incubation, cells were washed once and stained for flow cytometry as described earlier.

For the NaP stimulation experiment (**Fig 17**), CD4 T cells were cultured at 1 × 10^6 cells / mL in R10 supplemented with 250 U/ mL IL-2 at 37°C/5% CO_2 and split to 1 × 10^6 cells/mL every third day. CD4 stimulation was performed by adding 5 µL ImmunoCult Human CD3/CD28/CD2 T Cell Activator / 10^6 cells and, when specified, 10 mM sodium propionate for 24 h. K562 cells were

cultured as described earlier. Sorted NK cells were cultured at 2 × 10^6 / mL for 7 days in NKM_V2 (see **Table 14**) at 37 °C/5 % CO_2 prior to co-culture. For co-culture, 5 × 10^4 K562 or CD4 T cells were seeded and NK cells were added to reach an E:T ratio of 1:1 in a final volume of 200 µL in R10 supplemented with 100 U/ mL IL-2 (Roche) and 1 ng/ mL IL-15. Co-cultures were incubated for 16 h at 37 °C/5 % CO_2 with CD107a antibody. NK, CD4 and K562 only conditions were used as controls. After co-incubation, cells were washed once and stained for flow cytometry as described earlier.

Target cell lysis (as percentage) was determined using the following formula: *lysis=(1-(target cell count/average(target only cell count))*100* , where target cell count is the count of target cells in a given co-culture well and target only cell count is the count of target cells in control wells without effector cells.

2.9 Liver sample processing

Tumor-free liver samples were obtained from patients at the Asklepios Hospital Barmbek (AKB) undergoing extended liver resection due to liver metastases following colorectal cancer, liver adenoma, cholangiocellular carcinoma or hemangioma, and at the University Medical Center Hamburg-Eppendorf (UKE) from patients undergoing liver transplantation due to end-stage liver disease.

The liver tissue was stored in AD+++ (see **Table 14**) at 4 °C until processing. The liver tissue was cut into pieces of ~0.5 mm using a scalpel and tweezers in a 10 cm Petri dish. Liver pieces were transferred in a 50 ml tube and washed two times with washing medium (see **Table 14**) by pipetting up and down with a 25 mL pipette to wash off blood and fat. The washing medium was completely removed, and 5 mL/ g of prewarmed liver digestion solution (see **Table 14**) was added before incubating the tissue for 30 min at 37 °C in a water bath. Once single cells were detectable using light microscopy, the digestion was stopped by adding 25 mL of 4 °C wash medium and pipetting up and down with a 25 ml pipette. Digested cells were sterile filtered through 70 µm cell strainer and centrifuged at 300 × g for 5 min at 4 °C. The pellet was collected and washed twice with 4 °C wash medium. Digest was frozen at 1 × 10^7 cells/ vial using 1 mL recovery freezing medium or directly seeded.

2.10 Hepatocyte organoids culture and stimulation

Digested liver samples requiring seeding were centrifuged 5 min at 300 × g 4 °C and resuspended in 1 mL AD+++ in a 1.5 mL tube. The samples were centrifuged for 1 min on a desk centrifuge and resuspended in 4 °C HOM (see **Table 14**). 4 °C BME2 was added at a ¼ medium/BME2 ratio

with extreme caution to avoid air bubbles and 30 µL droplets were seeded per well on overnight-prewarmed 24 well plate. Droplets were incubated 2 min at 37 °C to harden BME2 and 500 µL of 37 °C HOM was added per well. The cells were maintained at 37 °C/5 % CO_2. Medium was exchanged every second or third day and passaging was performed when relative confluence in the droplet was reached.

Hepatocyte organoids harvesting was performed by removing the medium, adding 1 mL of 4 °C AD+++ to dissolve the BME2 gel and pulling up to 3 wells in a 15 mL tube on ice. The tube was filled with 4 °C AD+++ and pipetted up and down using a 10 mL pipette. Cells were centrifuged 5 min at 300 × g 4 °C, and the pellet was resuspended in 200 µL 4 °C AD+++ and transferred in a 1,5 mL tube.

Single cell suspension was obtained by vigorously pipetting up and down 100x with a P100 followed by a P10 pipette tips and filling up to 1 mL 4 °C AD+++. Single cell suspension was seeded at a ratio of 1:6 as described above or frozen by resuspending the content of 1 well in 1 mL recovery freezing medium and transferring 500 µL per cryovial.

Cryopreserved 1 mL-tubes containing cells in freezing medium were thawed in a 37 °C water bath under gentle agitation for 30-45 sec. Cells were then immediately resuspended in 9 mL warm AD+++ and centrifuged 5 min at 300 × g 4 °C. An additional wash with 10 mL AD+++ was performed followed by a 5 min at 300 × g 4 °C centrifugation before seeding as described above.

Hepatocyte organoids from AKB (*n*=3) and AIH LTX (*n*=3) donors were stimulated with IFNα+β, IFNγ, TNFα, Poly I:C and LPS according to **Table 9**.

2.11 Hepatocyte organoids flow cytometry and immunofluorescence staining

For flow cytometry staining, hepatocyte organoids were harvested as detailed above and single cell solutions were prepared by resuspending the harvested pellet in 500 µL 37 °C TrypLE Express and incubating at 37 °C for 10 min in a water bath, with mixing after 5 min. Once single cells were detectable using light microscopy, the digestion was stopped by adding 10 mL of 4 °C AD+++ and centrifuging 5 min at 500 × g and 4 °C. Single cells were stained for flow cytometry as described earlier. Antibodies used and corresponding dilutions are listed in **Table 7**.

For immunofluorescence staining, the published protocol of Dekkers *et al.* 2019 was used[277]: hepatocyte organoids were recovered from the 3D matrix and transferred to a 15 mL tube. Samples were then fixed and blocked, washed and immunolabeled overnight at 4 °C for the primary and secondary antibody stains. Samples were then transferred to a 1.5 mL tube,

resuspended in fructose-glycerol clearing solution, and placed on a slide. Secondary only and Hoechst only were as control stains.

2.12 Ethics

Donors recruited through the Healthy cohort Hansestadt Hamburg (HCHH) cohort provided written informed consent and the study was approved by the ethics committee of the Ärztekammer Hamburg (PV4780).

Donors recruited through the Translational Platform HIV (TP-HIV) Cohort by the German Center for Infection Research (DZIF) provided written informed consent and the study was approved by the ethics committee of the Ärztekammer Hamburg (MC-316/14).

Donors recruited through the Asklepios Hospital Barmbek (AKB) and University Medical Center Hamburg-Eppendorf (UKE) provided written informed consent and the study was approved by the ethics committee of the Ärztekammer Hamburg (PV4898, PV4081)

2.13 Data acquisition and analysis

The acquisition of flow cytometric data was performed with a BD LSR Fortessa and BD FACS fusion and the sorting of NK cells was performed with a BD FACS Aria fusion in the core facility Fluorescence Cytometry at the Leibniz Institute of Virology and analyzed using FlowJo software. Graphical and statistical analyses were performed using GraphPad Prism software (versions listed in **Table 13**). The Mann-Whitney test was used to estimate differences between unpaired sample groups. The Wilcoxon test was used to estimate differences between paired sample groups. The Fisher's exact test was used to estimate difference between two categorical variables. *P* values were not corrected for multiple testing.

Statistical parameters are stated in the results section as well as in each figure legend.

Immunofluorescence stainings were acquired on a Nikon Eclipse Ti2 and analysed using Fiji software (version listed in **Table 13**).

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Acknowledgements

This thesis has been a long journey and could not have been possible without help. I would like to take the opportunity to thank:

My parents, **Adrien** and **Malaury** for their love and support.

Baptiste and **Caro** for all the discussions, good chat and laughs that carried me through this thesis.

Angelique Hölzemer for giving me the opportunity to work under her supervision on this project. You were a wonderful mentor and made me learn a lot. Your dedication, knowledge, kindness openness to discussion and the help you provided at anytime despite your schedule were incredible.

Wilfredo Garcia-Beltran and his lab for the great discussions and help on this project.

Marcus Altfeld for welcoming me in his lab and the helpful comments during lab meetings.

Heike Hildebrandt and **Christin Illig** for their technical support. You are the reason this lab organization is flawless.

Leonore, Benedetta, Ana, Maria, Johannes, Gloria, Timo, Ole, Jenny, Kerri, Sarah, Pia, Nikolaos, Maya, Friederike, Deborah, Julia, Britta, Andrea for many wonderful moments and conversations. You are awesome people.

I warmly thank the many colleagues I have had the pleasure meeting at the Leibniz Institute of Virology. Thank you for the amazing time.

This thesis was supported through research funding by the State of Hamburg, Germany (LFF-FV78)

Eidesstattliche Versicherung

Declaration under oath

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

I hereby declare, under oath, that I have written the present dissertation on my own and have not used any resources or aids other than those acknowledged.

Hamburg, den 16.05.2023 Unterschrift