KIR3DS1 Directs NK Cell-mediated Control of Adenovirus and BK Polyomavirus Infection

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Table of Abbreviations

ADCC	Antibody-dependent cytotoxicity
ADP	Adenovirus death protein
AF647	Alexa Fluor 647
AIDS	Acquired immune deficiency syndrome
allo-HSCT	Allogeneic hematopoietic stem cell transplantation
AMP	Antimicrobial peptide
BKPyV	BK polyomavirus
BKVAN	BK polyomavirus-associated nephropathy
BMP	Bone morphogenetic protein
CAR	Chimeric antigen receptor
CCL	C-C motif chemokine
cGAS	Cyclic guanosine monophosphate adenosine monophosphate synthase
CMV	Cytomegalovirus
CXADR	Coxsackievirus and adenovirus receptor
CXCL	C-X-C motif chemokine
DAP	DNAX-activating protein
DDX41	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41
DPI	Days post-infection
dsDNA	Double-stranded DNA
E3/gp19K	E3/glycoprotein19K
EBV-HLH	Epstein-Barr virus associated hemophagocytic lymphohistiocytosis
ECM	Extracellular matrix
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
Fc	Fragment constant
FMO	Fluorescence minus one
GvHD	Graft-versus-host disease
HAdV5	Human adenovirus type 5
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIO	Human intestinal organoid
HIV	Human immunodeficiency virus
HLA-I	Human leukocyte antigen class I
HPV	Human papillomavirus

IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
IgA	Immunoglobulin A
IL	Interleukin
ISC	Intestinal stem cell
ISRE	IFN-stimulated response element
ITAM	Immunoreceptor tyrosine-based activation motif
K_d	Dissociation constant
KDDB	KIR and Disease Database
KIR	Killer cell immunoglobulin-like receptor
KSHV	Kaposi sarcoma-associated herpesvirus
LGR5	Leucine-rich-repeat-containing G-protein-coupled receptor 5
LILR	Leukocyte immunoglobulin-like receptor
LP	Lamina propria
LT antigen	Large tumor antigen
MHC	Major histocompatibility complex
MIC	MHC class-I-chain-related protein
mRNA	Messenger ribonucleic acid
NCR	Natural cytotoxicity receptor
Nef	Negative regulatory factor
NF-κB	Nuclear factor κB
NK cell	Natural killer cell
NKG2	Natural killer group 2
OC	Open conformer
PRR	Pattern recognition receptor
PSC	Pluripotent stem cell
RID	Receptor internalization and degradation protein
RPTEC	Renal proximal tubule epithelium
STAT	Signal transducer and activator of transcription
TA cell	Transit-amplifying cell
TAP	Transporter associated with antigen processing
TERT	Telomerase reverse transcriptase
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
ULBP	UL16-binding protein

UPR	Unfolded protein response
ZO-1	Zona occludens 1

Publications List

This thesis is presented in cumulative form based on the following publications.

Publication #1 J. M. Jung, W. Ching, M. E. Baumdick, H. Hofmann-Sieber, J. B. Bosse, T. Koyro, K. J. Möller, L. Wegner, A. Niehrs, K. Russu, M. Ohms, W. Zhang, A. Ehrhardt, K. Duisters, E. Spierings, A. Hölzemer, C. Körner, S. A. Jansen, S. Peine, I. Königs, M. Lütgehetmann, D. Perez, K. Reinshagen, C. A. Lindemans, M. Altfeld, M. Belderbos, T. Dobner, M. J. Bunders, KIR3DS1 directs NK cell–mediated protection against human adenovirus infections, *Sci. Immunol.* **6** (2021).

Publication #2 T. F. Koyro, E. Kraus, S. Lunemann, A. Hölzemer, S. Wulf, J. Jung, P. Fittje, F. Henseling, C. Körner, T. B. Huber, A. Grundhoff, T. Wiech, U. Panzer, N. Fischer, M. Altfeld, Upregulation of HLA-F expression by BK polyomavirus infection induces immune recognition by KIR3DS1-positive natural killer cells, *Kidney Int.* **99**, 1140–1148 (2021).

Abstract

Human adenovirus type 5 (HAdV5) and BK polyomavirus (BKPyV) are chronic viruses that cause severe infections in immunocompromised individuals. Reactivation of HAdV5 is a major threat for patients receiving allogeneic hematopoietic stem cell transplantation (allo-HSCT), while BKPyV reactivation after renal transplantation leads to the serious pathology BKPyV-associated nephropathy (BKVAN). Genetic epidemiological studies in patient cohorts consistently report associations of loci encoding for killer cell immunoglobulin-like receptors (KIRs) with protection from viral diseases. These KIRs are used by natural killer (NK) cells to recognize human leukocyte antigen class I (HLA-I) expressed on infected target cells and thus contribute to the control of viral infections. In this work, profiling of NK cell ligands on host cells infected with HAdV5 and BKPyV identified *de novo* induction of the non-classical HLA class I molecule HLA-F, a high affinity ligand for the activating KIR3DS1 receptor. The upregulation of HLA-F surface expression enabled recognition of HAdV5 and BKPyV host cells by KIR3DS1 and triggered activation of co-cultured KIR3DS1$^+$ reporter and primary NK cells. KIR3DS1$^+$ NK cells further showed enhanced cytotoxicity against HAdV5-infected intestinal epithelial cells derived from human intestinal organoids. Immunoimaging of tissue samples obtained from BKVAN patients exhibited elevated HLA-F protein levels and retrospective statistical analyses of a pediatric patient cohort revealed a protective association of the *KIR3DS1*$^+$/*HLA-Bw4*$^+$ compound genotype in HAdV reactivation after allo-HSCT. Considering the lack of safe antiviral medication for severe HAdV and BKPyV infections, the KIR3DS1–HLA-F immune axis constitutes a promising basis for developing effective immunotherapies.

1 Introduction

1.1 Innate immune system

The human immune system is an interactive network of cellular and humoral processes protecting the organism from invading microbial and viral pathogens. The defense against infection is organized in two fundamental immune responses determined by the specificity and speed of the reaction *(1)*. The adaptive, or acquired, immune response uses antibodies and lymphoid cells raised against specific pathogenic entities to protect the body from prolonged and re-occurring infections. The innate immune system emerged earlier in evolution and is characterized by its immediate response. It reacts to infections with a pre-programmed repertoire of biochemical agents including inflammatory cytokines and complement proteins and mobilizes several classes of innate immune cells *(2)*.

1.1.1 Natural killer cells in viral infection

A large proportion of the innate immune surveillance consists of innate immune cells from the myeloid lineage, including neutrophils and phagocytes, but it also features a distinct class of effector lymphocytes called natural killer (NK) cells. NK cells make up 10-15% of circulating lymphocytes in the human blood system and are minimally defined by the expression of the neural cell adhesion molecule (CD56), while not expressing the T cell co-receptor (CD3); albeit lineage markers change during NK cell development and vary across different body compartments *(3, 4)*. The term “natural killer” derives from cytotoxicity against malignant cells, yet newer studies highlight a broader involvement of NK cells in immune cell communication, tissue inflammation and homeostasis, and even suggest adaptive immune features *(5–8)*.

NK cells play a pivotal role in the early immune surveillance against viral pathogens *(9)*. Contrary to adaptive effector cells, the cytolytic activity of NK cells is fully regulated by pre-expressed immune-regulatory proteins *(10)*. This enables NK cells to recognize and eliminate host cells at early stages of infection, helping to confine viral replication and dissemination *(11, 12)*. NK cells can induce apoptosis in target cells *via* the Fas mechanism and by antibody-dependent cytotoxicity (ADCC) mediated by the FCγRIII receptor (CD16) *(5)*.

A third cytotoxicity mechanism is controlled through germline-encoded inhibitory and activating receptors, which are expressed in a stochastic and variegated pattern on the cell surface, resulting in a plethora of phenotypically and functionally distinct NK cell subsets *(13, 14)*. Through interaction with surface molecules on inspected target cells, these receptors transmit activating and inhibitory signals into the cell and the subsequent balance of integrated signals determines whether the NK cell is activated *(10, 15)*. A surplus of activating signals induces the release of cytoplasmic granules, which contain cytolytic mediators, perforin and serine proteases, that destroy the target cell. Activated NK cells can additionally respond to viral infection with the production of chemokines and cytokines *(5)*. NK cell-derived interferon-γ is an important mediator for early activation and maturation of immune cells and promotes cellular

resistance to infection *(16)*. The potential for cytokine production is loosely correlated with high expression of CD56 ($CD56^{bright}$) and low or lacking expression of CD16 ($CD16^{dim/neg}$) *(17)*. On the other hand, $CD56^{dim}CD16^{high}$ NK cells have an increased cytotoxic potential, which is associated with the expression of immunoglobulin-like receptors (KIRs) *(18)*.

1.1.2 Antiviral NK cell receptors

The interaction of an NK cell with a healthy cell usually results in inhibition of the NK cell ("tolerance", Fig. 1) *(13, 19)*. This is ensured through the interaction of inhibitory NK cell receptors with human leukocyte antigen class I (HLA-I) molecules, which are expressed by all nucleated cells under physiological conditions *(10, 20)*. HLA represents the human version of the major histocompatibility complex (MHC), which regulates the immune system by presenting antigenic peptides to immune cells *(21)*. The HLA-I-mediated inhibition of NK cells largely depends on the activity of inhibitory KIRs, more specifically, KIR2DL1/2/3/5 and KIR3DL1/2 *(11)*.

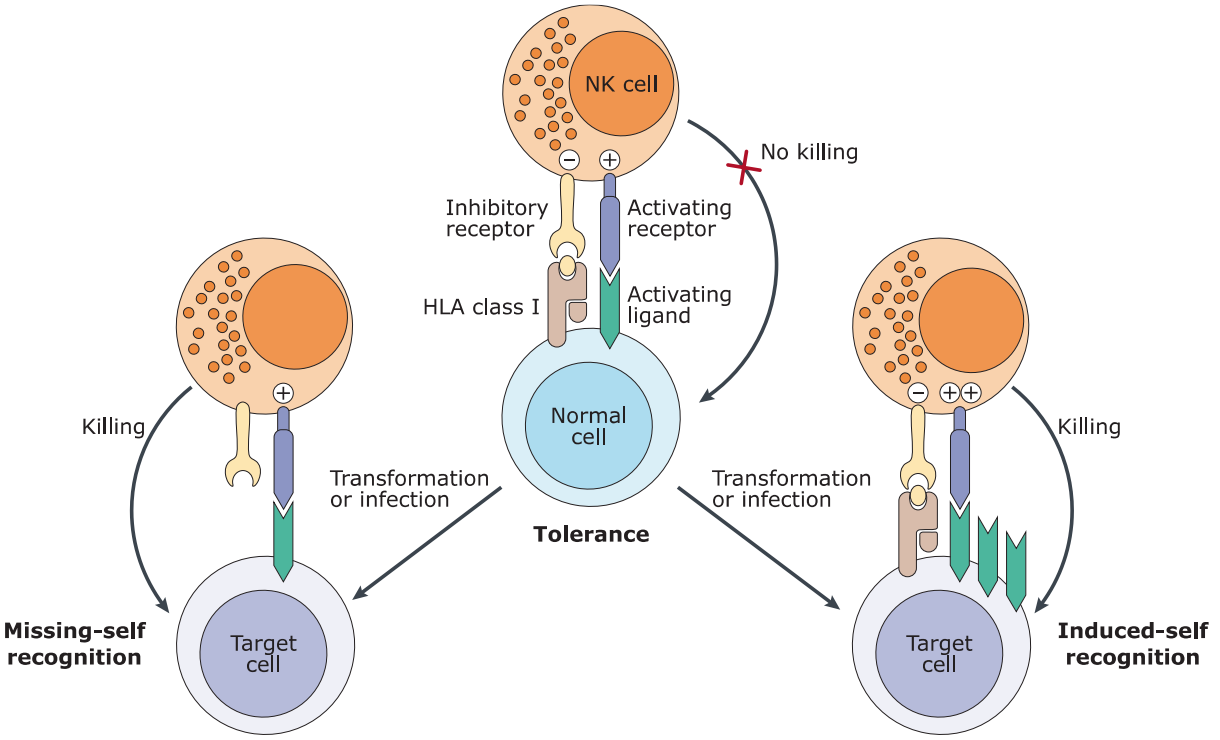

Fig. 1. Outcomes from NK cell inspection of normal and target cells. Tolerance (middle) describes a balanced state of inhibitory and activating receptor–ligand interactions, ensuring passivity of NK cells in physiological encounters. Transformation or infection can disrupt the tolerant state by establishing two forms of immune ligand dysregulation, both leading to net-activation of the NK cell and killing of the target cell. Missing-self recognition (left) results from lacking engagement of inhibitory receptors caused by a downregulation of HLA-I on the target cell. Induced-self recognition (right) describes a surplus of activating signals due to upregulated levels of activating ligands on the target cell, which stimulate the NK cells through activating receptors. Adapted from *(19)*.

The first digit in the KIR acronym indicates the number of extracellular immunoglobulin-like domains (2D and 3D) in the molecule, while the final digit specifies the gene number. The "L" indicates a "long" cytoplasmic tail, which contains immune-receptor tyrosine-based inhibitory motifs (ITIMs) transmitting inhibitory signals *(22)*. Another well-described inhibitory receptor is the heterodimer formed by CD94 and natural killer group 2A (NKG2A), a member of the C-

type lectin-like receptor family, which interacts with HLA-I *(23)*. In the event of missing HLA-I molecules on the target cell ("missing-self", Fig. 1), NK cells are activated due to lacking engagement of inhibitory receptors *(24)*. This is a contributing cause for graft-versus-host disease (GvHD) after allogeneic cell transplantation when the donor cells are missing the recipient's HLAs necessary to maintain NK cell inhibition *(25, 26)*.

Moreover, NK cells can be activated in a direct manner based on the interaction of activating receptors with stimulatory ligands upregulated on malignant or virus-infected host cells ("induced-self", Fig. 1) *(15)*. HLA-I-independent activating receptors include the family of Ig-like natural cytotoxicity receptors (NCRs: NKp30, NKp44, NKp46) and natural killer group 2D (NKG2D), a lectin-like receptor that has limited homology with the other family members NKG2A/C/E *(27)*. NKG2D is a relatively conserved glycoprotein expressed as a homodimer on the surface of cytotoxic T cells and on most NK cells *(28, 29)*. The transmembrane domain of NKG2D associates with the signaling adaptor molecule DNAX-activating protein 10 (DAP10), which stimulates activating signaling cascades in the effector cell *(30, 31)*. NKG2D binds to MHC class-I-chain-related protein (MICA/B) and to UL16-binding proteins (ULBP1-6) (Fig. 2) *(32)*; both ligand classes are homologous to HLA-I molecules and frequently upregulated by transformed and infected cells, eliciting "induced-self" responses by $NKG2D^+$ lymphocytes *(33)*. HLA-I-binding activating KIRs include KIR2DS1/2/3/4/5 and KIR3DS1, which are denoted with an "S" for "short" cytoplasmic tail *(22)*. Instead of ITIMs contained in the cytoplasmic domain, as with inhibitory KIRs, the truncated tail has a positively charged residue in the transmembrane domain, allowing interaction with the signaling adaptor DAP12. Following cross-linking of an activating KIR with its ligand, DAP12 transmits activating signals mediated by integrated immunoreceptor tyrosine-based activation motifs (ITAMs), stimulating cytokine production and cytotoxic degranulation *(30, 31)*.

Another class of NK cell receptors that interact with a variety of HLA-I molecules are leukocyte immunoglobulin-like receptors (LILRs) *(34)*. Like KIRs, LILRs provide either inhibitory or activating stimulation by engaging integrated ITIMs or associating with ITAM-bearing adaptor proteins *(35)*.

1.1.3 NK cell receptor interactions with HLA class I molecules

HLA-I is categorized into classical (HLA-A/-B/-C) and non-classical (HLA-E/-F/-G) molecules encoded in the MHC region p21.3 on chromosome 6 *(36)*. While the classical HLA-I loci comprise >20,000 alleles coding for >12,000 distinct proteins, for non-classical loci ~400 alleles and ~130 proteins have been annotated (The Immuno Polymorphism Database, as of June 2021) *(37)*. The affinity of KIR binding to HLA-I molecules is highly allotype-dependent. Among the various HLA-A and HLA-B molecules, KIR3DL1 recognizes specific HLA-A/-B allotypes that contain the serological motif Bw4 *(38, 39)*. HLA-Bw4 variants with isoleucine at amino acid position 80 (I80) have been shown to be bound by KIR3DL1 with higher affinity

(38). KIR3DL2 binds to HLA-A3 and HLA-A11, however, this interaction was found to be highly sensitive to the presence of specific peptides in the HLA peptide binding groove (Fig. 2) *(40–42)*. Therefore, HLA peptide loading represents an additional variable that establishes conditional KIR–HLA pairing and modulates binding affinities, for example by abrogating interactions through steric hindrance (peptide antagonism) *(43–46)*. Regarding their interaction with KIRs, HLA-C allotypes are distinguished through expression of either asparagine (HLA-C1) or lysine (HLA-C2) at amino acid position 80. Accordingly, KIR2DL2/3 and KIR2DS2 show higher affinity to the HLA-C1 motif, whereas KIR2DL1 and KIR2DS1 preferentially bind HLA-C2 *(47, 48)*.

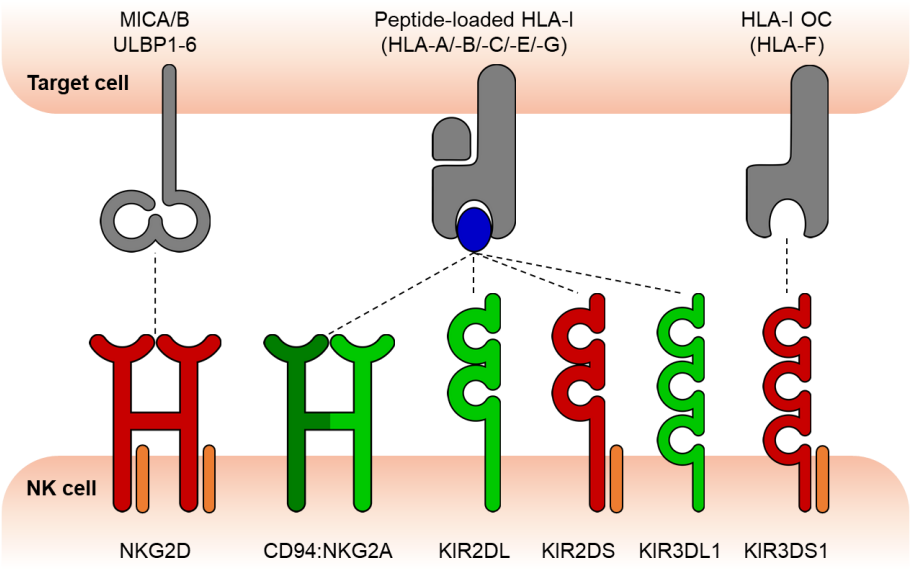

Fig. 2. Interactions between NK cell receptors and HLA-I(-like) molecules. MIC and ULBP are HLA-I-like molecules bound by the activating NKG2D receptor. Various NK cell receptors interact with HLA-A/-B/-C/-E/-G, which are predominantly expressed as trimeric structures consisting of a membrane-bound heavy chain, β2-microglobulin (β2m) and a peptide. HLA-F is more stable as an open conformer (OC) and bound by the activating KIR3DS1 receptor. Ligands in grey, peptide in blue, activating receptors in red, DNAX-activating protein (DAP) in orange and inhibitory receptors in green.

Like classical HLA-I, HLA-E is ubiquitously expressed in human tissues, albeit at much lower levels, and also presents self- and virus-derived peptides *(49, 50)*. Depending on the loaded peptide, HLA-E is bound either by the inhibitory CD94–NKG2A or the activating CD94–NKG2C/E heterodimer receptors (Fig. 2) *(51–53)*. The expression of HLA-G is restricted to certain tissues, for example the placenta, but is also upregulated under specific inflammatory conditions *(50)*. The most established link between HLA-G and NK cells is the interaction with inhibitory LILRs *(54)*. Several groups have reported binding of HLA-G by KIR2DL4, a peculiar receptor that mediates ITIM inhibition, while also associating with an activating Fc receptor γ protein *(55)*; however, the functionality of this interaction remains controversial *(37)*.

HLA-F differs from all other HLA-I molecules, showing the lowest polymorphism with only 45 annotated alleles and 6 proteins (The Immuno Polymorphism Database, as of June 2021). Comparative genetic analyses have shown that this low level of polymorphism is constant throughout human populations and primate species, indicating an unusual degree of

invariance *(56, 57)*. HLA-F shows moderate tissue restriction with elevated mRNA levels in the bone marrow, lymphoid tissues, blood, and gastrointestinal tract (The Human Protein Atlas, as of June 2021). Initial immunoregulatory studies have associated HLA-F with improved fertility and protection against amyotrophic lateral sclerosis, which has been partially connected to the recognition of HLA-F by the inhibitory receptors KIR3DL2 and LILRB1/2 *(58–62)*.

HLA-I is commonly expressed as a trimeric structure consisting of (i) a membrane-bound heavy chain with three extracellular domains (α1/2/3), (ii) a non-covalently associated β_2-microglobulin (β_2m) and (iii) an antigenic peptide of 8-10 amino acids bound in a groove formed by α1 and α2 (Fig. 2) *(63, 64)*. However, HLA-I can also be encountered transiently on cellular surfaces as empty heavy chains without β_2m and peptide, which is referred to as HLA open conformer (OC) *(65)*. The relevance of the peptide-free state of HLA-I molecules in immune regulation was first demonstrated through physical and functional interactions of HLA-F and other HLA-I OCs with KIR3DL2 and KIR2DS4 *(60)*.

HLA-F was long perceived as an MHC molecule that is unable to present peptides due to its partially open-ended peptide binding groove *(66, 67)*. A recently reported crystal structure showing HLA-F in complex with β_2m and peptide suggests that HLA-F does not anchor peptides at its N-terminus like other HLA-I molecules and is thus able to accommodate longer peptides of up to 30 amino acids *(62)*. Through interaction with the α3 domain and β_2m, LILRB1/2 can bind HLA-F in its peptide-associated trimeric form *(61, 62)*.

Nevertheless, evidence from several studies indicates that HLA-F is primarily expressed as OC in response to cellular stress and that HLA-F OCs are more stable than those of classical HLA-I molecules *(37, 62, 67)*. While HLA-F is mainly detected intracellularly in B cells, T cells, monocytes and NK cells under physiological conditions, surface protein levels increase rapidly following immune cell activation *(68, 69)*. The role of HLA-F surface expression in immune regulation was newly evaluated following reports of a high-affinity interaction of HLA-F (and other HLA-I) OCs with the activating KIR3DS1 receptor *(70, 71)*. It has been shown that the upregulation of HLA-F in infections with human immunodeficiency virus species 1 (HIV-1) and hepatitis C virus (HCV) elicits polyfunctional responses by KIR3DS1$^+$ NK cells, consisting of cytokine production, increased degranulation, and inhibition of viral replication *(70, 72–74)*. The KIR3DS1–HLA-F interaction may therefore represent an innate recognition mechanism triggered by the “induced-self” expression of HLA-F on infected host cells.

1.1.4 KIR and HLA genes in viral diseases

While some receptors, like NKG2D, are encoded by a single gene with little polymorphism, KIRs show remarkable diversity and rapid evolution in their ligand-binding domains *(75, 76)*. Genetic studies have attributed this dynamic to the co-evolution of KIR and HLA gene clusters, which constitute the most polymorphic regions in the human genome *(77)*. The polymorphism of HLA genes is highest within the peptide-binding α1/2 domains, which is due to constant

adaptation to environmental determinants, such as viral pathogens, in order to maintain the presentation of foreign antigens *(78–80)*.

The modulation of HLA-I conformation by viral peptides and the consequences for KIR recognition are well documented *(81, 82)*. It is assumed that KIR development mirrors the polymorphism of HLA-I genes in a form of genetic epistasis to preserve a necessary degree of receptor-ligand affinity *(83)*. This concept of natural selection of KIR–HLA combinations is supported by disease association studies correlating KIR and HLA genotypes of patients with the outcome of infectious, autoimmune, and inflammatory diseases *(77)*. The findings of these studies indicate a highly individualized host immune response to viral infections based on the genetically encoded network of KIR–HLA interactions *(81)*.

Table 1. Genetic associations of *KIR3DS1/L1* and *HLA-Bw4* loci with outcome in viral diseases listed by study release date. HIV: human immunodeficiency virus; AIDS: acquired immunodeficiency syndrome; HCV: hepatitis C virus; HBV: hepatitis B virus; HPV: human papillomavirus; EBV-HLH: Epstein-Barr virus associated hemophagocytic lymphohistiocytosis; BKPyV: BK polyomavirus; KSHV: Kaposi sarcoma-associated herpesvirus. KIR and Disease Database (KDDB) as of June 2021 *(84)*.

Viral pathogen	Reference	KIR and HLA	Observation
HIV	*(85–87)*	*KIR3DS1*/*HLA-Bw4I80*	Slower progression to AIDS Viral inhibition
	(85)	*KIR3DS1*	Faster progression to AIDS
	(86–89)	*KIR3DL1*/*HLA-Bw4*	Slower progression to AIDS Viral resistance Viral inhibition
	(90)	*KIR3DS1*/*KIR3DS1*	Viral resistance
HCV	*(91, 92)*	*KIR3DS1*/*HLA-Bw4*	Viral clearance
HBV	*(93, 94)*	*KIR3DS1*	Viral resistance Viral clearance
	(95)	*HLA-Bw4*	Viral susceptibility
HPV	*(96)*	*KIR3DS1*	Viral resistance
EBV-HLH	*(97, 98)*	*KIR3DS1*	Viral susceptibility
BKPyV	*(99)*	*KIR3DS1*	Viral resistance
Dengue virus	*(100)*	*KIR3DS1*/*KIR3DS1*	Viral clearance
	(100)	*KIR3DL1*	Viral susceptibility
KSHV	*(101)*	*KIR3DS1*/*HLA-Bw4*	Viral clearance

While activating KIRs tend to be protective against infectious diseases, they also correlate with increased inflammation and autoimmunity *(102)*. The locus for the activating KIR3DS1 receptor has been associated with both poor and beneficial outcome in a growing number of diseases, occasionally in combination with *HLA-Bw4* (Table 1). *KIR3DS1* is an allelic variant of *KIR3DL1* and although both alleles share high homology in their extracellular domains, there is only limited interaction of KIR3DS1 with HLA-Bw4 *(103, 104)*.

1.2 Chronic viruses

Viruses are obligate intracellular parasites that cause acute and chronic infections. In an acute infection, host and viral factors are in disequilibrium, which is resolved by death of the host or resolution of infection. As a third outcome, the infection reaches homeostasis, and a chronic viral state is established that causes no or minor pathology *(105)*. Chronic infections are usually directed to specific host cells or tissues (viral reservoirs), in which viral replication is restricted by the immune system *(106)*. When the host immune system is compromised, for example due to immunosuppressive therapy, persistent viruses can reactivate from their niche and cause invasive, uncontrolled infections. Notable chronic viral families causing severe infections in immunocompromised individuals include *Herpesviridae*, *Adenoviridae* and *Polyomaviridae* *(105)*.

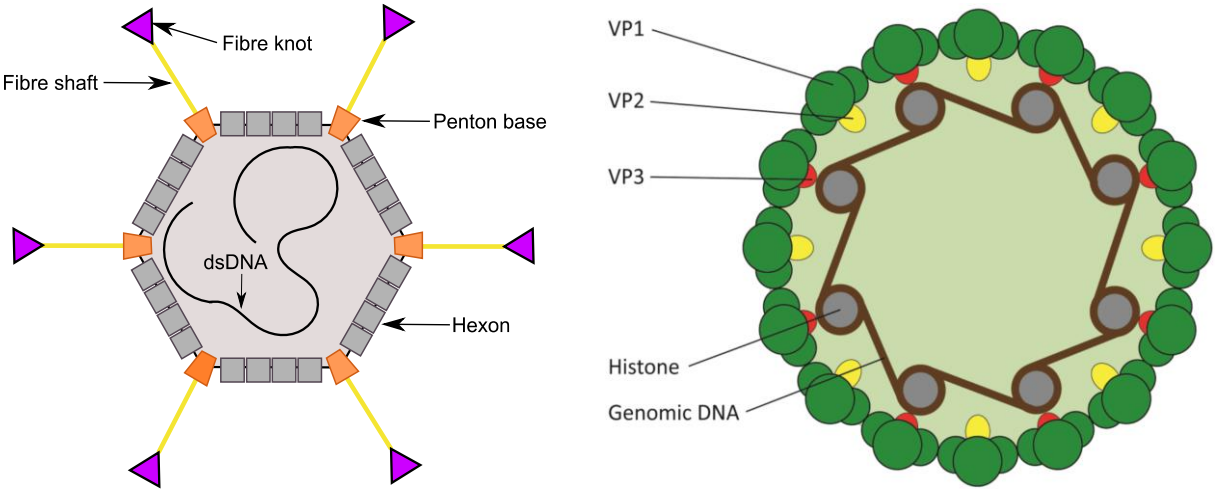

Fig. 3. Structure of human adenovirus (HAdV, left) and BK polyomavirus (BKPyV, right). Both viruses are characterized by a DNA genome and not having an outer lipid layer. For further information see chapters 1.2.1 and 1.2.2. Adapted from *(107, 108)*.

1.2.1 Human adenovirus

Human adenovirus (HAdV) was first isolated in 1953 from adenoids and tonsils of children. HAdV consists of a non-enveloped (no outer lipid layer) icosahedral nucleocapsid that contains a double-stranded (ds) DNA genome of 34-36 kbp (Fig. 3) *(109)*. The viral genome is divided into 9 distinct transcription units that are active at early (E1A, E1B, E2A, E3, E4 and L1), intermediate (pIX and IVa2) or late (major late transcription unit) stages of infection *(109)*. HAdV is classified into seven species (HAdV-A to HAdV-G) and 51 antigen-based serotypes, or >100 genotypes according to a newer genetic convention *(110, 111)*. HAdV infections occur worldwide and are directed to the eyes (conjunctiva), the respiratory and the gastrointestinal tract *(109)*. More specifically, viral replication occurs in the corneal epithelium and the epithelial linings of the lungs and gut *(112)*. This rather unspecific tropism derives from the ubiquity of the HAdV entry receptors, integrin and coxsackievirus and adenovirus receptor (CXADR), which are abundantly expressed cell adhesion proteins in polarized epithelium *(113, 114)*.

Several HAdV species can establish persistent infection in lung epithelium, brain tissue as well as in lymphoid tissues of the tonsils, adenoids and especially in the intestines *(115, 116)*. There is increasing evidence that the intestines are a main source for the onset of HAdV infections in immunosuppressed patients receiving allogeneic hematopoietic stem cell transplantation (allo-HSCT) *(117)*. In this context, intestinal shedding of viral particles before transplantation has emerged as a prognostic marker for the occurrence of HAdV reactivation *(118, 119)*. Additional risk factors for severe HAdV disease are young age and low counts of antiviral T cells *(120–122)*. Intestinal HAdV reactivation often occurs in the distal ileum, presumably due to the density of associated lymphatic tissue. Subsequently, invasive cytopathic infection of the intestinal epithelium causes enteritis, fever, and diarrhea *(123)*. Further dissemination into blood (adenoviremia) and other organs occurs within 100 days after HSCT and is associated with fatality rates as high as 50% *(124)*. Severe HAdV infections are treated by administering virostatic agents and with adoptive T cell transfer, however, these options are partly experimental and combined with severe side effects *(125–129)*. HAdV reactivation is also a considerable threat in solid organ transplantation, again affecting pediatric recipients more severely than the adult *(130)*. The prevalence of HAdV strains appears to be species-specific, with HAdV-C being the most common in HSCT patients. HAdV-A/-B are occasionally present, while other HAdV species are rarely encountered *(123, 131)*.

HAdV-C encodes seven gene products in its E3 transcription unit, of which most are involved in attenuating host immune responses, increasing the ability of the virus to establish persistence *(132)*. These products are E3/12.5K, E3/6.7K, E3/glycoprotein19K (E3/gp19K), adenovirus death protein (ADP), receptor internalization and degradation protein α and β (RIDα/β) and E3-14.7K *(133)*. RIDα/β form the RID complex, which prevents apoptosis through the so-called “death” ligands tumor necrosis factor (TNF), tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and Fas ligand by downregulating corresponding “death” receptors on the host cell and by blocking TNF and nuclear factor κB (NF-κB) activation *(134, 135)*. E3/6.7K together with the RID complex mediates downregulation of TRAIL receptors *(136)*. E3/14.7K protects the host cell from extrinsic apoptosis by blocking TNF-mediated cytolysis *(137)*. Apart from the E3 unit, the early proteins E1A and E1B/55K have been shown to antagonize type I interferon (IFN) expression during infection *(138, 139)*.

E3/gp19K is a type I transmembrane protein that primarily localizes in the endoplasmic reticulum (ER), which is facilitated by a linear dilysine motif and a conformational ER retrieval motif contained in the cytoplasmic tail *(140)*. The best-documented function of E3/gp19K is the reduction in killing of infected cells by cytotoxic T cells through downregulation of classical HLA-I molecules on the host cell surface *(132, 141)*. E3/gp19K inhibits HLA-I surface transport through direct binding of immature HLA-I molecules *via* its luminal domain, thereby retaining them in the ER *(142)*. This interaction is specific for HLA-A/-B and because E3/gp19K binds

the variable HLA-I α1/2 domains the binding affinity strongly varies between HLA allotypes *(143, 144)*. Moreover, E3/gp19K has been shown to impair maturation of HLA-I by binding to transporter associated with antigen processing (TAP), a component catalyzing peptide loading and release of HLA-I out of the ER *(145)*. Additional studies have demonstrated that E3/gp19K promotes evasion from NK cells by sequestering the NKG2D ligands MICA/B through direct binding to these HLA-I-like molecules *(146, 147)*. Based on its immunosubversive activities, E3/gp19K is increasingly recognized as a central factor for HAdV persistence *(148, 149)*.

1.2.2 BK polyomavirus

Human polyomaviruses are classified as ubiquitous oncoviruses belonging to the family of *Polyomaviridae*. BK polyomavirus (BKPyV) was isolated in 1972 from the urine of a renal transplant patient with the initials “B.K.”. Like HAdV, BKPyV is non-enveloped, exhibits an icosahedral shape and has a dsDNA genome of ~5 kb, which is considerably smaller than the HAdV genome (Fig. 3) *(150)*. The viral genome is divided into three main regions: early, non-coding and late. The early region encodes for small and large tumor (LT) antigen, two proteins involved in initiating viral DNA replication and stimulating cell cycle progression. The late region activates after DNA replication and codes for the three capsid proteins (Vp1/2/3) and the non-coding region produces transcription factors for early and late genes *(151)*.

Primary infection with BKPyV typically occurs during childhood and by adulthood, most people are seropositive *(152)*. For cell entry, BKPyV uses a sialic acid-containing glycoprotein, which is why infection can be limited by treatment with neuraminidase *(153, 154)*. Following initial infection, BKPyV remains latent in different tissues, mostly causing asymptomatic symptoms, but severe infections can cause pneumonitis, hepatitis, retinitis, and meningoencephalitis *(155)*. The most common site of persistent BKPyV infection is the tubular epithelium of the urogenital tract, where viral replication focuses on collecting ducts, renal calyces, and renal pelvis *(156, 157)*.

Like HAdV, BKPyV is an increasingly recognized pathogen in immunocompromised hosts, emerging through viral reactivation after immunosuppressive therapy or in individual immunodeficiency *(155, 158)*. Reactivation of renal BKPyV with subsequent invasive lytic infection is regarded as the underlying cause for hemorrhagic cystitis after allo-HSCT and BKPyV-associated nephropathy (BKVAN) after renal transplantation *(159, 160)*. The onset of BKVAN is often preceded by viral presence in patient urine and plasma, and the pathology is characterized by renal fibrosis resulted from inflammation und destruction of renal tubular cells *(161)*. Particularly after kidney transplantation, BKVAN represents a severe complication associated with markedly reduced graft survival and lacking availability of specific therapeutic options *(160, 162)*. Most commonly, immunosuppressive treatment is reduced to allow partial recovery of the recipient’s immune system, thereby increasing the risk of graft rejection *(163)*.

Despite the well-documented persistence of BKPyV in clinical settings, little is known about underlying mechanisms promoting immune evasion. There is no indication that BKPyV infection downregulates the expression of classical HLA-I molecules nor of the NKG2D ligands MICA/B and ULBP1/2 *(164)*. However, one study reported that BKPyV produces a microRNA that targets ULBP3, rendering host cells less vulnerable to NKG2D$^+$ NK cells *(164)*.

1.3 The intestinal epithelium

1.3.1 Intestinal mucosal immunity

The innate immune system includes the anatomical barriers of the human body, i.e. the skin and the mucosal membranes of the respiratory and intestinal tract. The main cellular component of the intestinal mucosa is a single layer of simple columnar epithelial cells lining the luminal surface of the gut. The intestinal epithelium is organized in two compartments, the intestinal villi and the crypts of Lieberkühn (Fig. 4A) *(165)*. The villus compartment contains a consortium of specialized intestinal epithelial cells (IECs) that primarily function as part of the digestive system. Enterocytes are particularly important for nutrient absorption and fluid exchange *(166)*. Enteroendocrine cells support digestion with the secretion of hormones that stimulate the production of bile and gastric acid *(166)*.

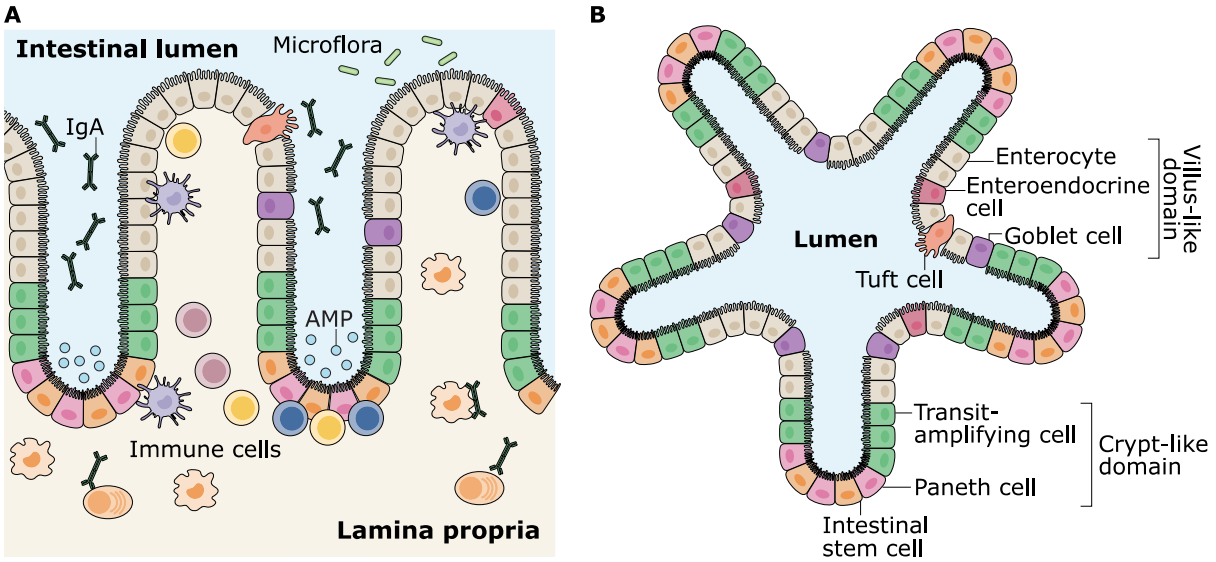

Fig. 4. The intestinal mucosa and human intestinal organoids (HIOs). (**A**) The epithelial lining separates the intestinal lumen from the lamina propria beneath, in which the gut-resident immune cells reside. The intestinal epithelial cells (IECs) are in simultaneous communication with the intestinal microflora and lymphatic tissues, and they produce antimicrobial peptides (AMPs) and immunoglobulin A (IgA) to neutralize microbial pathogens. (**B**) HIOs have villi and crypts, the two characteristic intestinal compartments that consist of distinct compositions of specialized IEC types. When embedded in basement membrane matrix, HIOs form an internal cavity (lumen), at which the apical brush boarder of the epithelium is facing. The basolateral side interacts with the extracellular matrix (ECM) proteins contained in the surrounding hydrogel. Adapted from *(167)*.

The intestinal mucosa is one of the body's most important interfaces for host-microbe interactions due to its large surface area that is covered by a dense intestinal microflora *(168)*. IECs are thus not only an important physical barrier to pathogens, but they also regulate microbial interactions with a protective mucus layer, which is produced by goblet cells *(169–171)*. In addition, IECs serve as immune sentinels against pathogens by sensing microbial

structures with innate pattern recognition receptors (PRRs), which trigger the production of a broad spectrum of antimicrobial peptides (AMPs) and the secretion of inflammatory cytokines to recruit immune cells *(172–174)*. Paneth cells react to direct microbial challenge with the production of α-defensins, an important class of AMPs with activity against non-enveloped viruses including HAdV and BKPyV *(175–177)*. Underneath the epithelial layer, separated by the basement membrane, lies the lamina propria (LP), a thin layer of connective tissue hosting the gut-resident immune cells (Fig. 4A) *(178)*. These include producers of immunoglobulin A (IgA), which has virus-neutralizing activity and is the main antibody found in mucous secretions *(179, 180)*. Except for the blood system and the lymphoid organs, the highest concentration of lymphocytes in humans is found in the intestines *(181)*.

1.3.2 Intestinal organoids

In order to maintain its integrity and functionality, the intestinal epithelium completely renews itself every few days *(182)*. The source for this rapid regeneration lies at the crypt base, where intestinal stem cells (ISCs) regularly divide to produce highly proliferative progenitors called transit-amplifying (TA) cells (Fig. 4B). As the nascent TA cells migrate out of the crypt towards the villus base, they gradually commit to the absorptive and secretory IEC lineages *(183)*. When exiting the crypt, proliferation ceases and the then fully differentiated IECs further migrate to the villus tip, performing their cellular functions until they are shed into the gut lumen (anoikis) *(165)*. During their migration out of the crypt, TA cells face increasing stimulation of the bone morphogenetic protein (BMP) and decreasing stimulation of the Wnt pathway due to opposing expression gradients of corresponding agonists and antagonists *(166)*. While BMP negatively regulates stemness, Wnt and Notch signaling are essential for preventing ISC differentiation *(182)*. In the crypts of the small intestine, ISCs are intercalated with Paneth cells, which are key producers of the Notch ligands DLL1/4, the Wnt ligand Wnt3 and additionally support the proliferation of ISCs with epidermal growth factor (EGF) *(165, 166)*.

In 2007, the genetic marker for ISCs leucine-rich-repeat-containing G-protein-coupled receptor 5 (*LGR5*) was identified *(184)*. Building on this discovery, the *ex vivo* culture of intestinal epithelium was developed, using the regenerative abilities of $Lgr5^+$ ISCs *(185, 186)*. Key adaptations for this technique were the embedment of ISCs in a hydrogel resembling the extracellular matrix (ECM) of the intestinal basement membrane and exogenous provision of Wnt3 and R-spondin, the physiological ligand of Lgr5 *(187, 188)*. Under these culture conditions, $Lgr5^+$ ISCs extracted from human tissue self-organized into organotypic spheroids, which were termed human intestinal organoids (HIOs, Fig. 4B) *(189)*. HIOs can be cultured longitudinally and show high resemblance to *in vivo* tissue, including the crypt-villus morphology and the capacity to form specialized IEC types *(190)*. Compared to the organogenesis based on embryonic and induced pluripotent stem cells (PSCs), HIO technology represented a new approach based entirely on adult stem cells *(191, 192)*. This

technique opened new prospects in stem cell research, regenerative medicine, drug testing and disease modeling of cancer and genetic disorders *(193, 194)*.

Furthermore, their close resemblance to physiological host tissue led researchers to use HIOs as an infection model to study host-pathogen interactions *(195, 196)*. First, groups microinjected *Helicobacter pylori* and *Salmonella enterica* into the lumen of gastric and small intestinal HIOs *(197, 198)*. In response to infection, HIOs mounted robust inflammatory and AMP responses, indicating their applicability for studying innate antimicrobial immunity. The authors used microinjection to incubate the enterobacteria on the apical epithelial organoid membrane, which in theory corresponds to the fecal-oral route of infection *in vivo*. A study by Co and colleagues described that the apical membrane lies in the spheroid interior, probably because the basolateral membrane self-organizes outward to interact with the surrounding ECM-like hydrogel *(199)*. Due to its manual nature, organoid microinjection has limited applicability for up-scaling procedures *(200)*. Co *et al*. adapted HIOs to hydrogel-free culture in suspension, thereby inducing the reversal of epithelial polarity so that the apical membrane faces the medium. Incubation experiments with different invasive enterobacteria showed a preferential entry through apical or basolateral HIO membranes, thus emphasizing the role of HIO polarity in enteric infection *(199)*.

The recent COVID-19 pandemic has raised interest in using organoids as a physiological *in vitro* model for viral infections *(201)*. The successful co-culture of HIOs with rotavirus and norovirus provided new insights into the pathophysiology of these enteric pathogens, which are inherently difficult to maintain in conventional cell lines *(202, 203)*. Shortly after, other groups reported productive infection of HIOs with human enterovirus and astrovirus *(204, 205)*. Recently, Holly and Smith used HIOs to culture laboratory and clinical HAdV strains, including HAdV species F, which replicates poorly in cell lines *(206, 207)*. They demonstrated that viral replication is restricted by type I/III interferon in HIO monolayers but not in the A549 cell line and identified a preferred tropism of HAdV type 5 (HAdV5) for goblet cells *(207)*. These studies have proven that HIOs facilitate viral culture, mount authentic innate immune responses, and provide physiological host cells, where traditional cell lines have failed. Therefore, HIOs constitute a highly beneficial model to study the infection with enteric viruses.

2 Hypotheses and Aims

Virology studies are conventionally performed in immortalized cell lines, which have limited cell composition and often display an unphysiological expression of immune ligands *(208, 209)*. Organoids are organ-like *in vitro* models that overcome this limitation and can be infected with viruses, positioning them as an ideal model for studying viral pathology *(210)*. HAdV and BKPyV cause severe disease in immunocompromised patients *(124, 154)*. Considering the suboptimal options to treat HAdV and BKPyV infections, the identification of new molecular targets is warranted *(125–129)*. Interestingly, several reports have associated the *KIR3DS1* locus with protection from viral diseases including BK polyomavirus-associated nephropathy and, additionally, HLA-F has been assigned to KIR3DS1 as a functional ligand *(70, 71, 99)*. HLA-F is upregulated on host cells infected *in vitro* with HIV-1 and HCV, leading to specific activation of KIR3DS1$^+$ NK cells *(70, 74)*.

It is thus hypothesized that human intestinal organoids can be infected with HAdV, allowing the assessment of immune ligand expression on infected intestinal epithelial cells. It is further hypothesized that HAdV and BKPyV infections induce HLA-F surface expression, which enables KIR3DS1$^+$ NK cells to recognize and neutralize infection.

Therefore, the aims of this thesis were:

1. To establish a physiological model for HAdV5 infection based on human intestinal organoids and to generate BKPyV-infected tubule cells.
2. To establish a flow cytometry protocol for the differential analysis of classical and non-classical HLA-I molecules and other NK cell ligands expressed on viral host and uninfected cells.
3. To examine the binding of NK cell receptors to respective ligands expressed on host cells following infection.
4. To co-culture HAdV5 and BKPyV host cells with primary NK cells to assess effector cell activation, host cell killing and viral inhibition.

3 Discussion

The focus of this dissertation was the NK cell recognition of host cells infected with HAdV and BKPyV. Profiling of NK cell ligands on the host cell surface revealed an upregulation of HLA-F, which enabled binding of the innate activating KIR3DS1 receptor. KIR3DS1$^+$ NK cells challenged with host cells infected with either virus induced NK cell degranulation. Using HAdV5-infected intestinal organoids, killing of infected cells by NK cells and improved viral clearance were furthermore demonstrated. The KIR3DS1–HLA-F recognition was put into clinical perspective by the observation of (i) increased HLA-F expression in BKVAN patient biopsies and (ii) a protective effect of the *KIR3DS1* gene in HAdV infection after pediatric allo-HSCT.

3.1 KIR3DS1–HLA-F recognition in controlling viral infection

The results of this thesis demonstrated that KIR3DS1$^+$ NK cells mount stronger responses against HAdV5 and BKPyV infection than KIR3DS1$^-$ NK cells *(211, 212)*. In both infections, a discernible degree of degranulation by KIR3DS1$^-$ NK cells was observed. KIR3DS1-independent NK cell activity was to be expected, considering the upregulation of NKG2D ligands in HAdV and of MICA *(212)* and MICB *(213)* in BKPyV infection, which likely triggered activation of NKG2D$^+$ NK cells; not to mention additional unidentified receptor–ligand interactions *(211–213)*.

Like NKG2D and its ligands, KIR3DS1 and HLA-F are characterized by an unusually low polymorphism. This implies that their interaction is conserved, in contrast, for example, to their highly diversified inhibitory counterparts KIR3DL1 and HLA-Bw4. Furthermore, the tight regulation of surface expression of HLA-F is reminiscent of the stress-inducible ligands MIC and ULBP *(214, 215)*. Genetic invariability and controlled expression indicate high homology between KIR3DS1–HLA-F and the well-studied conserved interaction of NKG2D with its ligands. It thus stands to reason that KIR3DS1–HLA-F categorizes as an “induced-self” mechanism for immune recognition of viral host cells. The fact that KIR3DS1 binds to HLA-F in its OC form further supports the notion of a conserved mechanism as this makes the interaction independent of peptide variability. Importantly, this would prevent viruses in abrogating the interaction using peptide antagonism, unless they were to force peptide loading on HLA-F in order to reduce OC formation.

In addition to HAdV and BKPyV, KIR3DS1–HLA-F NK cell recognition of host cells has been described for infections with HIV-1 and HCV *(70, 74)*. All four viruses encode mechanisms to subvert the host immune response and consistent with this, each of them can establish persistent infection in humans. They all block surface expression of NKG2D ligands to different extents, protecting them from T and NK cell recognition *(216)*. Furthermore, HAdV and HIV-1 are well-characterized in the dysregulation of classical HLA-I molecule presentation on host cells, and specific HIV-1 strains even interfere with the expression of the non-classical ligands

HLA-G/-E *(217–219)*. Considering the efforts to escape HLA-I immune surveillance, it is striking that HLA-F expression is not repressed by any of these viruses, leaving them vulnerable to KIR3DS1 recognition. Even adenoviral E3/gp19K, a versatile binder of HLA-I structures, showed no effect on HLA-F expression levels on HAdV5 host cells *(211)*. Although HIV-1 shows some degree of KIR3DS1 evasion at late stages of infection, it is unclear whether this involves regulation of HLA-F *(70)*. In conclusion, there is much to indicate that KIR3DS1–HLA-F recognition is not a considerable immune pressure for these viruses.

The rapid co-evolution of KIR and HLA gene clusters showcases that microbial infection can accelerate the variance of host factors *(81)*. In contrast, the low polymorphism of HLA-F and KIR3DS1 indicates that this immune axis is comparably stable. This apparently weak reciprocal pressure between viruses and KIR3DS1–HLA-F may partially be explained by the comparably low carrier frequency of activating KIR loci in human populations. The frequency of *KIR3DS1* carriers varies between 10 and 50% across different ethnicities, indicating a lower immune pressure for viral pathogens compared to the abundant inhibitory KIR loci and lectin-like NKG receptors, which mentioned viruses show obvious evasion strategies against *(220)*. The limited frequency of the *KIR3DS1* locus may be ascribed to adverse associations in infectious (influenza, syphilis) and other diseases (Table 2), in which KIR3DS1 may cause considerable immunopathology *(221–223)*. Therefore, one could speculate that regulation of HLA-F is not required to maintain viral fitness due to a sufficient number of hosts not encoding KIR3DS1.

Table 2. Genetic associations of the KIR3DS1 locus with cancers and autoimmune and inflammatory diseases listed by study release date. IBD: inflammatory bowel disease; GvHD: graft-versus-host disease. KIR and Disease Database (KDDB) as of June 2021 *(84)*.

Disease	**Reference**	***KIR* and *HLA***	**Observation**
Type 1 diabetes	*(224, 225)*	*KIR3DS1*	Increased susceptibility
Cervical neoplasia	*(226)*		
Multiple sclerosis	*(227)*		
Ankylosing spondylitis	*(228–230)*		
Kaposi sarcoma	*(101, 231)*	*KIR3DS1*(/*HLA-Bw4*)	
Rheumatoid arthritis	*(232)*	*KIR3DS1*	
IBD	*(233)*		
Hepatocellular carcinoma	*(234)*	*KIR3DS1*/*HLA-Bw4I80*	Decreased susceptibility
Hodgkin’s lymphoma	*(235)*	*KIR3DS1*	
Allo-HSCT	*(236)*		Decreased GvHD

The lack of viral interference with HLA-F surface expression poses a strong argument for developing antiviral immunotherapies based on KIR3DS1–HLA-F recognition. The finding that HLA-F was specifically upregulated on HAdV5 and BKPyV host cells and in BKVAN tissue

samples supports this proposal *(211, 212)*. Lunemann *et al*. have suggested a therapeutic approach using soluble chimeric constructs of the KIR3DS1 binding domain fused to the Fc domain of human IgG1 (KIR3D1-Fc). KIR3D1-Fc has been demonstrated to be effective in controlling HCV infection and in binding hexon$^+$ and LT$^+$ host cells *in vitro (74, 211, 212)*. Administration of KIR3D1-Fc could help the patient's immune system to tag HLA-F$^+$ viral host cells and to destroy them through ADCC facilitated by recognition of the Fc domain by the CD16 Fc receptor expressed on cytolytic lymphocytes *(5)*. Importantly, this concept would be applicable for KIR3DS1$^-$ individuals, overcoming the limited genetic predisposition for KIR3DS1 in human populations.

The observation that donor cells containing the *KIR3DS1* gene were protective against HAdV in allo-HSCT recipients suggests the involvement of KIR3DS1$^+$ effector cells in fighting infections in immunocompromised patients *(211)*. HSCT of KIR3DS1$^+$ donor cells could be examined as a clinical guideline to prevent infections with HAdV and other opportunistic viruses. This strategy could be augmented with a risk assessment of viral reactivation based on shedding diagnostics *(118, 119, 162)*. Nevertheless, implications of KIR3DS1 in immunopathology need to be taken into account. While gene copies of *KIR3DS1* are associated with less graft-versus-host disease (GvHD), it has been shown that GvHD significantly increases when *HLA-Bw4*$^-$ patients received *KIR3DS1*$^+$/*L1*$^+$ donor cells *(236–238)*. The antiviral activity of NK cells may be particularly important in HSCT because their reconstitution (<1 month post-HSCT) is considerably faster compared to T and B cells (3-4 months post-HSCT) *(239, 240)*. However, the KIR-based activity of NK cells could be limited due to a premature CD56^{bright}NKG2A$^+$ phenotype reported early after HSCT, showing reduced effector functions *(241, 242)*.

Furthermore, adoptive transfer of KIR3DS1$^+$ effector cells could generally be considered to treat difficult to control viral infections. Recent clinical studies have shown an increasing efficacy of adoptive cell therapy using T cells specific against herpesviruses, HAdV and BKPyV *(243–245)*. Effector cells targeting HLA-F$^+$ viral host cells could be produced through *in vitro* expansion of autologous KIR3DS1$^+$ NK cells (Fig. 5) *(246)*. Moreover, recent advances have been made in the genetic engineering of immune cells to redirect their target specificity. A handful of FDA-approved tumor therapies use T cells that express a chimeric antigen receptor (CAR) consisting of an antigen-recognizing ectodomain and an activating transmembrane domain *(247)*. A growing number of pre-clinical and clinical studies are investigating the usage of CAR-NK cells (Fig. 5), which show logistic and clinical benefits compared to CAR-T cells *(248)*. CAR-NK cells cause less GvHD in allogeneic transplantation than CAR-T cells and have a limited circulating lifespan, thus reducing the risk of off-target effects and cytokine release syndrome *(249)*. Moreover, in light of a phase I clinical trial involving a CAR using NKG2D as target-recognizing ectodomain and CD3ζ and DAP10 as activation mediators, the

development of KIR3DS1-based CAR-effector cells may show promise for the future *(250)*. Adoptive transfer of KIR3DS1$^+$ effector cells may thus be a promising option to fight acute viral diseases and to protect vulnerable patients from the onset of viral infections.

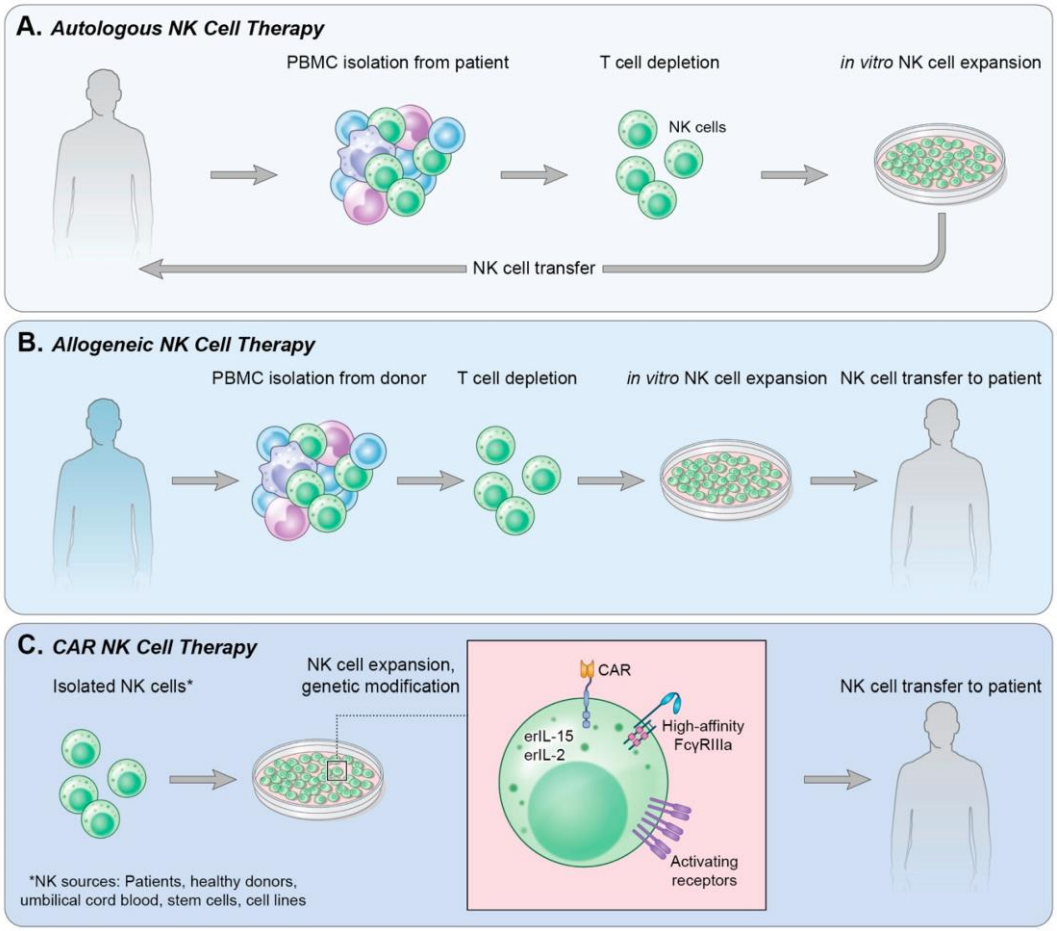

Fig. 5. Options for adoptive therapy using NK cells. (**A**) For autologous NK cell therapy, the patients' endogenous immune cells are used for minimal alloreactivity. NK cells are extracted from isolated peripheral blood mononuclear cells (PBMCs) and expanded *in vitro* assisted by cytokines and feeder cells. Following phenotypic assessment, NK cells are reinfused into the patient. (**B**) The process of allogeneic NK cell therapy is identical to that in (**A**), only that a healthy donor is used as source for immune cells. (**C**) Various sources can provide NK cells for CAR-NK cell therapy (see asterisk). NK cells are genetically modified to express recombinant CAR and cytokines, expanded *in vitro*, and transferred to the patient. Image taken from *(251)*.

Nevertheless, more basic research on KIR3DS1 and HLA-F is needed to broaden the understanding of its functions in immune regulation. A systematic assessment of HLA-F in infections with clinical HAdV and BKPyV strains is warranted, as is the involvement of KIR3DS1$^+$ NK cells in controlling other microbial infections. Apart from targeting viral host cells, KIR3DS1$^+$ NK cells might have completely unexplored roles in regulating other HLA-F$^+$ cells. NK cells not only positively regulate other immune cells, but they also control and eliminate over-stimulated macrophages, dendritic, T and B cells *(252)*. The fact that activated immune cells and cancers express surface HLA-F strongly suggests immune recognition by KIR3DS1, the possible consequences of which were documented in the numerous clinical associations (Table 2) *(68, 69, 253–261)*. Again, taking NKG2D as a receptor with many parallels to KIR3DS1, the direct involvement of NKG2D–ligand recognition in targeting stressed immune

cells and regulating autoimmunity makes it conceivable that the KIR3DS1–HLA-F mechanism has similar functions *(262, 263)*. Therefore, KIR3DS1$^+$ NK cells may cause beneficial outcomes in GvHD and specific cancers by controlling HLA-F$^+$ immune and malignant cells. On the other hand, KIR3DS1, like other activating KIRs, likely causes immunopathology in specific inflammatory and autoimmune diseases due to persisting and off-target activity of NK cells *(102)*. As with all new therapeutic approaches, the mentioned favorable and unfavorable aspects of KIR3DS1-mediated immune activation will require in-depth analysis and careful consideration.

3.2 *KIR3DS1*/*HLA-Bw4* genotype in protection from viral diseases

Over the years, the compound genotype *KIR3DS1*/*HLA-Bw4* has been associated with protection against an increasing number of viruses: HIV, HCV, HAdV and BKPyV (without *HLA-Bw4*) *(85, 91, 99, 211)*. The group of Mary Carrington furthermore reported reduced infection with the herpesvirus cytomegalovirus (CMV) and bacterial pathogens in HIV-positive individuals *(264)*. The authors proposed that *KIR3DS1*/*HLA-Bw4* not only confers resistance against HIV, but also provides general defense from infection with opportunistic pathogens, which is consistent with protection from BKPyV and HAdV reactivation *(99)*.

Whether KIR3DS1 and HLA-Bw4 act in concert or independently in their protective function is highly debated *(37)*. An obvious explanation for the joint occurrence of KIR3DS1 and HLA-Bw4 is their direct interaction facilitating recognition of infected HLA-Bw4$^+$ cells through KIR3DS1. The ectodomains of KIR3DS1 and KIR3DL1 differ in only six amino acid residues, and yet this abolishes binding of KIR3DS1 to HLA-Bw4 while increasing binding to HLA-F *(70, 71)*. It has been shown that certain HIV-1-derived peptides can overcome the steric hindrance of KIR3DS1 binding to HLA-Bw4, suggesting that this interaction is in principle possible *(265)*. Moreover, an *in vitro* study by Alter *et al*. demonstrated that KIR3DS1$^+$/KIR3DL1$^-$ NK cells effectively inhibit HIV-1 replication in primary CD4$^+$ T cells with *HLA-Bw4*$^+$ genotype *(266)*. However, these results remain unconfirmed for other viruses. According to a second model, HLA-Bw4 enhances KIR3DS1–HLA-F interactions by increasing HLA-F surface levels on infected cells. Evidence for this theory was provided by Daniel Geraghty's group reporting heterodimer formation of HLA-I and HLA-F OCs in *cis*, leading to mutual stabilization *(67, 267)*. Finally, KIR3DS1–HLA-F and KIR3DL1–HLA-Bw4 interactions could function independently, but in synergy with each other, to protect the host from viral infection. This notion is supported by the protective contribution of the *KIR3DL1* gene in combination with *HLA-Bw4* in HIV infection *(86–89)*. NK cell activation mediated by KIR3DL1 requires loss-of-recognition of HLA-Bw4, and indeed HLA-Bw4 is downregulated on HAdV5 host cells *(211)*. Although there is variance depending on the clone of the HIV protein negative regulatory factor (Nef), HLA-B is also downregulated on HIV host cells *(268–270)*. Interestingly, the protective association of *KIR3DS1* in BKPyV infection is independent of *HLA-Bw4* (unlike for HAdV and HIV) and no

significant association was found for the *KIR3DL1*$^{+}$/*HLA-Bw4*$^{+}$ compound genotype *(99)*. At the same time, combined HLA-I expression (assayed with a pan-antibody against HLA-A/-B/-C) was not downregulated by infection, suggesting that KIR3DS1–HLA-F alone can sufficiently control viral infection and that KIR3DL1–HLA-Bw4 is an independent additive stimulus for NK cells *(212, 271)*. However, this theory is limited by the observations that HCV control requires *KIR3DS1*$^{+}$/*HLA-Bw4*$^{+}$ loci, while HCV, like BKPyV, does not downregulate combined HLA-I *(74, 272)*. An examination of increased protection from BKPyV disease through *KIR3DS1*$^{+}$/*HLA-Bw4*$^{+}$ compared to *KIR3DS1* alone, as well as allotype-specific assessment of HLA-Bw4 expression in BKPyV and HCV infection may further clarify the relationship between KIR3DS1, KIR3DL1 and HLA-Bw4 in viral diseases. Finally, additive NK cell activation is likely to require co-expression of KIR3DS1 and KIR3DL1 on the same effector cell, but the existence of such an NK cell subset has not been definitively proven.

3.3 Immune surveillance of classical HLA-I in viral infection

Chronic viruses have evolved sophisticated immune evasion mechanisms to persist in host organisms. One major strategy is the selective downregulation of classical HLA-I molecules on the surface of host cells to evade recognition by HLA-I-surveilling T and NK cells. HAdV and lab strains of HIV-1 preferentially downregulate HLA-A/-B over HLA-C allotypes, which is mainly due to a higher binding affinity of the accessory proteins HIV Nef and HAdV E3/gp19K to HLA-A/-B *(132, 273)*. The resulting HLA-I profile maximizes dual escape from T and NK cells, firstly because the preservation of HLA-C maintains inhibition of NK cells *via* KIR2DL receptors. Of note, capsid proteins of HIV, HCV and HAdV (core protein, p24 and hexon, respectively) contain peptide epitopes that further increase NK cell inhibition by enhancing binding of KIR2DL3 to corresponding HLA-C–peptide complexes *(274–276)*. Secondly, downregulation of HLA-A/-B drastically reduces stoichiometric interaction with T cell receptors because they are much higher expressed on cellular surfaces than HLA-C *(49)*.

While BKPyV showed no downregulation of combined HLA-I expression, this thesis largely confirmed the above-described dysregulated state of HLA-I in HAdV infection *(211, 212)*. It is interesting to note that in HIOs, HLA-A2 (locus *HLA-A*0201*) was more strongly affected by E3/gp19K-mediated downregulation than HLA-A3 (locus *HLA-A*0301*), while wt HAdV5 infection clearly downregulated HLA-A3 in HEK293 cells *(211)*. In fact, HLA-A3 expression on wt-infected hexon^{+} IECs is effectively unaltered compared to the uninfected mock control. These results are in line with surface plasmon resonance analyses attributing the binding of E3/gp19K to HLA-A*0201 a lower dissociation constant (K_d) than the interaction with HLA-A*0301 *(143)*. Wt HAdV5 infection furthermore resulted in clear downregulation of HLA-Bw4 and HLA-Bw6 molecules on IECs *(211)*. While regulation of HLA-Bw6 might have no impact on NK cell recognition as there are no receptor interactions described, regulation of HLA-Bw4 is highly relevant, considering the interaction with the inhibitory KIR3DL1 receptor and genetic

associations in infections with HIV, HCV, HBV and HAdV (Table 1 and chapter 3.2). Boudreau *et al*. described a KIR3DL1-dependent NK cell response to primary $CD4^+$ T cells infected with HIV-1 and showed that $KIR3DL1^+$ NK cell activation strongly correlates with the expression of specific HLA-B allotypes *(277)*. The HIO donor expressing HLA-Bw4 exhibited an *HLA-B*4402*/*HLA-B*5801* genotype and both allotypes have moderate to high association with most KIR3DL1 subtypes; the *HLA-B*5801* locus encodes for an isoleucine (HLA-Bw4I80) with high affinity to KIR3DL1 *(211, 277)*. It seems plausible that HAdV5-mediated downregulation of HLA-Bw4 triggers "missing-self" recognition by $KIR3DL1^+$ NK cells and whether this is the case is worth investigating in co-culture experiments.

There are two additional noteworthy observations when comparing HLA-I expression between HAdV5-infected IECs and HEK293 cells *(211)*. Firstly, HLA-I expression on $hexon^+$ IECs infected with $19K^{stop}$ HAdV5 is generally higher than on infected HEK293. Secondly, in all HLA stainings, except HLA-C, HLA-I expression on $hexon^-$ bystander IECs was ~1.5-fold higher than in the mock control. The overall increased HLA-I expression on IECs may be explained by a stronger innate immune response mounted by HIOs. HAdV infection *in vivo* is associated with the release of the cytokines tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), IL-6, IL-12 and IFN-γ *(278)*. Infections with HAdV3/7 in mice confirmed the induction of TNF-α, IL-1β and IFN-γ as part of the acute inflammatory response in the murine airway epithelium *(279)*. IFN-γ and type I IFNs (-α/-β/-λ) are strong inducers of HLA-I expression, which is due to transcriptional regulation through IFN-stimulated response element (ISRE) present in the promotor region of all classical and non-classical *HLA-I* genes *(280)*. Increased HLA-I expression on IECs may therefore be a result of IFN production by HIOs in response to HAdV5 infection, which is a possible explanation for the rescue of HLA-A3 expression measured on $hexon^+$ IECs infected with wt HAdV5 *(211)*. Indeed, the capacity of IECs to produce mentioned IFNs has been demonstrated in infectious settings *(281–284)*. Consistent with these observations, a study on rotavirus infection in HT-29 IECs reported upregulation of HLA-I expression on bystander cells, which was partially linked to IFN-α signaling *(285)*. Moreover, uninhibited HLA-I expression by $19K^{stop}$ HAdV5-infected IECs stands in contrast to HEK293 infections and is indicative of a physiological host cell response to increase cross presentation of viral peptides to the immune system. Taken together, these results underline the importance of E3/gp19K in dampening HLA-I surface expression in HAdV infection and provide justification for the activities of E1A and E1B/55K to repress type I IFN production during infection *(138, 139)*.

The apparent lack of HLA-I downregulation by BKPyV is unusual for a chronic virus and may pose an important link for the strong control of this pathogen by antiviral T lymphocytes *(212, 286)*. Compared with HAdV and HIV, BKPyV is less studied concerning attenuation of HLA-I expression, so there might be yet undiscovered clinical strains with this ability. The observation

that LT$^+$ tubule cells upregulated HLA-I may likewise be explained by IFN stimulation and increased presentation of immunogenic BKPyV peptides *(212)*.

3.4 Regulation of HLA-F surface expression in viral infection

The increased HLA-I expression by hexon$^-$ bystanders over mock cells stands in stark contrast to equal and low expression of HLA-F *(211)*. These results may be explained by the tight regulation of HLA-F surface expression. In addition to ISRE, *in vitro* gene reporter analyses of the HLA-F promotor region have identified regulatory modules for the transcription factor NF-κB *(287)*. Kumar *et al*. have provided evidence for a link between NF-κB induction by viral infection and the upregulation of HLA-F surface expression in AV-3 epithelial cells infected with Japanese encephalitis virus *(288)*. The authors further described an induction of HLA-F mRNA transcription in cells stimulated with exogenous IFN-β, IL-1β and TNF-α, a product of NF-κB activation *(288)*. Studies on innate immune reactions to adenoviral vectors have reported nuclear translocation of NF-κB in epithelial cells early after cellular uptake *(289)*. The group of Daniel Muruve identified the interaction of adenoviral penton protein with host cell integrins as an important stimulus for the NF-κB pathway and the MAP kinases p38 and ERK; the latter of which promote IFN production and thereby affect HLA-I expression (see chapter 3.3) *(290, 291)*.

Following viral attachment and internalization, the viral capsid is depackaged, exposing additional subviral structures to innate PRRs. There is some evidence that TLRs are involved in intracellular sensing of HAdV structures. TLR9 seems particularly likely to be involved because it senses non-methylated CpG-rich dsDNA, such as is contained in the HAdV5 genome *(292)*. Although TLR9 sensing has been successfully connected to infection of epithelial HeLa cells with HAdV species B, TLR9 recognition of HAdV5 was marginal *(292)*. The authors speculated that this is due to the attachment of HAdV species B to CD46, whereas HAdV5 and other species C strains utilize CXADR for cell entry *(113)*. One study performed in epithelial A549 cells showed that the binding of HAdV5 fiber to CXADR triggers ERK and NF-κB activation, which is highly reminiscent of the immune reaction to the penton–integrin interaction described above *(293)*. The fiber–CXADR interaction and subsequent NF-κB and IFN stimulation therefore poses one likely mechanism for the HAdV5-induced increase in HLA-F surface expression.

Several lines of investigation have furthermore shown that the DNA sensors DEAD (Asp-Glu-Ala-Asp) box polypeptide 41 (DDX41) and cyclic guanosine monophosphate adenosine monophosphate synthase (cGMP-AMP synthase, short cGAS) recognize cytosolic HAdV DNA, eliciting a robust IFN-β response *(294)*. Activation of the NF-κB pathway can also occur in an indirect way from the activity of E3/gp19K. The continuous retrieval of HLA-I molecules in the ER leads to an accumulation (“overload”) of proteins, triggering NF-κB activation through the unfolded protein response (UPR) pathway *(295)*. However, HLA-F surface expression in

infection with 19K^{stop} was similar to wt HAdV5 infection, indicating that E3/gp19K alone does not strongly contribute to HLA-F upregulation *(211)*.

Taken together, these observations illustrate that the host cell response to HAdV infection depends on several decisive factors such as host cell and HAdV type, route of viral internalization, trafficking pathways and subcellular location. Overlapping activation pathways furthermore suggest that the systems for innate sensing of HAdV are partially redundant *(291)*. The exact mechanisms by which HLA-F surface expression is regulated are still unknown. Singular stimulation with exogenous IFN-γ/-β, IL-1β and TNF-α induces HLA-F gene expression, and all of these cytokines are upregulated in HAdV infection *(66, 288, 294)*. However, applying singular stimuli does not necessarily result in concomitant cell surface transportation *(66)*. A specific combination of internal and external stimuli is a plausible explanation for HLA-F expression on HAdV host cells, whereas the absence of HLA-F on bystander cells may be a sign of insufficient endogenous stimulation. It has been shown in epithelial HeLa cells that HLA-F primarily localizes in the ER and Golgi *(214)*. In a speculative sequence of action, HAdV infection of an IEC first stimulates the innate sensing network, driving the production of HLA-F and its accumulation in the export machinery. Subsequently, a specific combination of cytokine stimulation and accumulated intercellular stress, such as deterioration of subcellular compartments by viral replication, cause the release of the HLA-F pool and transportation to the cell surface.

It is interesting to note that in BKPyV-infected tubule cell cultures, LT$^-$ bystander cells showed an over 2-fold increase in HLA-F surface expression compared to mock *(212)*. Most of the knowledge about the innate immune response to BKPyV derives from the analysis of renal inflammation in BKVAN. Increased response markers include IL-6/-8, C-C motif chemokine (CCL) 2, CCL5 and C-X-C motif chemokine 10 (CXCL10) *(152)*. Astonishingly, a transcriptomic analysis of primary BKPyV infection in human primary kidney epithelial cells identified the upregulation of only two inflammatory genes, pentraxin 3 (*PTX3*) and *MICB (213)*. Besides IL-6, CXCL10 is also a common response marker in HAdV infection and associated with NF-κB activation *(296)*. Common cytokine secretion and the fact that both viruses are detectable for viral DNA sensors form possible parallels in how these viruses induce HLA-F surface expression. The observed difference in HLA-F expression on HAdV and BKPyV bystander cells may also be explained by differing time points of analysis *(211, 212)*. Whereas the analysis of HAdV5-infected HIOs was conducted 3 days post-infection (DPI), BKPyV-infected tubule cell cultures were measured 10 DPI. Therefore, it is likely that the accumulated stimulus by antiviral immune mediators was increased in tubule cells, possibly bringing HLA-F to the surface of LT$^-$ cells.

The mechanisms by which HLA-F surface expression is upregulated by viral infection remain incompletely understood. Kumar *et al*. have demonstrated the involvement of NF-κB activation

in virus-induced HLA-F upregulation by treating host cells with molecular NF-κB inhibitors *(288)*. IFN and NF-κB inhibitors could be implemented for future experiments on HLA-F surface expression in HAdV and BKPyV infection *(297)*. Alternatively, host cells that are inherently unresponsive for the mentioned stimuli could be used, for example signal transducer and activator of transcription (STAT)-deficient gastric adenocarcinoma cells, which exhibit blocked IFN signal transduction and can be infected by HAdV *(298)*. Finally, IFN-unresponsive host cells (and HIOs) can be genetically engineered by overexpressing the V protein of the paramyxovirus simian virus, which blocks type I IFN stimulation by targeting STAT *(299)*.

3.5 Prospects of organoid applications in virology

The usage of organoids for the investigation of HAdV5 infection has resulted in a number of deviating observations from results in conventional cell lines *(211)*. The differentiation of stem cell HIOs using Wnt3-free medium induced the expression of genetic markers for Paneth cells, enterocytes, goblet cells and enteroendocrine cells. This represents the full range of IEC types present *in vivo* that can serve as potential host cells for HAdV. In their examination of HAdV5 infection in HIO monolayers, Holly and Smith identified an increased infection rate in the goblet cell population *(207)*. However, goblet cells constitute a minor portion in the cellular composition of HIOs (~1%) and the intestinal epithelium (<10%), and infection rates in HIOs exceeding 20% point to additional tropism *(207, 300)*. The observation of moderate infection in HIOs that were maintained in stem cell medium further suggests that HAdV5 entry and replication is not limited to differentiated IECs of the villus compartment but also comprises crypt-residing TA, Paneth and ISCs *(211)*. Holly and Smith further investigated HIO monolayer infection with HAdV41, reporting a resistance to defensin 5 and that HAdV41 has no preference for goblet cells *(207)*. HAdV types 40 and 41 form the enteric HAdV species F, which is a leading cause for acute gastroenteritis in infants and young children worldwide *(301–303)*. Notably, species F does not encode E3/gp19K and has shorter fiber proteins than species C, which possibly explains lacking persistent capacities and prevalence in acute infections *(117, 132)*. Instead of gp19K, HAdV40/41 express the E3 proteins 19.4K and 31.6K, which are conspicuously missing in other species, but are possibly involved in attenuating the immune response as compensation for missing gp19K activity *(132)*. New preliminary data acquired from HAdV41 infections of intestinal epithelial HCT116 cells suggest that 19.4K and 31.6K play a role in sequestering MICB surface expression *(304)*. Nevertheless, the growth of HAdV40/41 is restricted in standard cell lines and heterocellular HIOs might be a more suitable model to examine the pathology and immune evasion mechanisms of these clinically highly relevant strains *(206)*. To investigate the tropism of different HAdV strains, infected HIOs could be stained with antibodies against specific IEC types and then analyzed by whole-mount immunofluorescence microscopy. Infected IECs could be visualized with antibodies against HAdV or by using a fluorescent HAdV reporter strain, such as HAdV5/mCherry *(211)*, and the

quantification of co-localizing viral and IEC proteins may help to assess preferences in host cell infection.

In addition to providing a variety of host cells, HIOs offer the benefit of three-dimensional architecture and compartmentalization. The infection experiments performed with HIOs presenting the basolateral or apical membrane outward led to the finding that the entry receptor for HAdV5 CXADR is primarily expressed on the apical side of HIOs and that this is associated with a strongly increased infection rate *(211)*. In line with immunofluorescence data on polarized colorectal carcinoma epithelium (T84 cells), CXADR co-localized with zona occludens 1 (ZO-1) at the apical poles of epithelial tight-junctions in HIOs *(114)*. Although other studies have shown that HAdV infection is very limited from the apical side of human airway epithelium, the route of infection is less clear for the intestinal epithelium *(305, 306)*. It is possible that spherical organoids do not accurately recapitulate the polarity of physiological intestinal epithelium and that the permissiveness of apical-out HIOs is an *in vitro* artifact. Whether this is the case could be examined by imaging CXADR in HIO monolayers, which form correctly polarized epithelium *(307)*.

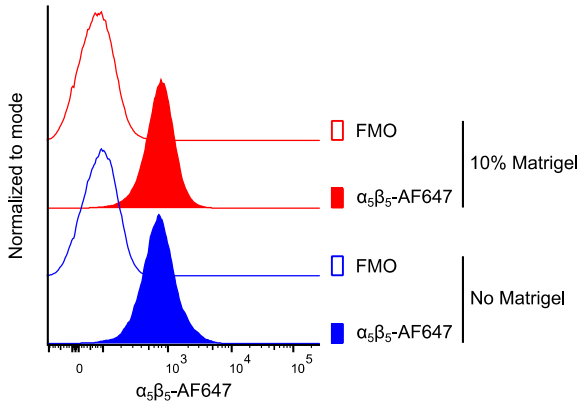

Fig. 6. Organoid-derived IECs express α5β5 integrin. Suspension organoids were cultured for 5 days in differentiation medium with 10% (v/v) Matrigel or without. IECs were stained with LIVE/DEAD dye (Invitrogen) and with anti-α5β5 labeled with Alexa Fluor 647 (AF647, Biolegend catalogue# 920006) or without (fluorescence minus one, FMO). n = 1 organoid donor. Cells were recorded with BD LSRFortessa II and analyzed using FlowJo 10.7 software (BD Biosciences).

A study by Zabner *et al*. on $\alpha_5\beta_5$ integrin, which is used as secondary receptor by HAdV2/5, provides an alternative explanation for the inefficient infection of apical airway epithelium *(305)*. After attaching to CXADR with its fiber protein, internalization of HAdV is catalyzed by the interaction between integrin and an RGD peptide contained in the penton protein *(308)*. The authors found that $\alpha_5\beta_5$ integrin is missing on mature apical airway epithelium and is expressed on the basolateral side, hampering infection with HAdV2 vectors *(305, 306)*. Flow cytometry of apical-out HIOs showed that $\alpha_5\beta_5$ integrin is abundantly expressed on IECs, including when cultured without ECM (Matrigel) (Fig. 6). This provides evidence for efficient viral internalization in IECs, although spatial expression should be examined to further determine the significance of $\alpha_5\beta_5$ integrin expression in HAdV5 infection of apical-out HIOs.

IECs contained in HIOs showed a more realistic and perhaps more physiological profile of immune ligands than HEK293 cells *(211)*. HEK293 like all other transformed and cancer cell lines contains mutations or transgenic material inhibiting apoptosis in continuous culture. This typically entails physiological changes that often include the stable expression of induced-self ligands *(208, 209)*. Consistent with this, HEK293 cells showed high baseline expression of MICA/B and ULBP1/2/5/6, and while this enabled the study of downregulation effects by E3/gp19K, it stands in stark contrast to absent NKG2D ligand expression on HIO-derived IECs *(211)*. In fact, HAdV5 infection of IECs revealed a *de novo* expression of surface NKG2D ligand, which has previously been highlighted by two studies showing NKG2D-dependent NK cell activation in response to HAdV5 E1A activity and E1-deleted adenoviral vectors *(309, 310)*. It is interesting to note that HEK293 cells stably express E1A, which could explain the constitutive expression of NKG2D ligands on these cells *(309)*. The observation that infected IECs expressed higher HLA-F levels than infected HEK293 is another potential argument for improved physiology and is possibly linked to the robust innate immune reactions by organoids following microbial challenge *(181, 210, 211)*. Much of the data on innate immunity to HAdV was generated with adenoviral vectors due to the interest in safety and applicability. Future studies should not only use clinical full sequence wt viruses, but also focus on infecting organoids to generate physiological immune responses. The quantification of the organoid transcriptome following viral infection by RNA sequencing could provide an overview of the inflammatory response with improved resemblance to *in vivo* immunopathology.

As a final point, organoids have the genetic identity of the original tissue donor, which includes varying HLA allotypes. The assessment of individual HLA allotype expression in HAdV5 infection of two HIO donors with distinct HLA-I dispositions allowed interesting observations in the individual HLA-I allotype regulation as a function of E3/gp19K activity (see chapter 3.3) *(211)*. While HLA-based studies in cell lines are restricted by the genetic HLA disposition of the original cell material, the generation of an HLA-typed organoid library enables studies of unlimited HLA allotype combinations. This feature is particularly interesting for co-culture experiments with immune cells because it allows HLA/KIR matching of organoid and immune cell donors, reducing undesirable alloreactivity *(181)*.

The BKPyV infections in this work were performed in RPTEC/TERT1, a cell line developed from renal proximal tubule epithelium (RPTEC) and immortalized through overexpression of human telomerase reverse transcriptase (TERT) *(311)*. As of late, unmanipulated cellular models of the kidney epithelium are available that are referred to as kidney organoids or tubuloids when developed from PSCs or adult stem cells, respectively *(312)*. Tubuloids can be generated from urine, which, as discussed for HIOs above, provides the opportunity to create an immunogenetic donor library for immune studies and HLA-matched co-culture experiments. Furthermore, tubuloids are an unexplored, but potentially formidable model for primary BKPyV

infections with high potential for investigations on viral tropism, pathophysiology, and innate immune responses of infected tubule cells.

4 Conclusion

This research showed that human intestinal organoids can be efficiently infected with HAdV5. Following infection, infected and uninfected intestinal epithelial cells were analyzed in the expression of immune ligands and successfully co-cultured with primary NK cells. Therefore, human intestinal organoids are a suitable model for studying the pathology of HAdV5 infection and immune cell responses. These results hold promise for utilizing organoids in future virology studies, including the investigation of viral tropism and entry in epithelial tissues, the *in vitro* culture of viruses including those difficult to maintain such as norovirus and rotavirus and the infection of organoids derived from other organs with matching pathogens, for example the infection of tubuloids with BKPyV.

Furthermore, it was demonstrated that HAdV5 and BKPyV infection induces the upregulation of HLA-F surface expression, leading to improved recognition and viral control by KIR3DS1^{+} NK cells. Despite being a potent attenuating protein for classical HLA-I molecules, HAdV5 E3/gp19K showed no effect on HLA-F expression. The fact that HLA-F was primarily upregulated on infected cells and less on uninfected bystanders suggests that HLA-F may be a suitable therapeutic target. Molecular and cellular tools that use the ligand-binding domain of KIR3DS1 to recognize HLA-F could be engineered to preferentially target infected cells. This could aid the immune system in fighting difficult to control viral infections, importantly, HAdV and BKPyV infections in immunocompromised patients.

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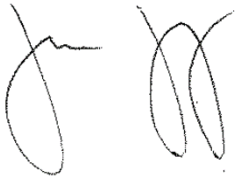
Eidesstattliche Versicherung

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 18.01.2022 Unterschrift



Appendix

Participation in publications

Publication #1 “KIR3DS1 directs NK cell-mediated protection against human adenovirus infections”, DOI: 10.1126/sciimmunol.abe2942[1]
I was involved in the design and realization of the experiments, data analysis and presentation and writing of the manuscript.

Publication #2 “Upregulation of HLA-F expression by BK polyomavirus infection induces immune recognition by KIR3DS1-positive natural killer cells”, DOI: 10.1016/j.kint.2020.12.014[2]
I contributed to the rebuttal of this paper by acquiring and analyzing additional experimental data.

Hamburg, den 18.01.2022 Unterschrift Betreuer

1. From J. M. Jung, W. Ching, M. E. Baumdick, H. Hofmann-Sieber, J. B. Bosse, T. Koyro, K. J. Möller, L. Wegner, A. Niehrs, K. Russu, M. Ohms, W. Zhang, A. Ehrhardt, K. Duisters, E. Spierings, A. Hölzemer, C. Körner, S. A. Jansen, S. Peine, I. Königs, M. Lütgehetmann, D. Perez, K. Reinshagen, C. A. Lindemans, M. Altfeld, M. Belderbos, T. Dobner, M. J. Bunders, KIR3DS1 directs NK cell–mediated protection against human adenovirus infections, *Sci. Immunol.* **6** (2021). Reprinted with permission from AAAS.
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KIR3DS1 directs NK cell–mediated protection against human adenovirus infections

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Human adenoviruses (HAdVs) are a major cause for disease in children, in particular after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Currently, effective therapies for HAdV infections in immunocompromised hosts are lacking. To decipher immune recognition of HAdV infection and determine new targets for immune-mediated control, we used an HAdV infection 3D organoid system, based on primary human intestinal epithelial cells. HLA-F, the functional ligand for the activating NK cell receptor KIR3DS1, was strongly up-regulated and enabled enhanced killing of HAdV5-infected cells in organoids by KIR3DS1$^+$ NK cells. In contrast, HLA-A and HLA-B were significantly down-regulated in HAdV5-infected organoids in response to adenoviral E3/glycoprotein19K, consistent with evasion from CD8$^+$ T cells. Immunogenetic analyses in a pediatric allo-HSCT cohort showed a reduced risk to develop severe HAdV disease and faster clearance of HAdV viremia in children receiving *KIR3DS1*$^+$/*HLA-Bw4*$^+$ donor cells compared with children receiving non–*KIR3DS1*$^+$/*HLA-Bw4*$^+$ cells. These findings identify the KIR3DS1/HLA-F axis as a new target for immunotherapeutic strategies against severe HAdV disease.

INTRODUCTION

Human adenoviruses (HAdVs) are a major cause of infections in humans (*1*). Several mucosal tissues can be infected with HAdV serotypes, including the intestine, lungs, and eyes. However, almost all HAdV serotypes replicate in the intestine, which is a prime site for HAdV infection and reactivation (*2*–*4*). In particular, children and immunocompromised individuals are at risk of developing serious HAdV complications (*5*), and HAdV accounts for more than 10% of hospitalizations for severe childhood gastroenteritis (*6*, *7*). In the setting of pediatric allogeneic hematopoietic stem cell transplantation (allo-HSCT), HAdV reactivation can be a life-threatening disease, characterized by intestinal inflammation, hepatitis, and respiratory failure, resulting in 8 to 26% mortality (*8*–*10*). HAdV species C, which includes type 5 (HAdV5), is the most frequently identified HAdV species after allo-HSCT because of effective immune evasion and persistence (*11*). At present, effective therapies for HAdV without severe adverse effects are lacking, which emphasizes the clinical need for new treatment strategies (*9*, *12*, *13*). We have recently shown that natural killer (NK) cells are the first cytotoxic lymphocytes populating the intestine in young children (*14*). NK cells are also among the first cells to recover after allo-HSCT and to enter mucosal tissues (*15*, *16*). Therefore, NK cells may provide critical natural protection against viral infections and could be harnessed to treat severe HAdV infection in immunocompromised children.

The recognition of HAdV-infected cells by T and NK cells depends on the expression of ligands on the cell surface, in particular human leukocyte antigen (HLA) molecules (*17*, *18*). Upon recognition of infected cells, the combined signaling downstream of activating and inhibitory receptors, which are subject to genetic variability, regulates NK cell responses (*17*, *18*). HAdV5 encodes the immune regulatory E3/glycoprotein19K and, like HAdV in general, can persist in tissues by altering the expression of ligands regulating immune recognition by CD8$^+$ T and NK cells (*2*, *4*, *11*, *19*–*21*). Studies using immortalized cells showed that E3/glycoprotein19K can bind and retain HLA class I molecules in the endoplasmic reticulum, thereby preventing presentation of viral peptides and killing of infected cells by CD8$^+$ T cells (*20*, *21*). E3/glycoprotein19K also suppresses surface presentation of major histocompatibility complex class I chain–related proteins A and B (MICA/B), promoting escape from NKG2D$^+$ NK cells (*22*). Because of their malignant transformation, cell lines based on immortalized cells have an altered baseline expression profile of immune ligands for T and NK cells, affecting immune recognition and skewing potential results from infection

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experiments compared with events in primary cells in vivo (*23*, *24*). Furthermore, homogeneous cell lines do not recapitulate the cellular heterogeneity characteristic for physiological tissues. The organoid technology based on primary human epithelial cells provides the opportunity to study viral infections in cell cultures based on primary human tissue cells (*25*–*27*), and intestinal organoids can be infected with HAdV (*28*). Here, we implemented a human intestinal organoid system to investigate the mechanisms by which NK cells can recognize and kill HAdV5-infected intestinal cells. To provide in vivo support of the identified mechanisms regulating NK cell–mediated control of HAdV in vitro, we assessed factors associated with severe HAdV disease in a cohort of children that received allo-HSCT.

RESULTS

Human intestinal organoids provide a robust model for HAdV5 infection

Three-dimensional (3D) intestinal organoids were generated from epithelial cells derived from human small intestinal (ileum) tissues. Intestinal epithelial cells were first cultured in a basement membrane extract (Matrigel) and medium including Wingless-type mouse mammary tumor virus integration site family member 3A (Wnt-3a), R-spondin-1, and Noggin to promote proliferation (*29*). A heterocellular system that resembles the composition of the human intestinal epithelium was obtained by adapting medium conditions to allow cellular differentiation (*29*), and organoids were further grown without scaffold to enable contact between organoids and virions in medium. This protocol allowed generation of organoids with the structure and cellular heterogeneity of the human intestine, including enterocytes, goblet cells, and enteroendocrine cells (Fig. 1, A and B). The long-term culture of 3D organoids in suspension has been shown to affect their polarity (*30*), which may have consequences for viral attachment and infection. Organoids cultured in basement membrane extract (Matrigel) present their basolateral side, whereas organoids cultured in suspension within 24 to 48 hours flip and present the apical side (*30*). Visualization of the apical side of 3D organoids by antibody staining of zonula occludens-1 (ZO-1) showed that organoids cultured in suspension flipped and presented their apical side compared with organoids cultured in Matrigel (Fig. 1C).

Building on these previous studies, we assessed whether organoid polarity affected HAdV infection. This is of relevance because primary HAdV infection occurs from the apical side. HAdV reactivation, on the other hand, is believed to originate from viruses in latently infected lymphocytes residing in the intestinal lamina propria that infect intestinal epithelial cells from the basolateral side (*3*, *31*), resulting in high viral load in stool and diarrhea as early clinical manifestations of severe HAdV reactivation (*3*, *32*, *33*). HAdV5 uses the coxsackievirus and adenovirus receptor (CXADR) as primary receptor for cell entry (*34*). 3D microscopy of intestinal organoids revealed that CXADR was expressed on both the apical and basolateral side, although to a lesser extent at the basolateral side (Fig. 1D). To assess whether apical or basolateral exposure of intestinal organoids to HAdV5 affected infection kinetics, intestinal organoids presenting the basolateral side (embedded in Matrigel) or presenting the apical side (cultured in suspension) were infected with an HAdV5/mCherry virus. Infection was detected in both models, although infection of organoids presenting the apical side was more efficient compared with organoids presenting the basolateral side (Fig. 1E). We assessed whether the lower infection rates in the basolateral side-presenting organoids were due to Matrigel surrounding the organoids during incubation with the viral inoculum. However, complete organoids freshly isolated from Matrigel and thereby presenting the basolateral side during a 1-hour infection incubation in suspension also showed lower infections compared with disrupted organoids presenting both apical and basolateral side (fig. S1A). Furthermore, 3D organoids cultured in expansion medium enriching for stem cells and embedded in Matrigel could also be infected with HAdV5 (fig. S1B). Thus, the human intestinal epithelium provides several populations of target cells that can be infected by HAdV5 from the lumen as well as from the lamina propria. Because of its high efficiency, we subsequently used the apical infection model of organoids in differentiation medium to assess which ligands can mediate immune recognition of virus-infected cells.

The HAdV5/mCherry strain used to visualize infection of intestinal organoids lacks the immune regulatory E3/glycoprotein19K; therefore, we implemented a flow cytometric assay detecting adenoviral proteins to assess the immune regulatory effects of E3/glycoprotein19K. We used wild-type (WT) and E3/glycoprotein19K knockout (19K^{stop}) HAdV5 in 3D organoids (fig. S2), which allowed us to quantify infection at the single-cell level by measuring adenoviral hexon protein expression. Intestinal organoids infected with WT and 19K^{stop} HAdV5 showed increased frequencies of hexon-positive infected cells up to 4 days after infection (Fig. 1, E and F, and fig. S1C). The optimal time point to obtain the largest absolute numbers of viable infected cells for expression analyses of ligands for NK cells was 3 days after infection and used in the subsequent experiments.

HAdV5-infected organoids up-regulate ligands for NKG2D

Ligands for the activating NK cell receptor natural killer group 2D (NKG2DL) are generally expressed at low levels under homeostatic conditions and can be up-regulated in viral infections, allowing recognition of infected or transformed cells by NK cells (*35*). NKG2DL include UL16-binding proteins (ULBP) and MICA/B, which were previously suggested to be down-regulated by E3/glycoprotein19K (*22*). We therefore assessed the consequences of HAdV5 infection for expression of NKG2DL in organoids. Baseline expression of NKG2DL (combined antibody pool against MICA/B, ULBP1, and ULBP2/5/6) by uninfected epithelial cells was nearly absent nor induced in hexon-negative uninfected cells derived from HAdV5-infected intestinal organoids (Fig. 2, A and B). In contrast, NKG2DL expression was significantly increased on HAdV5-infected (hexon-positive) epithelial cells in intestinal organoids. NKG2DL expression was up-regulated 1.7-fold [interquartile range (IQR): 1.4 to 1.8, $P = 0.004$] on epithelial cells infected with WT HAdV5, and 2.0-fold (IQR: 1.7 to 2.2, $P = 0.004$) by 19K^{stop} HAdV5, which is significantly higher than WT HAdV5 ($P = 0.01$). In comparison, human embryonic kidney (HEK) 293 cells exhibited high NKG2DL expression at baseline, which was actually reduced by WT HAdV5 infection compared with 19K^{stop} HAdV5 (fig. S3B). The absent expression of NKG2DL on epithelial cells in organoids at baseline, followed by significant de novo induction by HAdV5 infection, demonstrated the advantage of the primary cell–based 3D intestinal organoid system to study virus-induced NK cell receptor ligand expression over the conventional use of cell lines.

HAdV5 modulates the expression of classical HLA molecules in infected intestinal organoids

Having established human intestinal organoids as a powerful system to investigate the effect of HAdV5 infection on the expression of

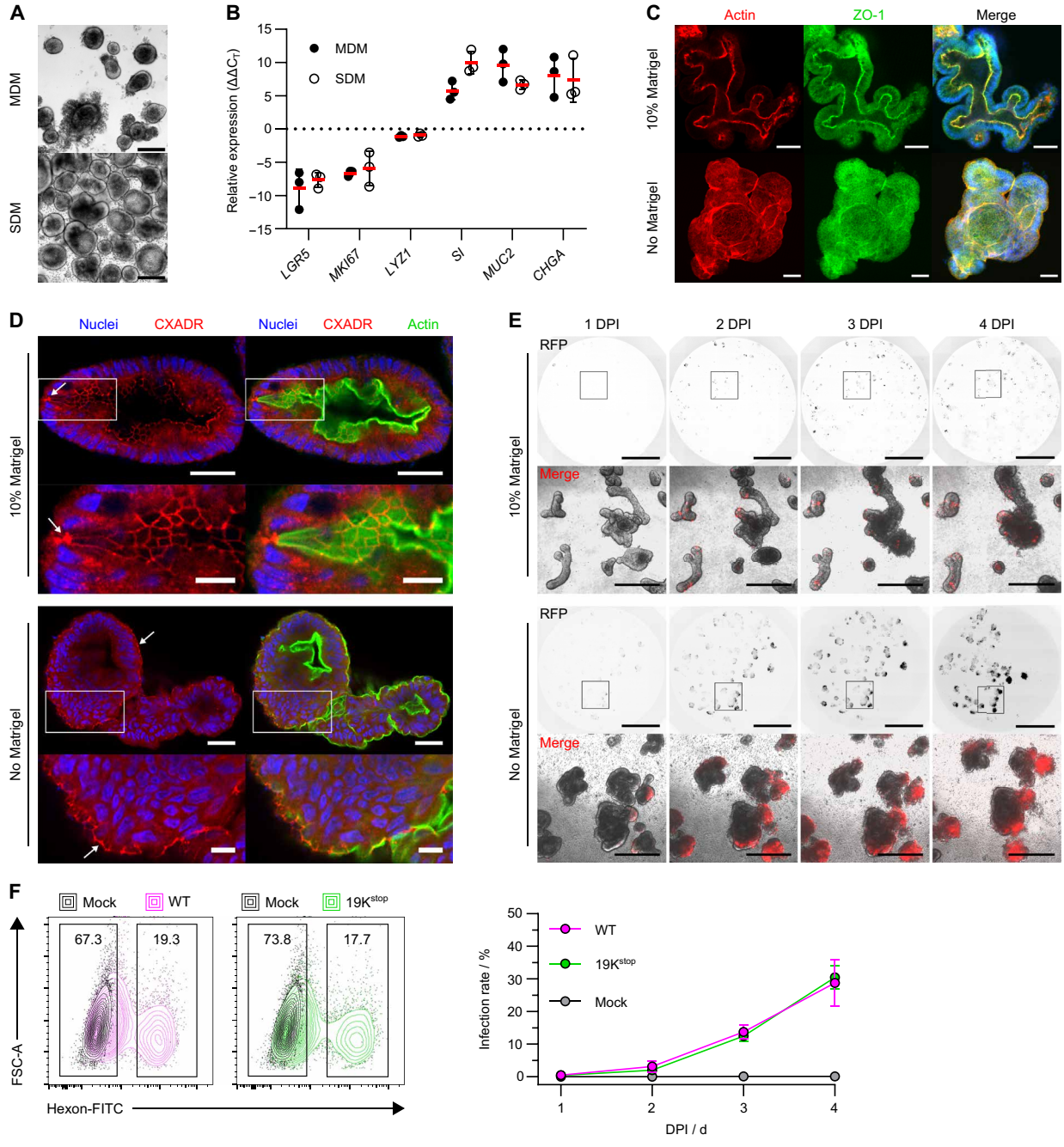

Fig. 1. Efficient HAdV5 infection of human intestinal organoids. (**A**) Human intestinal organoids generated from small intestinal tissue samples (ileum) in differentiation medium with Matrigel (MDM) and in suspension (SDM). Scale bars, 200 μm. (**B**) mRNA expression of hallmark genes for differentiated intestinal epithelial cell types in intestinal organoids cultured in MDM and SDM relative to epithelial cells in expansion medium enriched for intestinal stem cells ($n = 3$ donors; bars depict mean and SD). *LGR5* (intestinal stem cells), *MKI67* (proliferation), *LYZ1* (Paneth cells), *SI* (enterocytes), *MUC2* (goblet cells), *CHGA* (enteroendocrine cells). (**C**) Orientation of 3D intestinal organoids in low (10%) concentration Matrigel, presenting the basolateral side or in suspension presenting the apical side visualized by immunofluorescence staining for nuclei (blue), actin (red), and ZO-1 (green). Scale bars, 50 μm (images representative of organoids derived from two donors). (**D**) Immunofluorescence microscopy of the HAdV receptor CXADR (red), actin (green), and nuclei (blue) in intestinal organoids. Images representative of organoids derived from two donors. White arrows indicate areas with increased signal intensity. Scale bars, 30 μm (in images) and 10 μm (in insets). (**E**) Microscopy of intestinal organoids grown in low (10%) concentration Matrigel or suspension infected with HAdV5/mCherry with an MOI of 10 [DPI, days post infection; RFP, red fluorescent protein as inverted signal resulting in black pixels showing infected organoids; Merge, RFP in red (infection) overlaid with bright-field image]. Representative images from two organoid donors. Scale bars, 2 mm (RFP) and 0.5 mm (Merge). (**F**) Left panel shows gating of infected (hexon-positive) and uninfected (hexon-negative) cell subsets of intestinal organoids grown without Matrigel in suspension analyzed by flow cytometry 3 DPI with WT and 19K^{stop} HAdV5 with an MOI of 10. FSC-A, forward scatter area; FITC, fluorescein isothiocyanate. Right panel shows time course assessment by flow cytometry of epithelial cells derived from organoids infected under the same conditions as in the left panel. Infection rate corresponds to frequencies of hexon-positive cells as shown in gates of left panel. Each data point represents three independent cultures from two donors. Bars depict mean and SD.

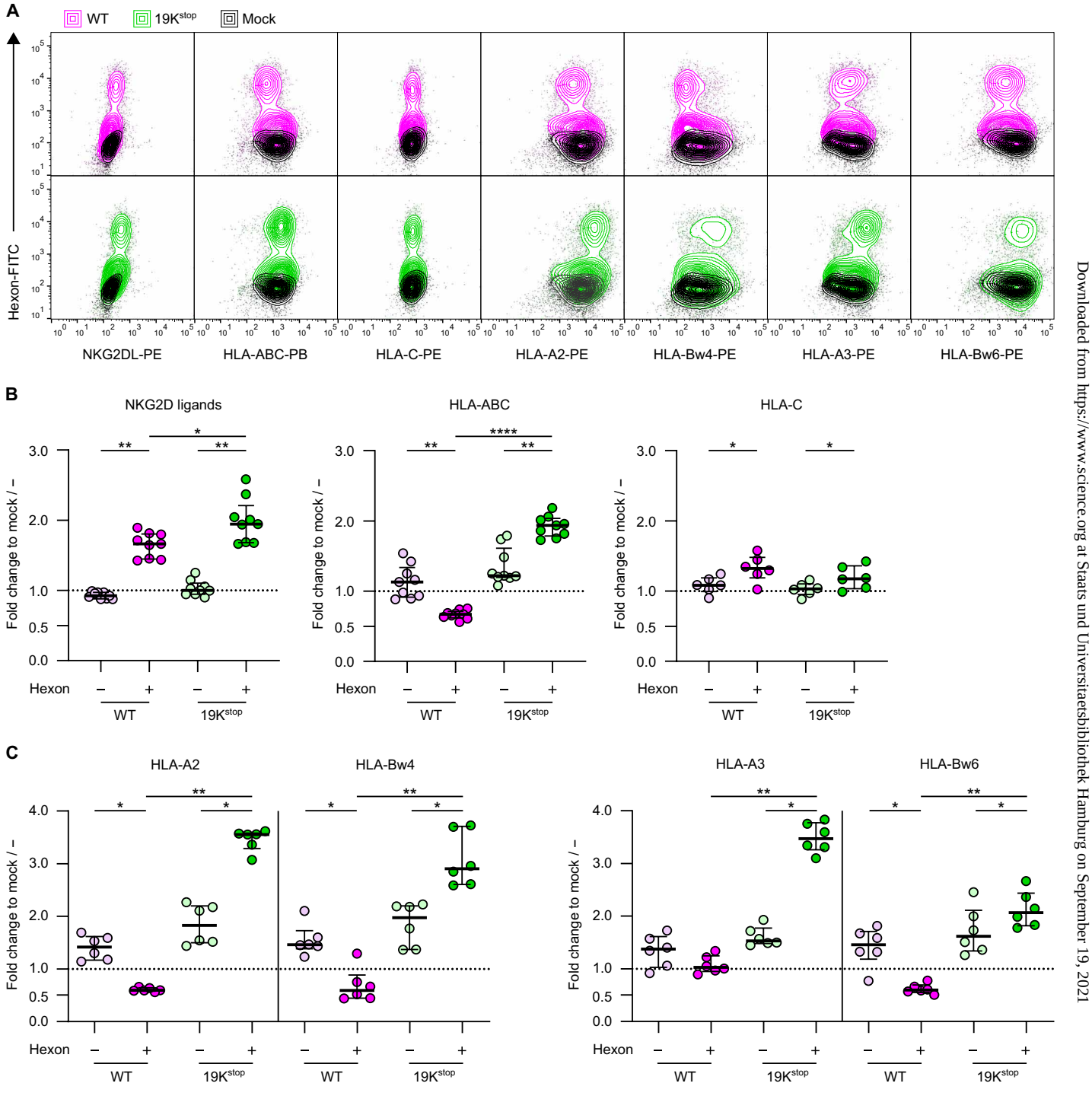

Fig. 2. HAdV5 dysregulates the expression of classical HLA class I molecules in intestinal organoids and up-regulates NKG2D ligands. (**A**) Flow cytometric analyses of WT and 19K^{stop} HAdV5–infected epithelial cells and uninfected controls (mock), showing adenoviral hexon–dependent expression of NKG2D ligands [antibody combination against MICA/B, ULBP1, and ULBP2/5/6, all phycoerythrin (PE)–conjugated] and classical HLA class I. (**B**) Fold change in pan-ligand expression based on MFI measured as in (A) by infected (hexon-positive) and uninfected (hexon-negative) epithelial cells (mock = 1.0) (NKG2DL and HLA-ABC: $n = 9$ from three donors; HLA-C: $n = 6$ from two donors). (**C**) Fold change in singular HLA allotype expression measured as in (A) by infected (hexon-positive) and uninfected (hexon-negative) epithelial cells (mock = 1.0). Left and right graphs show expression of two organoid donors with different HLA-A and HLA-B allotypes ($n = 6$ for each donor). All HAdV5 infections were performed with an MOI of 10. Bars depict median and IQR. Hexon-positive and hexon-negative subsets were compared using Wilcoxon matched-pairs signed rank test. Mann-Whitney *U* test was performed for comparing WT and 19K^{stop} HAdV5 samples. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

NK cell receptor ligands, we determined the consequences of WT and 19K^{stop} HAdV5 infection on the expression of additional ligands for NK cell receptors. HLA class I molecules comprise a large range of molecules serving as ligands for inhibitory KIRs (*17*, *18*). Pan-HLA class I expression was significantly reduced in WT HAdV5–infected organoids, and this reduction was lost in 19K^{stop} HAdV5–infected organoids, in which significant up-regulation of HLA class I was observed (Fig. 2, A and B, and fig. S4A). The reduction in pan-HLA class I expression after HAdV5 infection was largely driven by changes in HLA-A and HLA-B, as changes in HLA-C1/C2 expression were small and unaffected by 19K^{stop} HAdV5 (Fig. 2, A and B). This was confirmed for individual HLA-A and HLA-B molecules, using HLA-A– and HLA-B–specific antibodies on intestinal organoids typed for HLA class I (Fig. 2, A and C). WT HAdV5 infection of intestinal organoids resulted in down-regulation of HLA-A (HLA-A2 in particular), HLA-Bw4 (B*4402/B*5801 alleles), and HLA-Bw6 (B*1801/B*4001 alleles) molecules compared with uninfected organoids, whereas 19K^{stop} HAdV5 infection resulted in significantly higher HLA expression. In line with our observations for NKG2DL, the changes in HLA class I expression after HAdV5 infection in organoids were more robust compared with infections of HEK293 cells, emphasizing the benefit of 3D organoids for these type of analyses compared with transformed cell lines (fig. S3C). Overall, these data show that the immune-modulatory effects of HAdV5 E3/glycoprotein19K are tailored toward down-regulation of HLA-A and HLA-B, which generally reduces CD8$^+$ T cell–mediated immune pressure, whereas HLA-C molecules that serve as ligands for inhibitory KIRs are maintained on the surface of HAdV5-infected epithelial cells.

HAdV5-infected organoids strongly up-regulate HLA-F irrespective of E3/glycoprotein19K

Recently, HLA-F, a nonclassical HLA class I molecule, was identified as the principal ligand for the activating NK cell receptor KIR3DS1, promoting viral control (*36*–*38*). Mock-infected cells in organoids had low HLA-F expression, and HLA-F was not up-regulated on hexon-negative epithelial cells in infected organoids (Fig. 3A and fig. S4A). Expression of HLA-F was, however, strongly and significantly up-regulated on hexon-positive cells in infected organoids, irrespective of E3/glycoprotein19K (median fold change WT = 4.7, IQR: 3.1 to 6.1; median fold change 19K^{stop} = 4.0, IQR: 2.8 to 4.4; both $P < 0.01$) (Fig. 3A). Immunofluorescence showed expression of HLA-F and the HAdV hexon–interlacing pIX protein in 3D HAdV5–infected intestinal organoids, confirming HLA-F expression in intact human 3D epithelial structures (Fig. 3B). In contrast, HLA-E, which was previously shown to be up-regulated by HAdV3 (*39*), remained largely unchanged after infection (fig. S4B). Together, our detailed analyses identified HLA-F, the ligand for the activating NK cell receptor KIR3DS1, to be strongly up-regulated on HAdV5-infected cells compared with other ligands, but not on bystander cells. E3/glycoprotein19K did

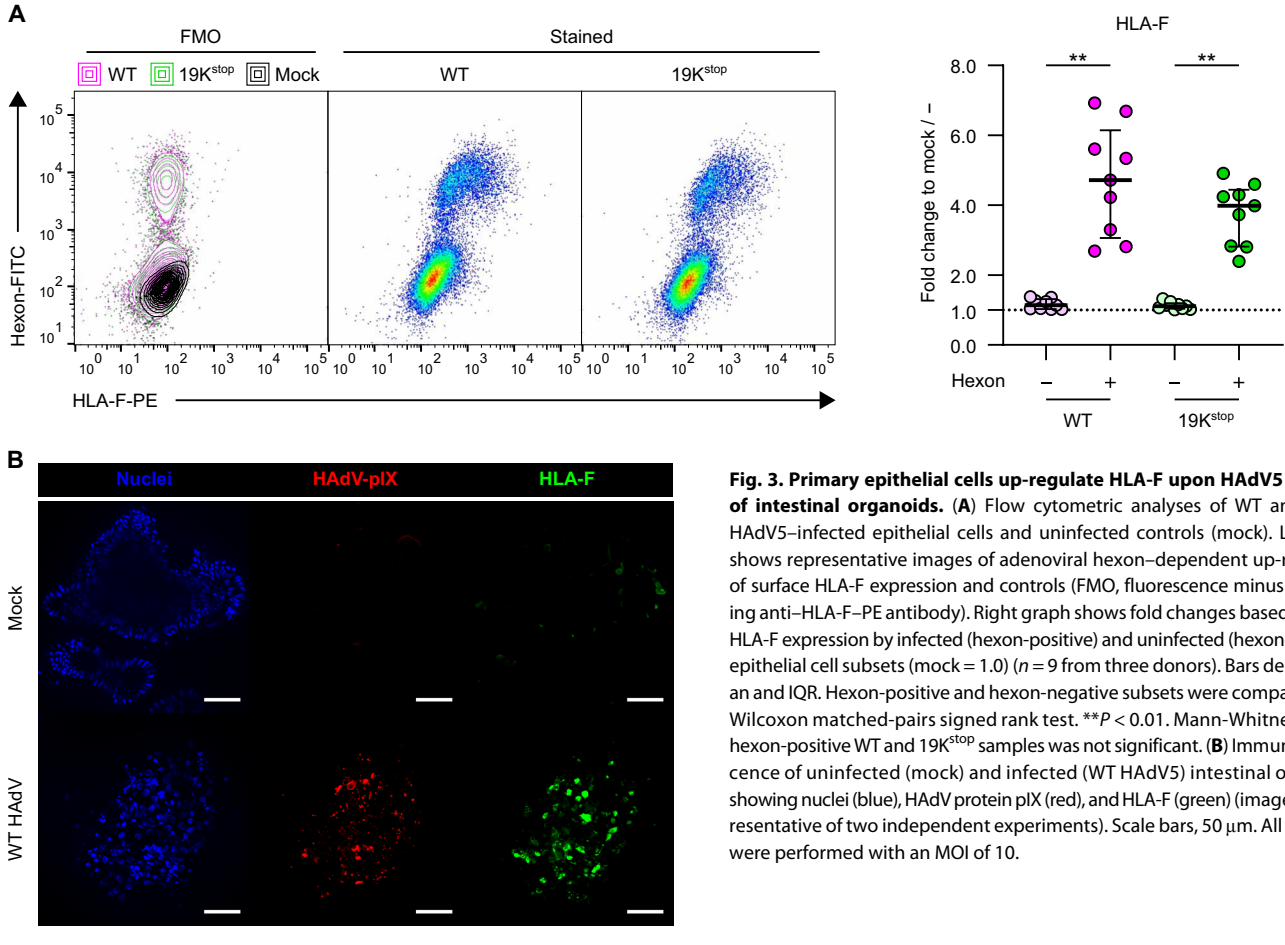

Fig. 3. Primary epithelial cells up-regulate HLA-F upon HAdV5 infection of intestinal organoids. (**A**) Flow cytometric analyses of WT and 19K^{stop} HAdV5–infected epithelial cells and uninfected controls (mock). Left graph shows representative images of adenoviral hexon–dependent up-regulation of surface HLA-F expression and controls (FMO, fluorescence minus one, lacking anti–HLA-F–PE antibody). Right graph shows fold changes based on MFI in HLA-F expression by infected (hexon-positive) and uninfected (hexon-negative) epithelial cell subsets (mock = 1.0) ($n = 9$ from three donors). Bars depict median and IQR. Hexon-positive and hexon-negative subsets were compared using Wilcoxon matched-pairs signed rank test. $^{**}P < 0.01$. Mann-Whitney *U* test of hexon-positive WT and 19K^{stop} samples was not significant. (**B**) Immunofluorescence of uninfected (mock) and infected (WT HAdV5) intestinal organoids, showing nuclei (blue), HAdV protein pIX (red), and HLA-F (green) (images are representative of two independent experiments). Scale bars, 50 μm. All infections were performed with an MOI of 10.

not affect HLA-F expression, identifying HLA-F as a potential robust target for immunotherapies directed against HAdV5 infection.

HAdV5-induced HLA-F results in specific killing of infected cells by KIR3DS1$^+$ NK cells

To determine whether HAdV5-induced up-regulation of HLA-F results in effective recognition by KIR3DS1$^+$ NK cells, we first assessed KIR3DS1 binding to HAdV5-infected epithelial cells. Hexon-positive cells from HAdV5-infected organoids showed significantly enhanced binding to soluble KIR3DS1-Fc [median fluorescence intensity (MFI) = 365, IQR: 350 to 436] compared with hexon-negative cells (MFI = 94, IQR: 64 to 162; $P < 0.01$) and mock controls (MFI = 102, IQR: 54 to 113; $P < 0.0001$) (Fig. 4A). KIR3DS1-Fc binding was furthermore significantly reduced upon blocking with anti–HLA-F antibody ($P = 0.03$) (Fig. 4A). To determine whether HLA-F expressed on HAdV5-infected intestinal organoids functionally activates KIR3DS1$^+$ cells, we used KIR3DS1ζ Jurkat reporter cells (J3DS1$^+$) transduced with a chimeric construct of the KIR3DS1 receptor fused to the cytoplasmic domain of CD3ζ (*36*). In this system, receptor cross-linking induced by binding of HLA-F results in up-regulation of the activation marker CD69 (*36*). J3DS1$^+$ and untransduced Jurkat cells (J3DS1$^-$) were coincubated with epithelial cells from uninfected and WT HAdV5–infected organoid cultures. Significantly higher percentages of CD69$^+$ J3DS1$^+$ cells were detected upon incubation with HAdV5-infected compared with uninfected cells (median WT = 21.3%, IQR: 20.8 to 23.4; median mock = 15.2%, IQR: 14.9 to 15.5; $P < 0.001$) (Fig. 4B). This effect was mediated by KIR3DS1, as CD69 expression by J3DS1$^+$ cells was significantly higher compared with J3DS1$^-$ cells coincubated with infected epithelial cells (median % CD69 = 12.4%, IQR: 11.9 to 12.6; $P < 0.001$) (Fig. 4B). Blocking J3DS1$^+$ cells with anti-KIR3DS1 or epithelial cells with anti–HLA-F antibody significantly reduced the frequencies of CD69$^+$ J3DS1$^+$ cells upon coincubation with HAdV5-infected epithelial cells (anti-KIR3DS1, $P = 0.0006$; anti–HLA-F, $P = 0.003$), confirming the KIR3DS1/HLA-F interaction in a live cell model (Fig. 4B).

Next, we assessed the functional response of primary NK cells to HAdV5-infected epithelial cells. Epithelial cells from HAdV5-infected 3D organoids were coincubated with primary human NK cells, and CD107a expression by KIR3DS1$^+$ and KIR3DS1$^-$ NK cells was used as a readout for degranulation. KIR3DS1$^+$ NK cells exhibited a significantly higher HAdV5-induced expression of CD107a compared with KIR3DS1$^-$ NK cells from the same donor (Fig. 4C and fig. S5A). CD107a expression by NKG2A$^+$ was slightly elevated compared with NKG2A$^-$ NK cells, indicating that HAdV5, unlike HAdV3, does not mediate NK cell inhibition by up-regulating HLA-E/NKG2A interactions (fig. S5, A and B).

NK cell–mediated viral control is exerted by killing of virus-infected cells. Therefore, we examined the cytotoxicity mediated by KIR3DS1$^+$ NK cells by measuring the lactate dehydrogenase (LDH) release in cocultures with HAdV5-infected cells. Primary KIR3DS1$^+$ and KIR3DS1$^-$ NK cells were fluorescence-activated cell sorting (FACS)–sorted from peripheral blood mononuclear cells (PBMCs) of the same donor and cultured with epithelial cells from uninfected and HAdV5-infected organoids. HAdV-specific lysis was calculated by subtraction of the release of uninfected from infected cocultures, adjusted for maximum and spontaneous LDH release of epithelial cells to control for cell survival. KIR3DS1$^+$ NK cells achieved significantly higher specific lysis of HAdV5-infected epithelial cells than KIR3DS1$^-$ NK cells, as determined by enhanced LDH release ($P = 0.03$) (Fig. 4D), demonstrating superior killing capacity of HAdV5-infected cells by KIR3DS1$^+$ NK cells. This was confirmed by a stronger decrease in hexon-positive cells in the cultures with KIR3DS1$^+$ NK cells compared with KIR3DS1$^-$ NK cells (fig. S5C). Similarly, supernatants of cocultures of epithelial cells from HAdV5-infected organoids with KIR3DS1$^+$ NK cells contained lower HAdV5 DNA copy numbers compared with cultures with KIR3DS1$^-$ NK cells (fig. S5D). From one child undergoing abdominal surgery for reconstruction of a congenital malformation (noninflammatory/noninfectious disease), a large intestinal tissue sample was obtained to generate both organoids and to isolate sufficient numbers of intestinal KIR3DS1$^+$ and KIR3DS1$^-$ NK cells to perform the lysis assay in a unique autologous intestinal tissue–based system. Autologous intestinal KIR3DS1$^+$ NK cells showed superior killing of HAdV5-infected epithelial cells compared with KIR3DS1$^-$ NK cells [NK cell–specific cytotoxicity, 9.7 versus 3.6% ($n = 1$)], providing further support that KIR3DS1 mediates enhanced control of intestinal HAdV5 infection in children. Together, HAdV5 infection–induced HLA-F expression mediated enhanced recognition and killing of infected cells by primary KIR3DS1$^+$ NK cells.

KIR3DS1/*HLA-Bw4* is a protective genotype against severe HAdV disease in children receiving allo-HSCT

A large cohort of pediatric allo-HSCT recipients with detailed clinical and HLA-KIR genotyping data provided the opportunity to translate these in vitro findings showing enhanced killing of HAdV5-infected epithelial cells by KIR3DS1$^+$ NK cells to in vivo observations in children. We determined whether allo-HSCT with a donor with a *KIR3DS1*$^+$/*HLA-Bw4*$^+$ genotype was protective against HAdV reactivation in the recipient. It is well established that the *HLA-Bw4* allele is required to harness the protective effect of KIR3DS1$^+$ NK cells in viral infections such as HIV (*40*), probably by licensing KIR3DS1$^+$ NK cells via KIR3DL1 (*41*). In total, 367 allo-HSCTs performed in 341 children were included. Demographic characteristics are described in table S2. In this cohort, 34 HSCTs (9.3%) were complicated by clinically relevant HAdV viremia (>1000 copies ml^{-1}). Overall mortality within the cohort was 24%, whereas among the children with HAdV reactivation, mortality was 32% ($P = 0.21$). Comparing the risk of HAdV viremia between HSCTs from donors with *KIR3DS1*$^+$/*HLA-Bw4*$^+$ genotype with donors with all other genotypes (non–*KIR3DS1*$^+$/*HLA-Bw4*$^+$) (*41*), recipients of *KIR3DS1*$^+$/*HLA-Bw4*$^+$ donor cells had a lower risk of high HAdV viremia (Fig. 4E, Kaplan-Meier $P = 0.13$). To adjust for confounding variables that may affect HAdV severity and were associated with HAdV viremia in univariate analyses (table S2), a multivariate Cox proportional hazards model was generated. This model, including sex, number of consecutive transplants, and diagnosis as covariates, also suggested a reduced risk for high HAdV viremia in pediatric allo-HSCT patients receiving donor cells with a *KIR3DS1*$^+$/*HLA-Bw4*$^+$ genotype compared with non–*KIR3DS1*$^+$/*HLA-Bw4*$^+$ genotypes ($P = 0.09$) (fig. S5E). Next, we investigated whether the time to HAdV clearance, defined as two consecutive negative HAdV polymerase chain reaction (PCR) tests, was affected by the donor *KIR3DS1*$^+$/*HLA-Bw4*$^+$ genotype. These analyses furthermore illustrated that severe HAdV reactivation in children receiving *KIR3DS1*$^+$/*HLA-Bw4*$^+$ donor cells (3 of 68; 4.4%) was reduced compared with non–*KIR3DS1*$^+$/*HLA-Bw4*$^+$ (31 of 299; 10.4%), supporting the findings above (Fig. 4F). Furthermore, children receiving *KIR3DS1*$^+$/*HLA-Bw4*$^+$ donor cells achieved faster

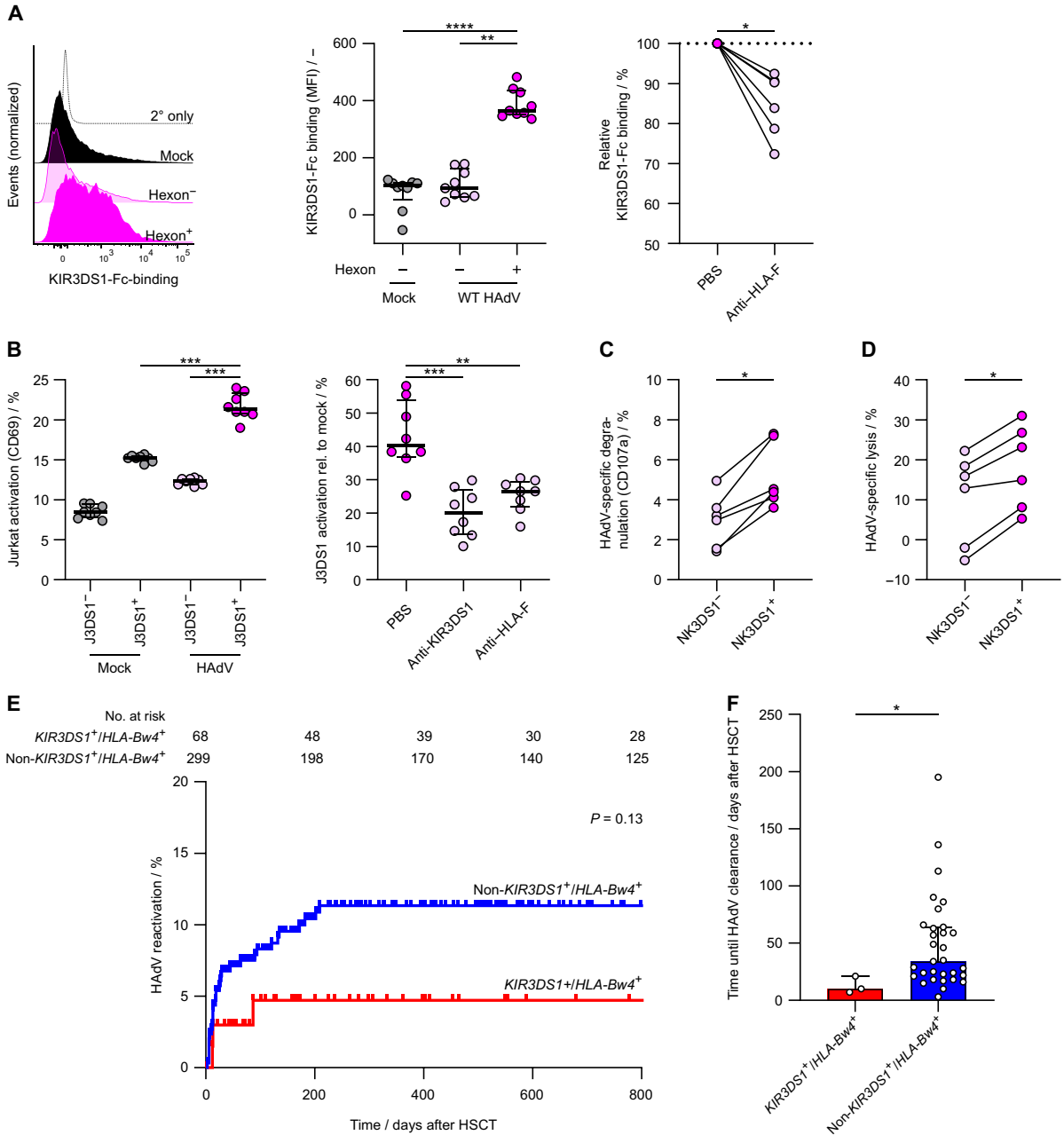

Fig. 4. KIR3DS1$^+$ NK cells have superior capacity to kill HAdV5-infected epithelial cells, and severe adenoviremia is reduced in children receiving *KIR3DS1*$^+$/*HLA-Bw4*$^+$ allo-HSCT. (**A**) Flow cytometric analyses of KIR3DS1-Fc binding to infected (hexon-positive) and uninfected (hexon-negative) epithelial cells derived from WT HAdV5–infected (MOI = 10) and uninfected intestinal organoids (left). Representative plots of KIR3DS1-Fc binding to WT HAdV5–infected, uninfected, and mock-infected cells. 2° only: secondary antibody only. KIR3DS1-Fc binding to WT HAdV5–infected, uninfected, and mock-infected cells based on MFI ($n = 9$ from three donors) (middle). Bars depict median and IQR. Hexon-positive and hexon-negative subsets were compared using Wilcoxon matched-pairs signed rank test. Mann-Whitney *U* test was performed for comparing hexon-positive cells and mock. $^{**}P < 0.01$, $^{****}P < 0.0001$. Relative KIR3DS1-Fc binding to hexon-positive epithelial cells, epithelial cells were preincubated with PBS (set to 100%) or anti–HLA-F antibody ($n = 6$ from two donors) (right). Wilcoxon matched-pairs signed rank test; $^{*}P < 0.05$. (**B**) Activation of KIR3DS1ζ-transduced (J3DS1$^+$) and untransduced (J3DS1$^-$) Jurkat cells after coincubation with epithelial cells from WT HAdV5–infected (MOI = 10) or uninfected intestinal organoids (mock) presented as frequencies of CD69$^+$ reporter cells (left). Activity of J3DS1$^+$ reporter cells after coincubation with epithelial cells from HAdV5-infected intestinal organoids relative to coincubation with epithelial cells from uninfected organoids (right) ($n = 8$, from two independent experiments). Bars depict median and IQR. Mann-Whitney *U* test; $^{**}P < 0.01$, $^{***}P < 0.001$. (**C**) Frequencies of CD107a$^+$ KIR3DS1$^+$ or KIR3DS1$^-$ NK cells after coincubation with epithelial cells from HAdV5-infected (MOI = 20) or uninfected organoids presented as specific degranulation ($n = 6$ from three blood donors, two donors measured in duplicate coincubations in independent experiments). Wilcoxon matched-pairs signed rank test; $^{*}P < 0.05$. (**D**) NK cell–mediated cytotoxicity after coincubation with epithelial cells from HAdV5-infected organoids presented as specific lysis by subtracting cytotoxicity measured upon coincubation with epithelial cells from uninfected intestinal organoids ($n = 6$ from 4 donors). Wilcoxon matched-pairs signed rank test; $^{*}P < 0.05$. (**E**) Kaplan-Meier curve showing prevalence of HAdV reactivation in pediatric allo-HSCT recipients of *KIR3DS1*$^+$/*HLA-Bw4*$^+$ donor cells versus all other genotypes (non–*KIR3DS1*$^+$/*HLA-Bw4*$^+$). (**F**) Interval between HAdV reactivation and viral clearance in allo-HSCT recipients of *KIR3DS1*$^+$/*HLA-Bw4*$^+$ donor cells compared with non-*KIR3DS1*$^+$/*HLA-Bw4*$^+$ in days (bars depict median and 10 to 90% range; Mann-Whitney *U* test; $^{*}P < 0.05$).

viral clearance (10 days) compared with children receiving non–*KIR3DS1*$^+$/*HLA-Bw4*$^+$ donor cells (34 days; $P = 0.02$) (Fig. 4F). The protective effect of the *KIR3DS1*$^+$/*HLA-Bw4*$^+$ genotype was most pronounced in the first 8 weeks after allo-HSCT, coinciding with NK cell recovery after allo-HSCT (*15*, *16*). These data identify *KIR3DS1*$^+$/*HLA-Bw4*$^+$ as a protective genotype against HAdV reactivation in high-risk pediatric patients and provide further support of the clinical relevance of KIR3DS1-mediated protection against HAdV infection.

DISCUSSION

At present, effective therapies for HAdV disease in immunocompromised hosts are lacking, resulting in a high mortality in children with HAdV reactivation after allo-HSCT (*8*–*10*). Our studies identify HLA-F to be specifically up-regulated on human primary HAdV5–infected cells in intestinal organoids that recapitulate the human epithelium and demonstrate the clinical relevance of the KIR3DS1/HLA-F axis in protection against severe HAdV disease in children undergoing allo-HSCT. Harnessing the interaction between KIR3DS1 and HLA-F expressed by HAdV-infected cells could provide novel immunotherapeutic options in patients with HAdV reactivation after allo-HSCT or in patients with congenital immune deficiencies. Our findings also illustrate the strength of primary human tissue cell–based 3D organoids for the discovery of new targets for therapeutic strategies against viral infections in humans.

HAdV5 can evade antiviral CD8$^+$ T cell responses through the efficient down-regulation of HLA-A and HLA-B expression by the HAdV E3/glycoprotein19K. In contrast, our data show that HAdV5 appears to lack immune evasion strategies to suppress HLA-F expression, which serves as a ligand for the activating NK cell receptor KIR3DS1. Although structural variability of E3/glycoprotein19K exists between HAdV species B to E, the modulation of HLA class I molecule expression is similar (*42*, *43*) and was also observed in our study, with HLA-A/-B being the most affected and HLA-C the least. This differential modulation of the expression of HLA class I molecules by accessory viral proteins is reminiscent of immune evasion strategies used by other persistent viruses in an effort to maximize escape from CD8$^+$ T cell–mediated recognition while maintaining inhibition toward NK cells (*44*, *45*). NK cell responses can further be reduced by HLA-E, which provides an inhibitory signal to NK cells via NKG2A (*17*, *18*, *35*). Infections with specific HAdV species have been reported to increase HLA-E expression, promoting evasion from NK cell killing (*39*). HAdV5 did not affect HLA-E expression in intestinal organoids, nor were responses of NKG2A$^+$ NK cells impaired, suggesting that HLA-E–mediated inhibition of NKG2A$^+$ NK cells does not play a considerable role in this setting. In contrast, the expression of ligands for the activating NK cell receptor NKG2D was significantly induced by HAdV5 infection in intestinal organoids. NKG2DL expression was further enhanced upon infection with the 19K^{stop} variant of HAdV5, which is in line with previous studies showing interference of the adenoviral protein E3/glycoprotein19K with the expression of MICA/B (*22*). Last, the sequence of HLA class I–presented peptides can modulate the binding of inhibitory KIRs and thereby inhibit KIR$^+$ NK cells (*46*), and previous studies have shown that HAdV hexon–derived peptides presented by HLA-C can mediate binding of inhibitory KIRs (*47*). On the other hand, presentation of specific peptides by HLA-B57 has been shown to facilitate binding to KIR3DS1 (*48*), but it is unknown whether HAdV-derived peptides can activate KIR3DS1$^+$ NK cells in the context of HLA-B57. Together, HAdVs have evolved sophisticated immune evasion strategies. The strong up-regulation of HLA-F expression upon HAdV5 infection enabling superior killing of HAdV5-infected intestinal epithelial cells by KIR3DS1$^+$ NK cells provides a rationale for the development of novel immunological strategies to control severe HAdV5 reactivation.

Potential therapeutic strategies harnessing the protective KIR3DS1/HLA-F axis include preferential selection of *KIR3DS1*$^+$/*HLA-Bw4*$^+$ donors for allo-HSCT in children at risk for HAdV reactivation. Alternatively, KIR3DS1-hIgG fusion constructs that have been shown here to specifically bind to HAdV5-infected cells might provide a targeted approach for CD16-mediated activation of NK cells in patients receiving *KIR3DS1*$^-$ donor cells. Furthermore, recent small studies used the transfer of CD8$^+$ T cells specific against cytomegalovirus, Epstein-Barr virus, and HAdV to treat viral reactivation in patients undergoing HSCT (*49*–*51*). However, HAdV-induced down-regulation of HLA-A and HLA-B reduces the efficacy of CD8$^+$ T cell–mediated recognition, whereas HLA-F might provide an excellent target for KIR3DS1-expressing cytotoxic cells. Chimeric antigen receptor (CAR)–T and CAR-NK cells are increasingly used for immunotherapies, and the adoptive transfer of NK cells has been associated with reduced clinical complications compared with T cells (*52*–*56*). KIR3DS1$^+$ CAR-NK cells and expanded KIR3DS1$^+$ NK cells from healthy donors as adoptive transfer interventions could therefore provide new therapeutic strategies to control HAdV replication in immunocompromised patients. Together, we identified a protective role of KIR3DS1$^+$ NK cells sensing the induction of HLA-F expression upon HAdV5 infection in intestinal organoids and, in line with these in vitro data, demonstrated KIR3DS1-mediated control of severe adenoviremia in a pediatric cohort of allo-HSCT patients. These findings provide strong rationale to harness the KIR3DS1/HLA-F axis for future immunotherapeutic interventions for severe HAdV infection.

MATERIALS AND METHODS

Study design

The aim of this study was to identify immune ligands expressed by adenovirus-infected epithelial cells in 3D human intestinal organoids, which could serve as targets for NK cells to mediate control of adenovirus infection. We generated adenovirus-infected 3D intestinal organoids derived from adult and infant donors. Expression of ligands mediating NK cell recognition by adenovirus-infected epithelial cells was assessed using flow cytometry and immunofluorescence. This approach allowed to simultaneously assess coexpression of adenoviral proteins and immune ligands within the same organoid and directly compare infected and uninfected cells from the same donor and exposed to the same conditions. To determine whether adenovirus-specific ligand expression triggered NK cell receptors, we used a receptor-expressing reporter cell line (J3DS1$^+$) and a negative control (J3DS1$^-$). To test whether primary NK cells were similarly able to specifically respond to adenovirus-infected cells, NK cell degranulation upon coincubation with intestinal epithelial cells from adenovirus-infected organoids was assessed by flow cytometry. Receptor-specific NK cell–mediated killing of adenovirus-infected intestinal epithelial cells was tested by comparing specific lysis of adenovirus-infected cells by KIR3DS1$^+$ and KIR3DS1$^-$ NK cells from the same donor in cocultures. To investigate the in vivo implications of these in vitro experiments, we determined the protective

effect of the *KIR3DS1* genotype on severe adenovirus reactivation in children receiving allo-HSCT using regression analyses.

Tissue samples

Intestinal tissues were obtained with written informed consent from adult donors or the legal guardians of pediatric donors (table S1) with approval by the ethics committee of the Medical Association of the Freie Hansestadt Hamburg (Ärztekammer Hamburg) at Altona Children's Hospital and University Medical Center Hamburg-Eppendorf. Samples without signs of inflammation were collected during anatomical reconstruction from the final section of the small intestine (ileum). Experiments were performed in organoids derived from infant and adult donors.

Pediatric HSCT cohort

The Wilhelmina Children's Hospital and Princess Máxima Center for Pediatric Oncology maintain a routinely audited registry, collecting pseudonymized data of all patients who underwent an allo-HSCT. All patients and their caregivers provided written informed consent for analyses of clinical data for study purposes as approved by the local medical ethics committee (METC Utrecht). Clinical data from patients receiving allo-HSCT between March 1998 and February 2020 were included. Patients with detectable HAdV in plasma before allo-HSCT were excluded from the analysis. From the 383 eligible transplants, the donor HLA and KIR type were known in 367 who were included. HSCTs, for which no donor KIR type was available, were performed in the beginning of the cohort, which coincided with a lower frequency of HSCT to treat malignancies. This is consistent with trends over time in the use of allo-HSCT in children. Patients were transplanted according to international guidelines (*57*). Patient and transplantation characteristics are shown in table S2. Most of the patients received myeloablative conditioning, consisting of chemotherapy or total body irradiation. Follow-up included the quantification of HAdV DNA load in plasma on a weekly basis until T cell reconstitution [CD4 T cells >200 μl^{-1} (*58*)]. HAdV plasma load >1000 copies ml^{-1} was considered to be clinically relevant and indication for preemptive treatment with cidofovir (*58*).

Donor HLA and KIR typing of intestinal donors and pediatric HSCT cohort

DNA isolated from organoids was determined for HLA type using reverse sequence-specific oligonucleotide (SSO) typing with LABType XR typing kits (One Lambda) assessed by Luminex Flexmap 3D. Reaction patterns were analyzed by HLA Fusion software version 4.3 (One Lambda). HSCT donors were HLA- and KIR-typed as follows: Genomic DNA was isolated from blood using the MagNA Pure Compact System (Roche Diagnostics). HLA typing for the HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1, and HLA-DPB1 loci was executed by next-generation sequencing on the MiSeq (Illumina) using NGSgo-AmpX, LibrX, and IndX reagents (GenDX). Samples were molecularly typed for the presence or absence of 16 KIR genes using a PCR-SSO technique in combination with Luminex using commercial reagents (One Lambda) and following the instructions of the manufacturer. Tested KIRs included 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DP1, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DL2, 3DL3, 3DP1, and 3DS1.

Culture of human intestinal organoids

Intestinal tissues were washed with phosphate-buffered saline (PBS), the muscle layer was removed, and tissue was cut into segments of <0.5 cm^2. Tissue segments were incubated for 2 × 20 min at 4°C on a shaker in Iscove's modified Dulbecco's medium (IMDM; Gibco) supplemented with 5 mM EDTA (Sigma-Aldrich), 2 mM 1,4-dithiothreitol (Roth), 1% penicillin-streptomycin (Sigma-Aldrich), and 5% fetal bovine serum (FBS; Biochrom AG). The epithelial cell fraction was suspended in Matrigel diluted at 2:1 with Advanced Dulbecco's modified Eagle's medium (DMEM)/F12 containing 1% GlutaMAX, 10 mM Hepes (all Gibco), and 1% penicillin-streptomycin. Cells in Matrigel were seeded in 10-μl droplets on a 24-well plate (Greiner), covered with 0.5 ml of expansion medium [Advanced DMEM/F12 supplemented with 1% GlutaMAX, 10 mM Hepes, 1% penicillin-streptomycin, 2% B27 supplement, 1% N2 supplement (both Gibco), mouse epidermal growth factor (50 ng ml^{-1}; PeproTech), 1.25 mM *N*-acetyl-L-cysteine, 10 nM [Leu15]-gastrin, 10 mM nicotinamide, 10 μM SB202190 (all Sigma-Aldrich), 500 nM A83-01 (Tocris), and 10% homemade Noggin–, 20% R-spondin-1–, and 50% Wnt-3a–conditioned medium], and incubated at 37°C (*59*). Until the first medium change, 10 μM ROCK inhibitor (STEMCELL Technologies) was added. The medium was refreshed every 2 to 3 days, and mature organoids were passaged weekly by pipetting. For culture of organoids in suspension, Matrigel droplets containing organoids were detached with a spatula and incubated with ice-cold PBS containing 5 mM EDTA (Promega) for 1 hour at 4°C with gentle rocking to remove Matrigel. Organoids were suspended in 0.5 ml of differentiation medium (expansion medium without Wnt-3a, nicotinamide, and SB202190) and transferred to a 24-well low attachment plate (Corning). For studies assessing epithelial cell polarity and to maintain the 3D organoid structure with the basal side oriented outward, a low concentration of Matrigel (10%) was dissolved in the medium. The isolation of lamina propria–derived lymphocytes cells was described before (*14*). Briefly, the intestinal tissue remaining after detachment of the epithelial fraction used for organoids was minced and digested for 2 × 30 min at 37°C in IMDM supplemented with collagenase D (1 mg ml^{-1}; Sigma-Aldrich), 1% FBS, and deoxyribonuclease I (1000 U ml^{-1}; STEMCELL Technologies). Lamina propria–derived lymphocytes were purified, using 60% stock isotonic Percoll (VWR International) and PBS.

Cell lines

HEK293 cells [American Type Culture Collection (ATCC) CRL-1573] and A549 cells (ATCC CCL-185) were used for these studies. The generation of KIR3DS1ζ-transduced Jurkat reporter cells (based on ATCC clone E6.1) was described previously (*36*). HLA-negative β2m-knockout Jurkat cells were generated with CRISPR-Cas9. A chimeric gene construct consisting of the extracellular and the transmembrane domain of the KIR3DS1 receptor fused to the cytoplasmic domain of CD3ζ was cloned into the lentiviral pLVX transfer vector and transduced into β2m-knockout Jurkat cells. Jurkat transductants were supplemented with 20% FBS and selected with puromycin (1 μg ml^{-1}; InvivoGen).

Viruses

A previously identified WT strain of human adenovirus type 5 species C (HAdV5) was used in this study (*60*). On the basis of the sequence of the WT strain, the E3/glycoprotein19K knockout mutant $19K^{stop}$ was generated by inserting a premature stop codon (5′-TGAT-3′) and a frameshift at nucleotide 33 of the 19K open reading frame (*22*). The HAdV5/mCherry reporter strain was produced from bacmid H5pg4100 (*61*) by inserting the coding sequence for

the mCherry reporter protein upstream of the adenoviral DNA binding protein open reading frame. HAdV5/mCherry was titrated by flow cytometry in fluorescence-forming units per milliliter in A549 cells. The titer of unlabeled viruses was quantified by $TCID_{50}$ (median tissue culture infectious dose) in A549 cells and converted into plaque-forming units per milliliter. For infections of HEK293 cells, cells were grown to >90% confluence and infected in DMEM with a multiplicity of infection (MOI) of 20. Infected cells were harvested and analyzed by flow cytometry 2 days after infection. Intestinal organoids were infected after 2 to 3 days of suspension in differentiation medium. The MOI was calculated upon counting a single-cell suspension of organoids in one well using TrypLE (Gibco). Plaques and fluorescent units were assessed in A549 cells. At an MOI of 10, these titers convert into viral genome copies per infected epithelial cell as follows: HAdV5/mCherry: 6.4×10^5, WT HAdV5: 4.9×10^4, and $19K^{stop}$ HAdV5: 5.7×10^4. Whole 3D organoids were infected in Advanced DMEM/F12 with an MOI of 10 or 20 at 4°C for 1 hour (see legend for MOI used), and after washing, they were cultured in a low attachment plate in differentiation medium. Infected organoids were harvested and analyzed 3 days after infection unless stated otherwise.

Quantitative real-time PCR

Organoids were suspended in ice-cold TRIzol (Invitrogen), and RNA was extracted with alcoholic precipitation. RNA was transcribed into complementary DNA (cDNA) using qScript cDNA SuperMix (VWR International). Target and reference genes were amplified on the LightCycler 96 System (Roche) using the QuantiFast SYBR Green Kit (Qiagen), and the primers were listed in table S3. For calculating the relative gene expression by differentiated organoids (5 days in differentiation medium), target C_T values were normalized to reference genes (*GAPDH* and *SDHA*) and then normalized to gene expression by organoids in expansion medium using the $\Delta\Delta C_T$ method. HAdV DNA was quantified using the RealStar Adenovirus PCR assay (Altona Diagnostic, Hamburg, Germany) according to the manufacturer's recommendation. Briefly, automated nucleic acid extraction was performed from viral supernatants using a MagnaPure 96 system (Roche). Eluates were analyzed by LightCycler 480 II using second-derivative maximum method and quantitative HAdV reference material (supplied with the kit).

Immunofluorescence

Organoids were fixed in PBS/4% paraformaldehyde (PFA; Sigma-Aldrich), washed with 0.1% PBS-Tween (Th. Geyer), and further processed in PBS with 0.1% Triton X-100 and 0.2% (w/w) bovine serum albumin (PAA Laboratories). Organoids were incubated overnight with primary antibodies [anti–ZO-1 (Invitrogen), anti–HLA-F (BioLegend), anti-pIX (provided by H. Wodrich), and anti-CXADR (R&D Systems)] followed by overnight incubation with AF546-labeled anti-rabbit immunoglobulin G (IgG), AF647-labeled anti-mouse IgG, AF647-labeled phalloidin, and Hoechst 33342 (all Invitrogen). Organoids were covered with clearing solution (60% glycerol and 2.5 M fructose in distilled H_2O) and imaged with a Nikon 20× NA (numerical aperture) 0.45 Plan Fluor extra-long working distance objective on a Nikon spinning disk confocal microscope or on a Nikon A1 confocal laser scanning microscope. Organoids infected with HAdV5-mCherry were imaged on a Leica DMI6000 B fluorescence microscope equipped with a Leica DFC350 FX camera. Images were analyzed with ImageJ 1.5 software.

Flow cytometry

3D organoids or cell lines were dissociated to single cells with TrypLE (Gibco) for 15 min at 37°C. Cells were washed and incubated with LIVE/DEAD dye (Invitrogen). Surface molecules were incubated with antibodies or Fc fusion constructs (R&D Systems) listed in table S4. KIR-Fc fusion constructs were stained by incubating with labeled anti-IgG F(ab) fragments (Invitrogen). Cells were fixed with PBS/4% PFA at room temperature for 30 min. For staining adenoviral hexon in infected cells, the eBioscience intracellular staining buffer set (Invitrogen) was used with fixation/permeabilization concentrate diluted at 1:4. For blocking experiments, purified anti-HLA-F (25 μg ml^{-1}; BioLegend) or anti-KIR3DS1/KIR3DL1 (Beckman Coulter) was incubated on cells for 30 min. Cells were measured with BD LSRFortessa II and analyzed using FlowJo 10.7 software (BD Biosciences) with exclusion of doublet cells.

KIR3DS1ζ$^+$ Jurkat reporter cell assay

Infected and uninfected organoids were dissociated to single cells, and viable epithelial cells of a representative well were counted to enumerate the number of target cells. Jurkat reporter cells were incubated with epithelial cells at a reporter/target cell ratio of 1:5 for 5 hours at 37°C. Cells were analyzed by flow cytometry. Anti-CD45 (Invitrogen) was used to distinguish epithelial ($CD45^-$) and Jurkat cells ($CD45^+$), and reporter cell activity was assessed with anti-CD69 (BioLegend). Relative Jurkat activation in antibody-mediated blocking experiments was calculated by first subtracting and then dividing by the $CD69^+$ frequency resulting from coincubation with mock epithelial cells.

NK cell degranulation and target killing assay

To reduce allogeneic reactions in coincubation experiments, NK cell donors (table S1) were matched to organoid donors with regard to their HLA class I genotype. All donors used had heterozygous alleles encoding for the epitopes C1/C2 and Bw4/Bw6. PBMCs were isolated from whole blood by density gradient centrifugation using Biocoll (Biochrom AG). The NK cell–enriched fraction was captured with the EasySep Human NK Cell Isolation Kit (STEMCELL Technologies). Infected and uninfected organoids were dissociated to single cells with TrypLE (Gibco) as described above, and viable epithelial cells of a representative well were counted to enumerate the number of target cells. In degranulation experiments, enriched NK cells were incubated with epithelial cells at an effector/target cell ratio of 1:5 for 5 hours at 37°C in RPMI 1640 with 10% FBS. At the beginning of the coincubation, anti-CD107a antibody (BioLegend) was added. NK cells were identified by flow cytometry as viable $CD45^+$, $CD3^-$, $CD56^+$, $CD16^+$, and CD107a expression was measured in NK cell subsets identified as $KIR3DS1^+$, $KIR3DS1^-$, and $NKG2A^+$ or $NKG2A^-$ (fig. S5A). HAdV-specific degranulation was calculated by subtracting the frequency of $CD107a^+$ NK cells incubated with uninfected epithelial cells from the $CD107a^+$ frequency in coincubations with infected epithelial cells. For lysis experiments measuring LDH, PBMC-derived enriched NK cells or intestinal lamina propria–derived lymphocytes were sorted viable $CD3^-$, $CD14^-$, $CD19^-$, $CD56^+$, $CD16^+$ using a BD FACSAria Fusion, and the $KIR3DS1^+$ and $KIR3DS1^-$ fractions were separately captured. Infected and uninfected organoids were digested to single cells; viable epithelial cells were sorted and enumerated with a BD FACS Fusion and then incubated with sorted $KIR3DS1^+$ or $KIR3DS1^-$ NK cells at an effector/target cell ratio of 10:1 overnight (16 to 20 hours) at 37°C in RPMI

1640 with 10% FBS. Supernatants were analyzed using CyQUANT LDH Cytotoxicity Assay (Invitrogen), and absorbance was measured with a Safire2 microplate reader (Tecan Group). Supernatants were analyzed for viral load. The assessment of virus-specific NK cell–mediated cytotoxicity (HAdV-specific lysis) included controls for spontaneous (accounting for epithelial cell survival) and maximum LDH release by epithelial cells (*62*); HAdV-specific lysis = [(infected sample − spontaneous release)/(maximum release − spontaneous release) − (uninfected sample − spontaneous release)/(maximum release − spontaneous release)] × 100. Killing of hexon-positive epithelial cells was performed with enriched and sorted KIR3DS1$^+$ and KIR3DS1$^-$ NK cells as described above, but with an effector/target ratio of 2:1 and incubation time of 5 hours. Relative specific lysis was calculated by dividing the hexon-positive epithelial cell frequencies: coculture/infected control without NK × 100. Anti-CD45 was used to distinguish epithelial (CD45$^-$) and NK cells (CD45$^+$) for CD107a and hexon assessments by flow cytometry.

Statistical analysis

GraphPad Prism 8 (GraphPad Software) was used to analyze the in vitro data. Statistical significance of differences of experimental data was assessed as stated in legends, using nonparametric test (Mann-Whitney *U*) or Wilcoxon matched-pairs signed rank test for paired samples or Welch's test taking nonequal variance between groups into account. Values of $P < 0.05$ were considered significant. The probability of HAdV reactivation was calculated using the Kaplan-Meier estimate; the two-sided log-rank test was used for univariate comparisons between *KIR3DS1*/*HLA-Bw4* genotype versus the remaining genotypes. Cox proportional hazards models were used to adjust for additional variables associated with HAdV reactivation. Variables associated with HAdV >1000 copies ml^{-1} with a *P* value of <0.15 in univariable analyses (chi-square) were selected for the multivariable Cox proportional hazards model. The statistical analyses of the pediatric allo-HSCT cohort were performed using SPSS version 25 and R 4.0.3 software using the packages survival and cmprsk.

SUPPLEMENTARY MATERIALS

www.science.org/doi/10.1126/sciimmunol.abe2942
Figs. S1 to S5
Tables S1 to S4
Data file S1

View/request a protocol for this paper from *Bio-protocol*.

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KIR3DS1 directs NK cell–mediated protection against human adenovirus infections

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Armor against adenovirus

Reactivation of human adenovirus (HAdV) is a major cause of mortality in children and immunocompromised individuals after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Effective therapies for severe HAdV infections are lacking; therefore, new treatment strategies are required. Using a 3D intestinal organoid system, Jung *et al.* investigated how natural killer (NK) cells recognize and kill HAdV-infected intestinal epithelial cells. HAdV-infected intestinal epithelial cells strongly up-regulated HLA-F, the ligand for activating NK cell receptor KIR3DS1, which enabled enhanced recognition and killing by NK cells. Furthermore, HAdV-infected pediatric allo-HSCT recipients who received *KIR3DS1*/*HLA-Bw4* donor cells exhibited protection from severe HAdV infection and faster HAdV clearance. These findings suggest that the KIR3DS1/HLA-F axis is a promising target for treatment of severe HAdV reactivation after allo-HSCT.

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Supplementary Materials for

KIR3DS1 directs NK cell–mediated protection against human adenovirus infections

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The PDF file includes:

Figs. S1 to S5
Tables S1 to S4

Other Supplementary Material for this manuscript includes the following:

Data files S1

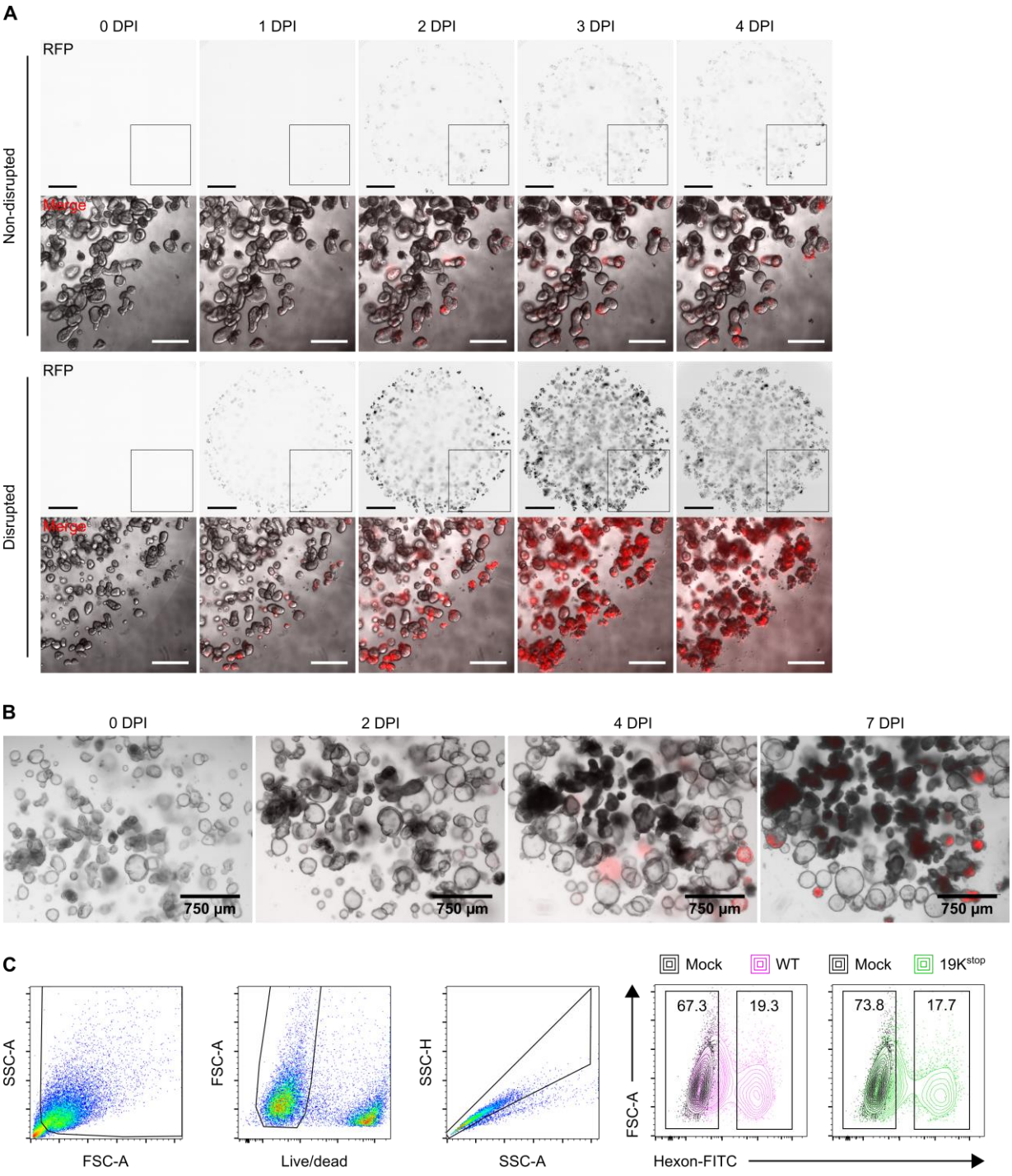


Fig. S1. HAdV5 infection in intestinal organoids. (**A**) Microscopic analyses of HAdV5-infected intestinal organoids, which were first cultured in differentiation medium and Matrigel, then infected in suspension with HAdV5/mCherry (MOI = 10) while intact (non-disrupted) or after pipetting (disrupted) and finally re-embedded into Matrigel (RFP, red fluorescent protein as inverted signal in black, Merge: RFP in red overlaid with bright-field image). Representative images from two donors. RFP scale bars are 2 mm, Merge scale bars are 0.5 mm. (**B**) Intestinal organoids cultured

and infected with HAdV5/mCherry (MOI = 10) in Matrigel and supplemented with expansion medium enriching for intestinal stem cells. (**C**) Gating strategy for flow cytometric analyses of infected (hexon-positive) and uninfected (hexon-negative) epithelial cells after infection of intestinal organoids in suspension with wildtype (WT) and 19K^{stop} HAdV5. Numbers indicate frequencies of cells within the gate.

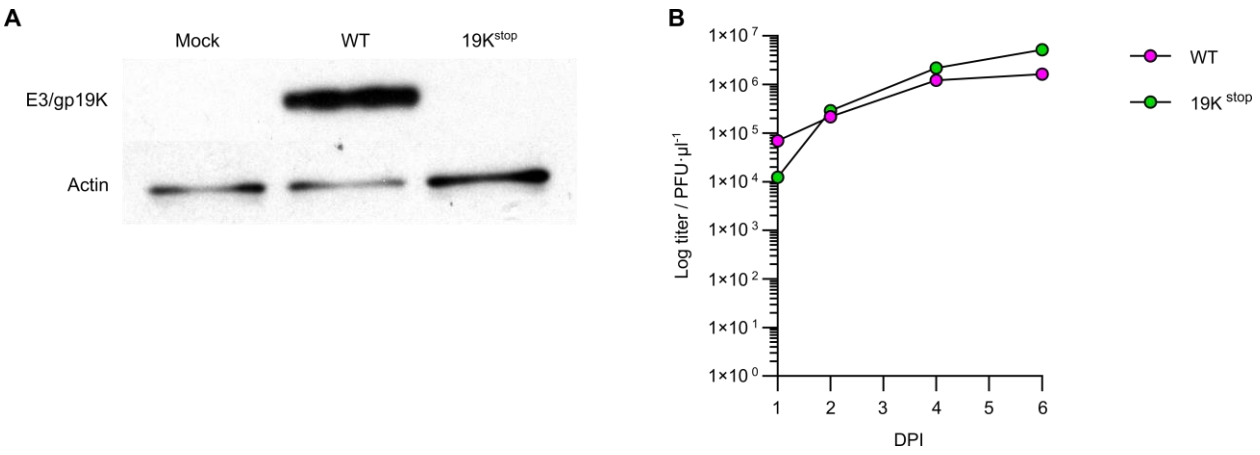


Fig. S2. Validation of the 19K^{stop} HAdV5 knockout strain. (**A**) Western blot of organoid protein lysates confirmed expression of E3/glycoprotein19K after infection with WT HAdV5 (MOI = 10) and absence of E3/glycoprotein19K in 19K^{stop}-infected (MOI = 10) cultures (*n* = 1). (**B**) $TCID_{50}$ assessment using cell lysates of infected organoids (MOI = 10) for infection of A549 monolayers showed increasing titers for WT and 19K^{stop} HAdV5 (*n* = 1 donor, 8 infections per data point).

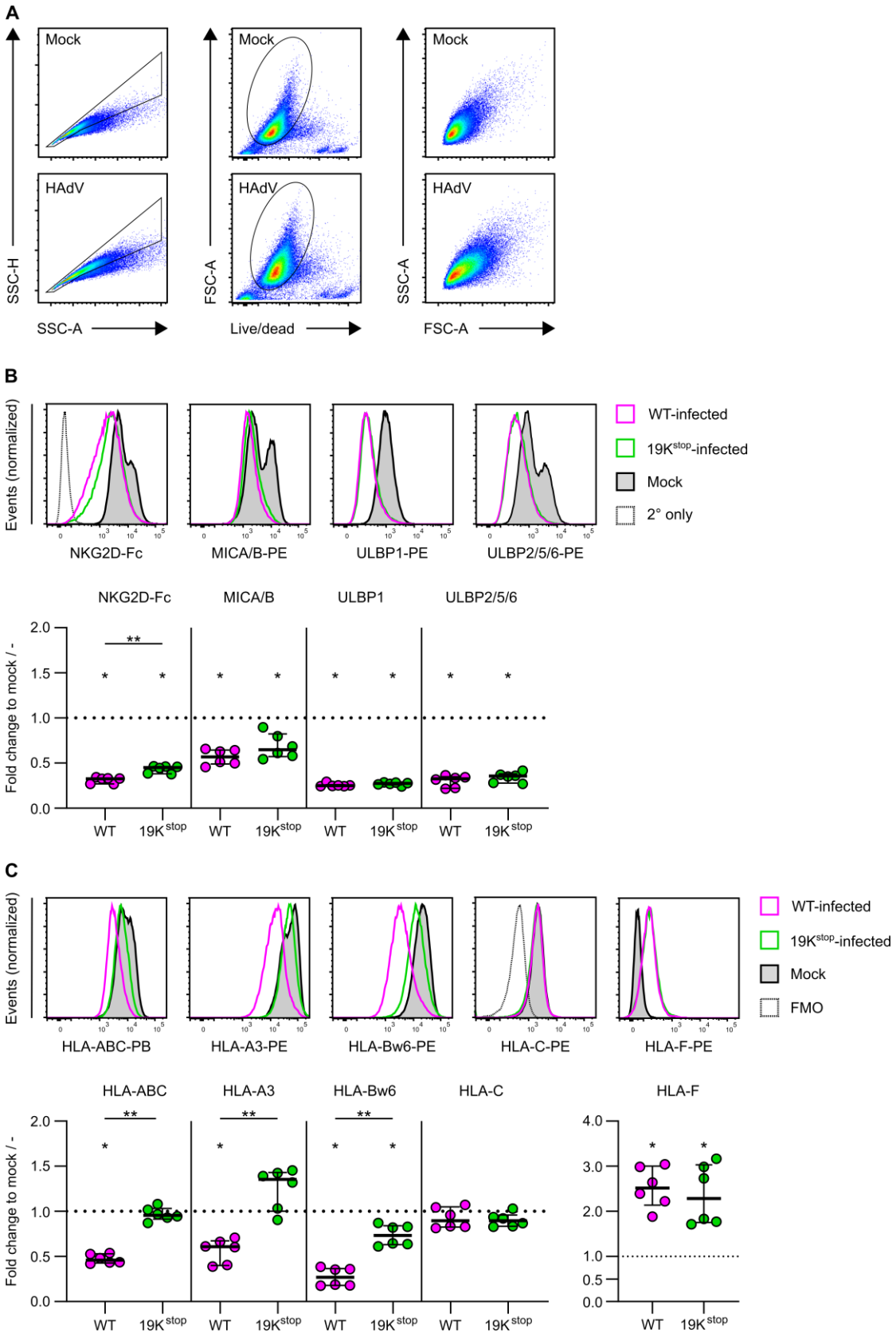

A
Mock
HAdV
SSC-H
SSC-A
FSC-A
Live/dead
SSC-A
FSC-A
B
Events (normalized)
NKG2D-Fc
MICA/B-PE
ULBP1-PE
ULBP2/5/6-PE
WT-infected
19K^stop-infected
Mock
2° only
NKG2D-Fc
MICA/B
ULBP1
ULBP2/5/6
Fold change to mock / -
WT
19K^stop
C
HLA-ABC-PB
HLA-A3-PE
HLA-Bw6-PE
HLA-C-PE
HLA-F-PE
FMO
HLA-ABC
HLA-A3
HLA-Bw6
HLA-C
HLA-F

Fig. S3. Regulation of ligands for NKG2D and HLA molecules by HAdV5 E3/glycoprotein19K in HEK293 cells. (**A**) Gating strategy of flow cytometric analyses of infected (HAdV5) and uninfected (mock) HEK293 cells, showing singlets (left), viable cells (middle) and FSC-SSC distribution. (**B**) Expression of NKG2D ligands by HEK293 cells identified by NKG2D-Fc fusion-construct binding and antibodies against MICA/B, ULBP1 and ULBP2/5/6 expressed as fold changes in median fluorescence intensity (MFI). The upper row shows representative flow cytometric analyses depicted as histograms of HEK293 cells infected with wildtype (WT, magenta) and $19K^{stop}$ HAdV5 (green) compared with uninfected HEK293 cells (mock, black), and cells incubated only with the secondary antibody (dotted line). In the lower row the ligand expression by infected cells is normalized to mock controls (mock = 1.0) (n = 6; bars depict median and IQR). (**C**) Expression of HLA molecules by HEK293 cells identified by antibodies against HLA-ABC, HLA-A3, HLA-Bw6 and HLA-C, showing downregulation of HLA-A and HLA-B by WT and differential regulation by $19K^{stop}$ HAdV5. HLA-F is upregulated after infection in HEK293 cells, however less than in primary epithelial cells in organoids. The upper row shows representative flow cytometric analyses depicted as histograms of HEK293 cells infected with WT (magenta) and $19K^{stop}$ HAdV5 (green) compared with uninfected HEK293 cells (mock, black) and cells incubated without anti-HLA antibody (dotted line; FMO, fluorescence minus one). In the lower row the HLA expression by infected cells is normalized to mock controls (mock = 1.0) (n = 6; bars depict median and IQR). WT and $19K^{stop}$ HAdV5 samples were compared with Mann-Whitney-*U* test indicated by underlined asterisks. Asterisks above groups denote differences of infected cultures to mock tested using Wilcoxon signed-rank test. *P < 0.05; **P < 0.01. All infections were performed with an MOI of 20.

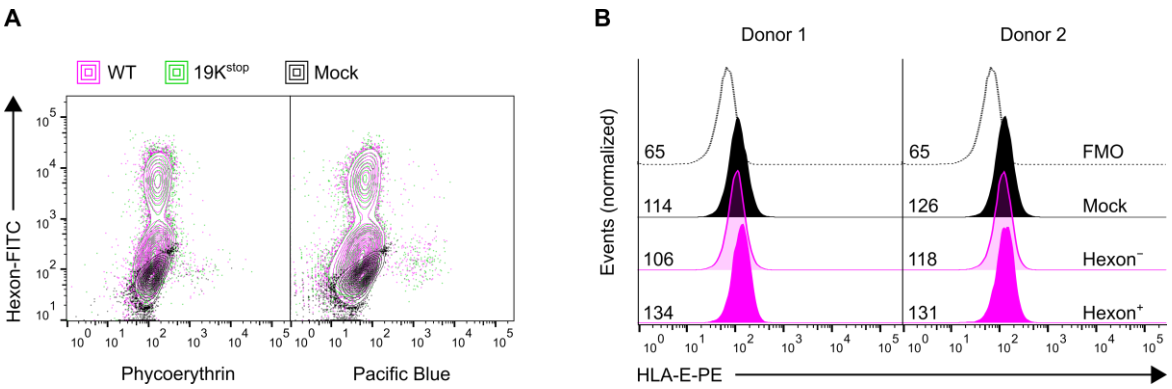
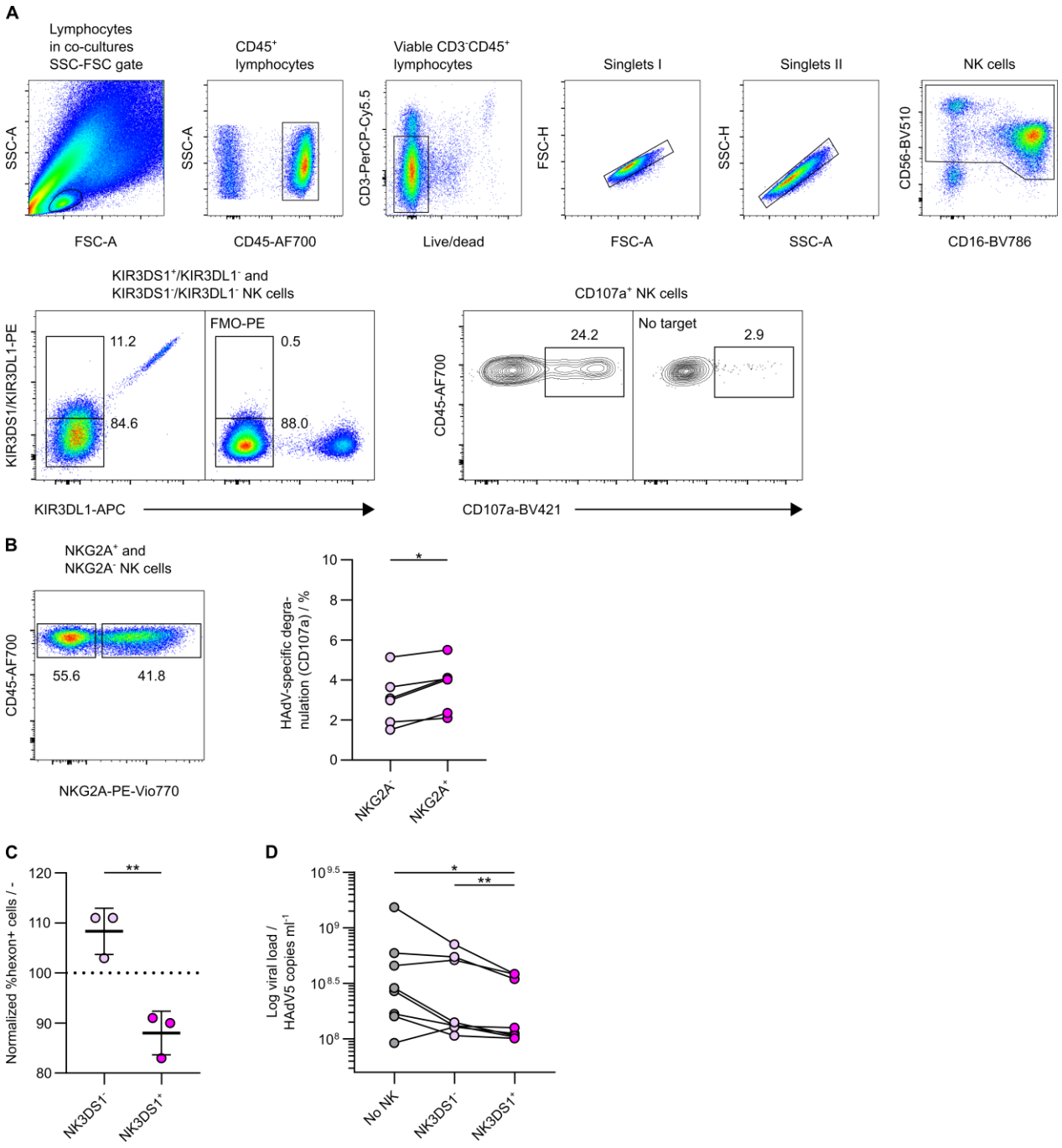


Fig. S4. Flow cytometric analyses of epithelial cells derived from HAdV5-infected organoids. (**A**) HAdV5-infected epithelial cell controls stained fluorescence-minus-one (FMO) for the parameters phycoerythrin (PE) and Pacific Blue (PB) shown in main Fig. 2 and 3 (MOI = 10). (**B**) Flow cytometric analyses of two intestinal organoid donors showing HLA-E expression by infected (hexon-positive) and uninfected (hexon-negative) epithelial cells in comparison to cells derived from uninfected (mock) organoids (MOI = 10) (FMO for PE). Numerical values are median fluorescence intensity (MFI).



	B	SE	Sig.
Sex	-1.14	0.43	0.008
HSCT #	0.51	0.39	0.19
Diagnosis category*	0.20	0.15	0.18
Genotype *KIR3DS1/HLA-Bw4*	-1.03	0.61	0.09

Fig. S5. Flow cytometric analyses of NK cells upon coincubation with epithelial cells from HAdV5-infected organoids and Cox regression analyses of risk factors for severe HAdV disease in pediatric allo-HSCT cohort. (**A**) Gating strategy for flow cytometric analyses of

CD107a expression by KIR3DS1$^+$ and KIR3DS1$^-$ NK cells as well as NKG2A$^+$ and NKG2A$^-$ NK cells, following coincubation with epithelial cells from uninfected and WT HAdV5-infected intestinal organoids. For gating of KIR3DS1$^+$ NK cells, a control unstained for anti-KIR3DS1/KIR3DL1 is shown (FMO-PE). Numbers indicate frequencies of cells within the gate. (**B**) Gating of NKG2A$^+$ and NKG2A$^-$ NK cells (left) and CD107a frequencies (right) after coincubation with epithelial cells from uninfected and WT HAdV5-infected intestinal organoids expressed as specific degranulation (n = 6 from three donors). Wilcoxon matched-pairs signed rank test; *P < 0.05. All infections were performed with an MOI of 20. (**C**) Normalized frequencies of hexon-positive epithelial cells derived from WT HAdV5-infected organoids (MOI = 10) after coincubation with KIR3DS1$^+$ or KIR3DS1$^-$ NK cells (bars depict means and standard deviations). Welch's t-test; **P < 0.01. (**D**) Viral loads in supernatants of HAdV5-infected epithelial cells cultured overnight alone (No NK) or together with KIR3DS1$^+$ or KIR3DS1$^-$ NK cells (n = 4 donors from 2 experiments). Wilcoxon matched-pairs signed rank test was performed between all data sets and significant comparisons are indicated; *P < 0.05, **P < 0.01. (**E**) Multivariate Cox proportional hazard model of risk for children receiving *KIR3DS1*$^+$*/HLA-Bw4*$^+$ donor cells versus all other genotypes (non-*KIR3DS1*$^+$/*HLA-Bw4*$^+$) to develop HAdV titer >1000 copies mL^{-1} upon allo-HSCT adjusted for sex, number of allo-HSCTs and diagnosis.

Table S1: Demographic data of the organoid and NK cell donors.

Cell type	Donor #	Age	Sex
Intestinal organoids	1	3 months	Male
	2	3 months	Female
	3	40 years	Male
	4	81 years	Male
NK cells	1	32 years	Female
	2	28 years	Male
	3	48 years	Female
	4	34 years	Male
	5	29 years	Female
	6	25 years	Male

Table S2: Demographic data of the pediatric allo-HSCT cohort.

	All HSCTs (*n* = 367)	HAdV reactivation (*n* = 34)	No HAdV reactivation (*n* = 333)	*P* value
Male sex (*n*, %)	217 (59)	27 (79)	190 (57)	0.02
Age at allo-HSCT (years, median ± range)	9.0 (0.2-23)	7.0 (0.4-18)	9.2 (0.2-23)	0.44
HSCT (*n*, %)				0.11
First	332 (91)	28 (82)	304 (91)	
Second	31 (8)	6 (18)	25 (8)	
Third	4 (1)	0 (0)	4 (1)	
Diagnosis (*n*, %)				0.12
Malignancy	194 (53)	13 (38)	181 (54)	
Immune deficiency	53 (14)	9 (26)	44 (13)	
Bone marrow failure	62 (17)	5 (15)	57 (17)	
Other	58 (16)	7 (21)	51 (15)	
Stem cell source (*n*, %)				0.70
Bone marrow	140 (38)	13 (38)	127 (38)	
Cord blood	222 (61)	20 (59)	202 (61)	
Peripheral blood	5 (1)	1 (3)	4 (1)	
Conditioning regimen (*n*, %)				0.49
Chemotherapy-based	340 (93)	33 (97)	307 (92)	
Total body irridation	27 (7)	1 (3)	26 (8)	
Serotherapy (*n*, %)	252 (69)	26 (76)	226 (68)	0.34

Table S3: Primers used for qPCR.

Primer	Sequence 5’-3’
GAPDH Fwd	CGGAGTCAACGGATTTGG
GAPDH Rev	TGATGACAAGCTTCCCGTTC
SDHA Fwd	AAGGTGCGGATTGATGAGTA
SDHA Rev	TTTGTCGATCACGGGTCTAT
LGR5 Fwd	GAATCCCCTGCCCAGTCTC
LGR5 Rev	ATTGAAGGCTTCGCAAATTCT
MKI67 Fwd	CTTGTTTGGAAGGGGTATTG
MKI67 Rev	TCATCAGGGTCAGAAGAGAA
LYZ1 Fwd	ACCCCAGGAGCAGTTAAT
LYZ1 Rev	GCCACCCATGCTCTAAT
SI Fwd	GGACACTGGCTTGGAGACAAC
SI Rev	TCCAGCGGGTACAGAGATGAT
MUC2 Fwd	GCATGGACGGCTGTTTC
MUC2 Rev	CAGCGGCCAGCGTTACA
CHGA Fwd	ACTCCGAGGAGATGAACGGA
CHGA Rev	TGGCTGCTCTGGTTCTCAAG

Table S4: Antibodies and staining agents used in this study.

	Clone	Fluorophore	Source	Catalogue#	LOT#	Dilution
Hoechst 33342	-	-	Invitrogen	H3570	-	1:2000
ZO1	Polyclonal Rabbit	Unconjugated	Invitrogen	40-2200	UB280595	1:100
HLA-F	3D11	Unconjugated	BioLegend	373202	B265354; B314352	1:50 (IF) 25 $\mu g \cdot ml^{-1}$ (block)
pIX	Polyclonal Rabbit	Unconjugated	Home-made	-	-	1:1000
CXADR	Polyclonal Rabbit	Unconjugated	R&D Systems	NBP1-88192	A80339	1:100
Phalloidin	-	AF647	Invitrogen	A22287	1941485	1:40
Rabbit IgG	-	AF546	Invitrogen	A10040	2020130	1:500 (ZO1) 1:1000 (pIX, CXADR)
Mouse IgG	-	AF647	Invitrogen	A32787	TJ271040	1:500
NKG2D-Fc	-	-	R&D Systems	1299-NK-050	FVV0618021	25 $\mu g \cdot ml^{-1}$
KIR3DS1-Fc	-	-	R&D Systems	4136-KR-050	PQG0817122; PQG1118081	25 $\mu g \cdot ml^{-1}$
F(ab')2 fragment	-	PE	Invitrogen	H10104	2045251A	1:50
MICA/B	6D4	PE	BioLegend	320906	B279674	1:20
ULBP1	170818	PE	R&D Systems	FAB1380P	AAJW0313021	1:20
ULBP2/5/6	165903	PE	R&D Systems	FAB1298P	LWE0512091	1:20
HLA-F	3D11	PE	BioLegend	373204	B263486	1:20
HLA-ABC	W6/32	PB	BioLegend	311418	B238174	1:50 (ECs) 1:100 (HEK293)
HLA-C	DT9	PE	BD Biosciences	566372	8200695	1:20
HLA-A3	GAP.A3	PE	eBioscience	12-5754-42	1997117	1:100
HLA-Bw6	REA143	PE	Miltenyi Biotec	130-099-835	5190315256	1:50 (ECs) 1:100 (HEK293)
HLA-A2	BB7.2	PE	BioLegend	343306	B266130	1:100
HLA-Bw4	REA274	PE	Miltenyi Biotec	130-103-847	5190715384	1:50
Hexon	1463	FITC	Bio-Rad Laboratories	OBT1824F	160802	1:100
CD45	HI30	PE	Invitrogen	12-0459-42	1998313	1:100
CD69	FN50	BV421	BioLegend	310930	B261486	1:100
KIR3DS1/L1	Z27.3.7	Unconjugated	Beckman Coulter	IM2748	20LIQ176	25 $\mu g \cdot ml^{-1}$ (block)
KIR3DS1/L1	REA168	PE	Miltenyi Biotec	130-104-533	5181010458; 5200113184	1:50
KIR3DL1	DX9	APC	BioLegend	312716	B290375	1:50
CD3	UCHT1	PerCP/Cy5.5	BioLegend	300430	B278331	1:100
CD14	HCD14	APC-Cy7	BioLegend	325620	B289555	1:100
CD19	HIB19	APC-Cy7	BioLegend	302218	B279663	1:100
CD56	NCAM16.2	BUV395	BioLegend	563554	0007566	1:100
CD45	2D1	AF700	BioLegend	368514	B248834	1:100
CD16	3G8	BV785	BioLegend	302046	B254002; B209737	1:100

CD45	2D1	AF700	BioLegend	368514	B248834	1:100
CD56	HCD56	BV510	BioLegend	318340	B303256	1:100
NKG2A	REA110	PE-Vio770	Miltenyi Biotec	130-105-647	5161201058	1:50
CD107a	H4A3	BV421	BioLegend	328626	B255007; B302917	1:100
E3/19K	TW1.3	Unconjugated	NIH/NIAID	NR-4546	-	1 $\mu g \cdot ml^{-1}$

Upregulation of HLA-F expression by BK polyomavirus infection induces immune recognition by KIR3DS1-positive natural killer cells

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BK polyomavirus-associated nephropathy is a common complication after kidney transplantation leading to reduced graft function or loss. The molecular pathogenesis of BK polyomavirus-induced nephropathy is not well understood. A recent study had described a protective effect of the activating natural killer cell receptor KIR3DS1 in BK polyomavirus-associated nephropathy, suggesting a role of NK cells in modulating disease progression. Using an *in vitro* cell culture model of human BK polyomavirus infection and kidney biopsy samples from patients with BK polyomavirus-associated nephropathy, we observed significantly increased surface expression of the ligand for KIR3DS1, HLA-F, on BK polyomavirus-infected kidney tubular cells. Upregulation of HLA-F expression resulted in significantly increased binding of KIR3DS1 to BK polyomavirus-infected cells and activation of primary KIR3DS-positive natural killer cells. Thus, our data provide a mechanism by which KIR3DS-positive natural killer cells can control BK polyomavirus infection of the kidney, and rationale for exploring HLA-F/KIR3DS1 interactions for immunotherapeutic approaches in BK polyomavirus-associated nephropathy.

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KEYWORDS: BK polyomavirus; BK polyomavirus–associated nephropathy; HLA-F; KIR3DS1; natural killer cells

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Translational Statement

BK polyomavirus–associated nephropathy is an emerging health problem due to increasing numbers of kidney transplantations, but currently no specific antiviral therapies are available, emphasizing the clinical need for new treatment strategies. The presence of Killer Cell Immunoglobulin-Like Receptor 3DS1 (*KIR3DS1*), encoding an activating receptor expressed on natural killer cells, has been associated with protection from BK polyomavirus–associated nephropathy. Here, we demonstrated that human leukocyte antigen class F, the ligand for KIR3DS1, was expressed on BK polyomavirus–infected cells both *in vivo* and *in vitro* and that KIR3DS1-positive natural killer cells could sense the expression of human leukocyte antigen class F on infected cells. These results provide a rationale to develop interventions harnessing the antiviral activity of KIR3DS1-positive natural killer cells in BK polyomavirus–associated nephropathy, including adoptive transfer of antiviral effector cells expressing KIR3DS1.

Human BK polyomavirus (human polyomavirus I; BKPyV) is an icosahedral, nonenveloped, double-stranded DNA virus that was isolated in 1971 and is serologically ubiquitous in human populations.[1] Primary infection with BKPyV occurs in early life, with antibodies against BKPyV detected in 70% by the age of 10.[1] After primary infection, BKPyV establishes an asymptomatic persistent infection of the genitourinary tract with intermittent virus shedding into the urine.[2] Reactivation of BKPyV in patients under immunosuppression leads to diseases of the kidney and the urinary tract, including hemorrhagic cystitis in patients who received stem cell transplantation and BKPyV-associated nephropathy (BKVAN) in patients receiving renal transplants.[3] After kidney transplantation, intermittent BKPyV viruria occurs in 25% to 30% and viremia in 10%

to 12% of patients.[4,5] BKVAN is characterized by destruction of kidney tissue leading to deterioration and eventually loss of the graft, and BKPyV reactivation has become an emerging health problem in recent years because of the increasing numbers of bone marrow and renal transplantations.[3] After kidney transplantation, ~10% of patients who experience BK viremia progress to BKVAN, of whom up to 50% lose their graft.[4] Furthermore, BKPyV reactivation has been detected in recipients of other solid organ transplants including heart, liver, lung, and pancreas.[6]

Treatment options for BKPyV reactivation are restricted because of the lack of available antiviral agents. Reducing immunosuppressive treatment after kidney transplantation represents the main therapeutic option, but increases the risk of graft rejection.[7] Immunotherapeutic agents might offer innovative approaches to treat BKPyV reactivation without lowering immunosuppressive therapy. However, they require a detailed understanding of the immune pathogenesis of BKPyV infection. The mechanisms enabling immunologic control of BKPyV infection are poorly understood.[3,8] Some studies have suggested a role of T cells and demonstrated that BKPyV-specific $CD4^+$ T cells recognize viral antigen.[9,10] Recent studies furthermore suggest a role of natural killer (NK) cells in BKVAN pathogenesis. One study demonstrated that the BKPyV-derived microRNA bkv-miR-B1-3p can downregulate UL16 binding protein 3 (ULBP3) expression, an important ligand for the activating NK cell receptor NKG2D, indicating a potential immune evasion strategy to reduce recognition by NK cells.[11] Furthermore, the presence of the gene encoding for the activating Killer Cell Immunoglobulin-Like receptor 3DS1 (KIR3DS1), that can bind to the nonclassical human leukocyte antigen class I (HLA-I) molecule human leukocyte antigen class F (HLA-F),[12] has been associated with protection from BKVAN.[13] Here, we investigated the consequences of BKPyV infection on the expression of ligands for NK cells and observed a significant upregulation of HLA-F in kidneys of patients with BKVAN and on BKPyV-infected renal tubular cells. Elevated HLA-F expression resulted in KIR3DS1 binding and enabled primary KIR3DS1-positive ($KIR3DS1^{pos}$) NK cells to recognize BKPyV-infected cells.

RESULTS

Flow cytometric detection of large T antigen allows the quantification of BKPyV infection of renal tubular cells

To determine the consequences of BKPyV infection on the expression of NK cell ligands on infected cells, we established an immunofluorescence and flow cytometry–based assay to assess the infection of renal tubule epithelial cells using the well-established human RPTEC/TERT1 cell line (telomerase reverse transcriptase–immortalized renal proximal tubular epithelial cells—in the following, called "tubule cells") and the rearranged Dunlop strain of BKPyV. To determine BKPyV infection, the expression of large T (LT) antigen was quantified, which represents the major early viral protein expressed during the BKPyV life cycle.[14] BKPyV-infected and identically treated (mock-infected) tubule cells were stained for LT antigen expression. No significant LT antigen expression was detected in mock-infected tubule cells, whereas BKPyV-infected tubule cells showed LT expression using immunofluorescence (Figure 1a). Furthermore, using multiparameter flow cytometry, LT antigen–positive (LT^{pos}) and –negative cells (LT^{neg}) were distinguished in tubule cells after BKPyV infection (Figure 1b). To investigate the kinetics of BKPyV infection, BKPyV-infected cells were assessed for LT antigen expression using flow cytometry over a period of 20 days postinfection. Infection rates increased over time, reaching up to 21% of LT^{pos} cells at 20 days postinfection (Figure 1c). Taken together, these data demonstrate that RPTEC/TERT1 cells are susceptible to BKPyV infection and that infection rates can be quantified over time through the assessment of LT antigen expression using flow cytometry.

HLA-F expression is strongly upregulated on RPTEC/TERT1 cells after infection with BKPyV

HLA-I molecules play important roles in the recognition of virus-infected cells by innate and adaptive immune cells, and viruses have evolved multiple immune evasion strategies to modulate HLA-I surface expression.[15] To determine whether BKPyV infection resulted in changes in HLA-I expression, we assessed the surface expression of HLA-I molecules, comparing normalized median fluorescence intensity (MFI) levels between BKPyV-infected LT^{pos} (BKPyV-inf/LT^{pos}) tubule cells, BKPyV-exposed but not BKPyV-infected LT^{neg} (BKPyV-exp/LT^{neg}) tubule cells, and mock-infected control cells. MFIs were normalized to MFIs of mock-infected controls. Mock-infected cells were cultured under the same conditions as BKPyV-exposed RPTE cells. Analysis was performed at day 10 after infection (Figure 2a). BKPyV-inf/LT^{pos} tubule cells upregulated human leukocyte antigens (HLA)–ABC, MHC class I chain–related gene A (MIC/A), and HLA-F compared with mock-infected controls (Figure 2a). The strongest upregulation was observed for HLA-F (3.6-fold compared with mock-infected controls; $P < 0.01$) followed by MICA (2.8-fold increase; $P < 0.01$) and HLA-ABC (1.4-fold increase; $P < 0.01$) (Figure 2b). We observed an upregulation of HLA-F, HLA-ABC, and MIC/A also on BKPyV-exp/LT^{neg} tubule cells; however, this upregulation was significantly lower than that on BKPyV-inf/LT^{pos} tubule cells. The upregulation of these molecules is potentially explained by the fact that a subset of BKPyV-exp/LT^{neg} tubule cells might already be infected with BKPyV but not yet express LT antigen. Taken together, these data demonstrated a significant modulation of NK cell ligands after BKPyV infection and in particular a significant upregulation of HLA-F expression on BKPyV-infected RPTEC/TERT1 cells.

HLA-F expression on BKPyV-infected RPTE cells increases binding of KIR3DS1 and activation of primary $KIR3DS1^{pos}$ NK cells

Given the upregulation of HLA-F expression on BKPyV-infected cells, we subsequently investigated whether elevated

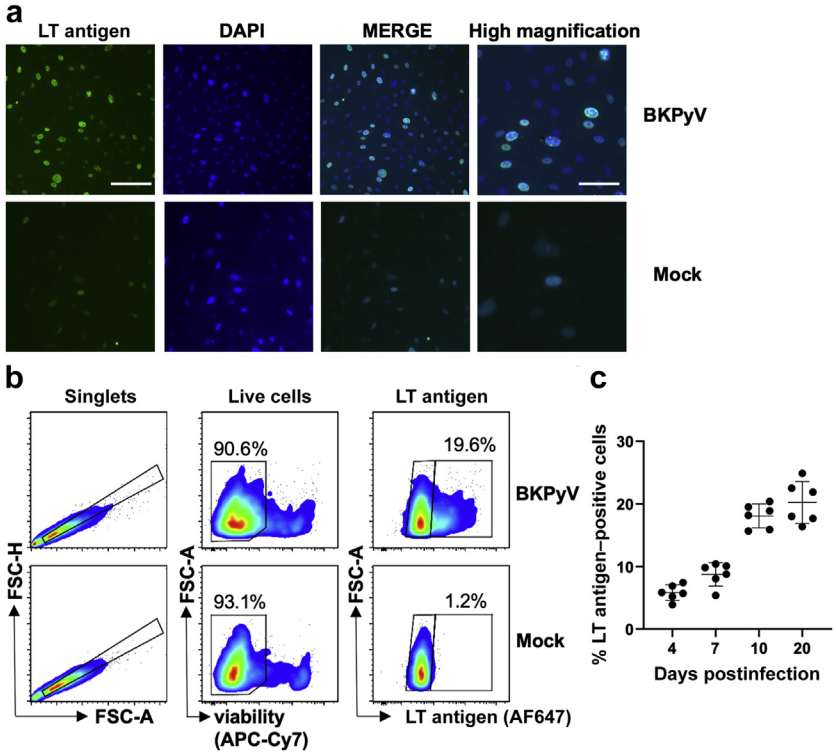

Figure 1 | Immunofluoresence and flow cytometry–based assay to detect and quantify BK polyomavirus (BKPyV) infection on immortalized renal proximal tubular epithelial cells (RPTEC/TERT1). (**a**) Immunofluorescence of infected (upper row) and mock-infected (lower row) RPTEC/TERT1. Staining for large T (LT) antigen and 4',6-diamidino-2-phenylindole (DAPI). Bar = 100 or 50 μm (high magnification). (**b**) Gating strategy used in multiparameter flow cytometry for BKPyV-infected (upper row) and mock-infected controls (lower row). After gating for single cells, live cells were identified using near-infrared vitality dye. Finally, LT antigen–positive cells, showing infection with BKPyV, were differentiated from uninfected cells. (**c**) Percentage of LT antigen–positive RPTEC/TERT1 cells during a 20-day course of BKPyV infection. FSC, forward scatter. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

HLA-F surface levels resulted in increased binding of the activating NK cell receptor KIR3DS1 and activation of KIR3DS1^{pos} NK cells. A soluble chimera of the extracellular domain of KIR3DS1 fused to the fusion construct (Fc) region of human IgG1 (KIR3DS1-Fc) was used to measure KIR3DS1 binding to BKPyV-infected cells.[11] We observed significantly increased binding of KIR3DS1-Fc to BKPyV-inf/LT^{pos} tubule cells compared with BKPyV-exp/LT^{neg} and mock-infected control cells (Figure 3a). BKPyV-inf/LT^{pos} cells had a 2.7-fold (MFI 14.027) increase in KIR3DS1-Fc binding compared with mock-infected controls (MFI 5.193) ($P < 0.01$). This binding was also significantly higher than that of KIR3DS1-Fc on BKPyV-exp/LT^{neg} cells (2.1-fold increase, MFI 10.693; $P < 0.01$), reflecting the differences in HLA-F surface levels (Figure 2a). Relatively higher binding of KIR3DS1-Fc to BKPyV-exp/LT^{neg} compared with mock-infected control tubule cells was consistent with increased HLA-F expression on BKPyV-exp/LT^{neg} tubule cells. These data demonstrate that the upregulation of HLA-F expression on BKPyV-infected tubule cells resulted in enhanced binding of the activating NK cell receptor KIR3DS1.

To further determine whether enhanced binding of KIR3DS1 also resulted in activation of KIR3DS^{pos} cells, we used KIR3DS1ζ^{pos} Jurkat reporter cells. In KIR3DS1ζ^{pos} Jurkat reporter cells, the extracellular domain of KIR3DS1 is fused to the CD3ζ chain, resulting in Jurkat cell activation upon engagement of the receptor through downstream CD3 ζ signaling. Reporter cell activation is quantified by measuring the surface expression of CD69 by flow cytometry.[12] Coculturing experiments of KIR3DS1ζ^{pos} Jurkat reporter cells with BKPyV-exposed tubule cells resulted in significantly increased activation of KIR3DS1ζ^{pos} Jurkat reporter cells compared with cocultures with mock-infected cells (Figure 3b). Furthermore, KIR3DS1ζ^{pos} Jurkat reporter cells were significantly stronger activated by BKPyV-exposed tubule cells compared with Jurkat cells not carrying KIR3DS1 (Figure 3b). Activation of KIR3DS1ζ^{pos} Jurkat reporter cells by BKPyV-exposed tubule cells was furthermore significantly reduced by addition of antibodies blocking KIR3DS1 (Figure 3c; $P < 0.001$) or HLA-F (Figure 3d; $P < 0.001$).

To assess the functional effect of increased HLA-F surface expression and KIR3DS1 binding during BKPyV infection on primary NK cell function, we performed CD107a degranulation assays, as lysosomal-associated membrane protein-1 (or CD107a) has been described as a suitable marker of NK cell degranulation. Sorted KIR3DS1^{pos} and KIR3DS1-negative NK cells from *KIR3DS1/DL1* heterozygous donors were cocultured with BKPyV-infected or mock-infected tubule

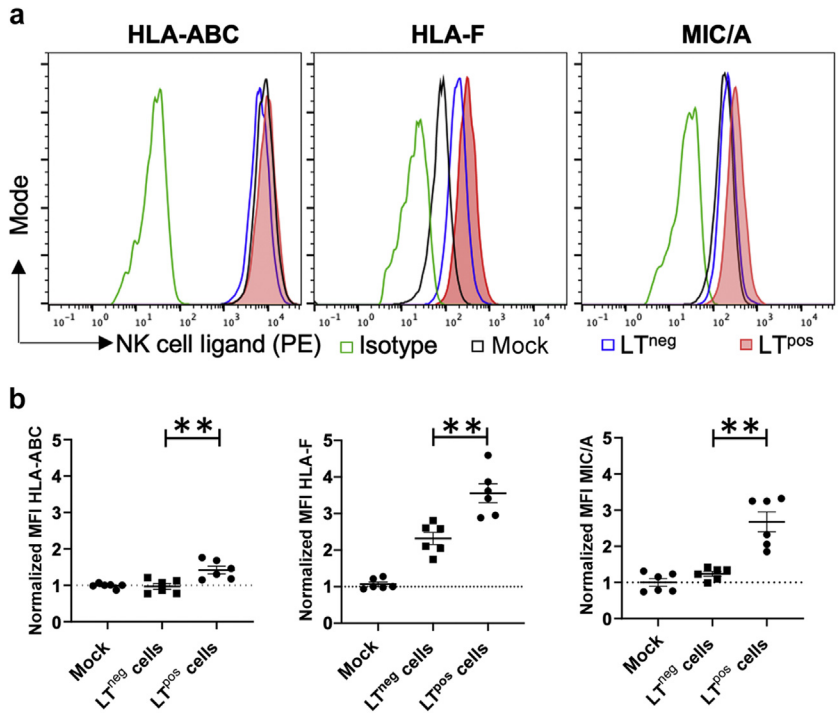

Figure 2 | Increased expression of human leukocyte antigen class F (HLA-F) on BK polyomavirus (BKPyV)–infected/large T antigen–positive (LT^{pos}) cells. (**a**) Histograms of natural killer (NK) cell ligands HLA-ABC, HLA-F, and MHC class I polypeptide-related sequence A (MIC/A). Expression on infected renal proximal tubular epithelial cells (RPTEC/TERT1) (LT^{pos}) cells is shown in red, on LT antigen–negative (LT^{neg}) cells in blue, and on mock-infected controls in black. Isotype controls are shown in green. PE, Phycoerythrin. (**b**) Normalized median fluorescence intensity (MFI) ratios are shown for each NK cell ligand comparing LT^{pos} cells with LT^{neg} cells and mock-infected controls. For normalization, MFIs of mock-infected control RPTEC/TERT1 cells were set to 1 and MFIs of LT^{pos} and LT^{neg} cells, respectively, are shown as *x*-fold of mock-infected cells. The Mann-Whitney test was used for statistical analysis when comparing 2 groups within 1 diagram. $^{**}P < 0.01$.

cells, and CD107a expression was quantified using flow cytometry (Supplementary Figure S1B). Infection with BKPyV caused a significantly increased CD107a expression on $KIR3DS1^{pos}$ compared with KIR3DS1-negative primary NK cells (Figure 3e). Although KIR3DS1-negative NK cells also showed increased degranulation (1.5-fold) after exposure to BKPyV-infected compared with mock-infected tubule cells, indicating a certain degree of KIR3DS1-independent activation, $KIR3DS1^{pos}$ NK cells showed a significantly higher increase in degranulation (2.4-fold) in response to BKPyV-infected tubule cells. Taken together, these results demonstrated that HLA-F expression on BKPyV-infected tubule cells results in significant binding of KIR3DS1 and activation of $KIR3DS1^{pos}$ primary NK cells.

Increased HLA-F expression in kidneys from BKPyV-infected individuals

To investigate whether BKPyV infection of kidney grafts also resulted in HLA-F expression *in vivo*, we analyzed paraffin-embedded kidney biopsies that had either been performed before kidney transplantation into the host ("zero biopsy") or after transplantation when BKVAN or graft rejection was clinically suspected. Of each group, 3 individuals were studied and included in the article. Immunohistochemistry was used to stain kidney biopsies for HLA-F expression. The "zero biopsy" revealed no HLA-F expression on renal tubule cells while endothelial cells showed some positivity for HLA-F (Figure 4a, left). A lack of HLA-F expression on renal tubule cells was also observed in biopsies from control patients with proven antibody-mediated (humoral) rejection (Figure 4a, middle). In contrast, in biopsies from patients with immunohistochemically proven BKVAN, tubular epithelial cells showed membranous expression of HLA-F, especially at the basolateral side (Figure 4a, right). Quantification of HLA-F–positive tubule cells showed significant more positive cells in BKVAN biopsy compared with zero biopsy or during humoral rejection (Figure 4b; $P < 0.0001$).

Because of sequence similarity between BKPyV and simian virus 40 (SV40), staining for SV40/LT antigen is used in clinical routine when BKPyV infection is suspected. Thus, we performed costaining for SV40 and HLA-F on paraffin-embedded kidney biopsies and observed that HLA-F was preferentially expressed on infected (SV40-positive) cells whereas SV40-negative tubule cells showed only very sparse positivity for HLA-F (Figure 4c). Statistical analysis revealed a strong correlation between SV40/LT antigen and HLA-F expression on these tubule cells ($R^2 = 0.724$; Figure 4c). Taken together, these data show that HLA-F is upregulated on tubule cells *in vivo* during BKVAN.

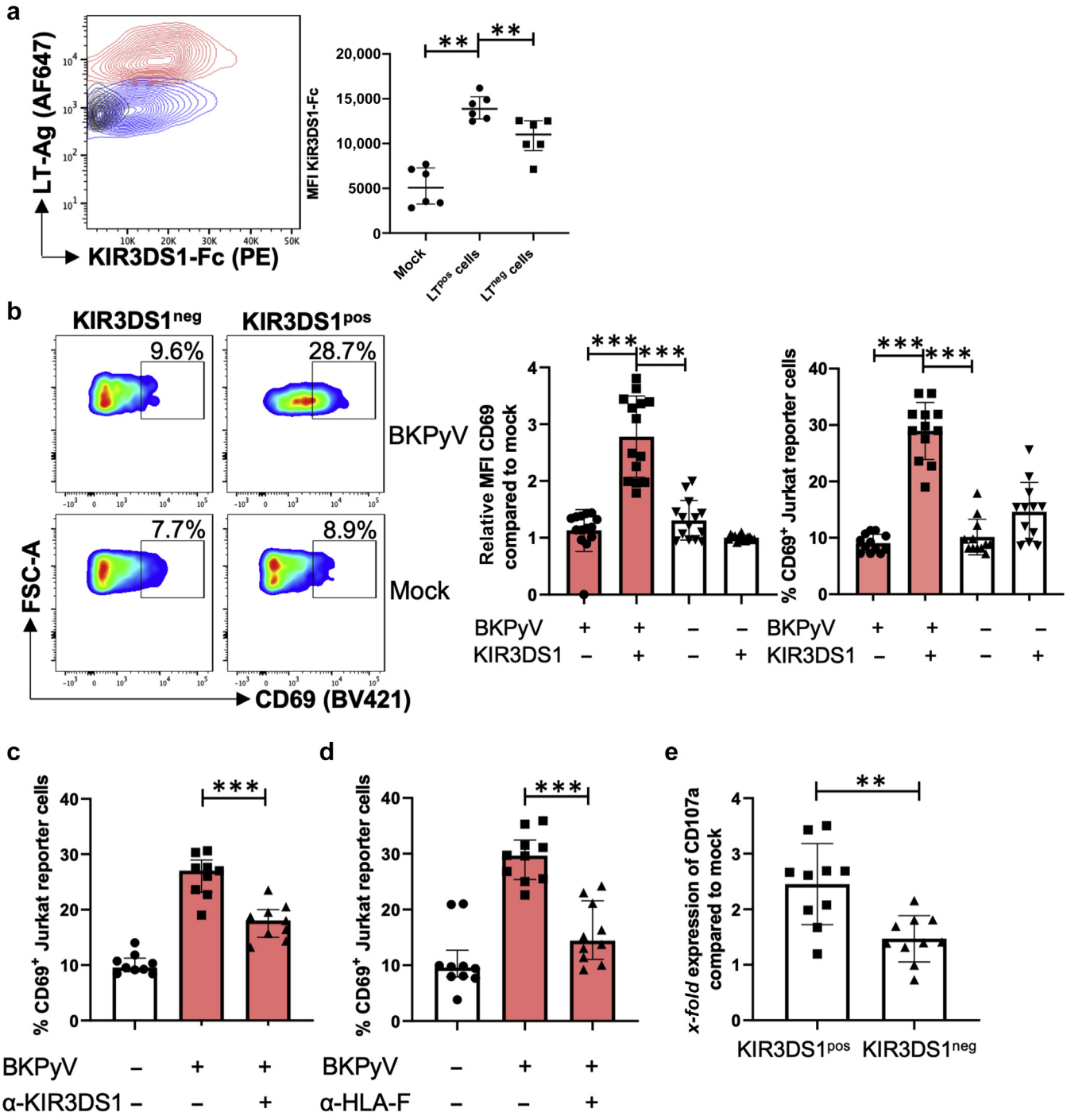

Figure 3 | Activation of Jurkat Killer Cell Immunoglobulin-like Receptor 3DS1 (KIR3DS1)ζ-reporter and KIR3DS-positive (KIR3DS^{pos}) primary natural killer (NK) cells upon co-cultivation with BK polyomavirus (BKPyV)–infected renal proximal tubular epithelial cells (RPTEC/TERT1). (**a**) Contour plots of KIR3DS1-fusion construct (KIR3DS1-Fc) binding to RPTEC/TERT1 cells. LT antigen (LT-Ag)–positive (LT^{pos}) cells are shown in red, LT-Ag–negative (LT^{neg}) cells in blue, and mock-infected control cells in black. Median fluorescence intensities (MFIs) are shown for each group, with $n = 6$ per group in total from 2 experiments. Statistical testing was performed using the Mann-Whitney test. $^{**}P < 0.01$. PE, Phycoerythrin. (**b**) Plots of CD69 expression on β-$_2$-microglobulin knockout Jurkat cells (left) or KIR3DS1-CD3ζ Jurkat reporter cells (right) after 5 hours of coincubation with infected (top) or uninfected (bottom) RPTEC/TERT1 cells. Relative CD69 MFI (normalized to mean MFI of β-$_2$-microglobulin knockout Jurkat cells coincubated with uninfected RPTEC/TERT1) and percentage of CD69$^+$ cells are shown for 4 conditions: infected RPTEC/TERT1 cocultured with β-$_2$-microglobulin knockout Jurkat cells (red bar; left) or KIR3DS1-CD3ζ Jurkat reporter cells (red bar; middle left) and uninfected RPTEC/TERT1 cocultured with KIR3DS1-CD3ζ Jurkat reporter cells (white bar; middle right) or β-$_2$-microglobulin knockout Jurkat cells (white bar; right). $n = 14$ in total from 3 independent experiments. Statistical testing was performed using the Mann-Whitney test. $^{***}P < 0.001$. (**c**) Coculture of uninfected (left) or infected (middle) RPTEC/TERT1 cells with KIR3DS1-CD3ζ Jurkat reporter cells. Blocking of the KIR3DS1/human leukocyte antigen class F (HLA-F) interaction using anti-KIR3DS1 antibodies (right). Red bars indicate coculturing with BKPyV-infected RPTEC/TERT1 cells. $n = 9$ technical replicates from 2 independent experiments. (Continued)

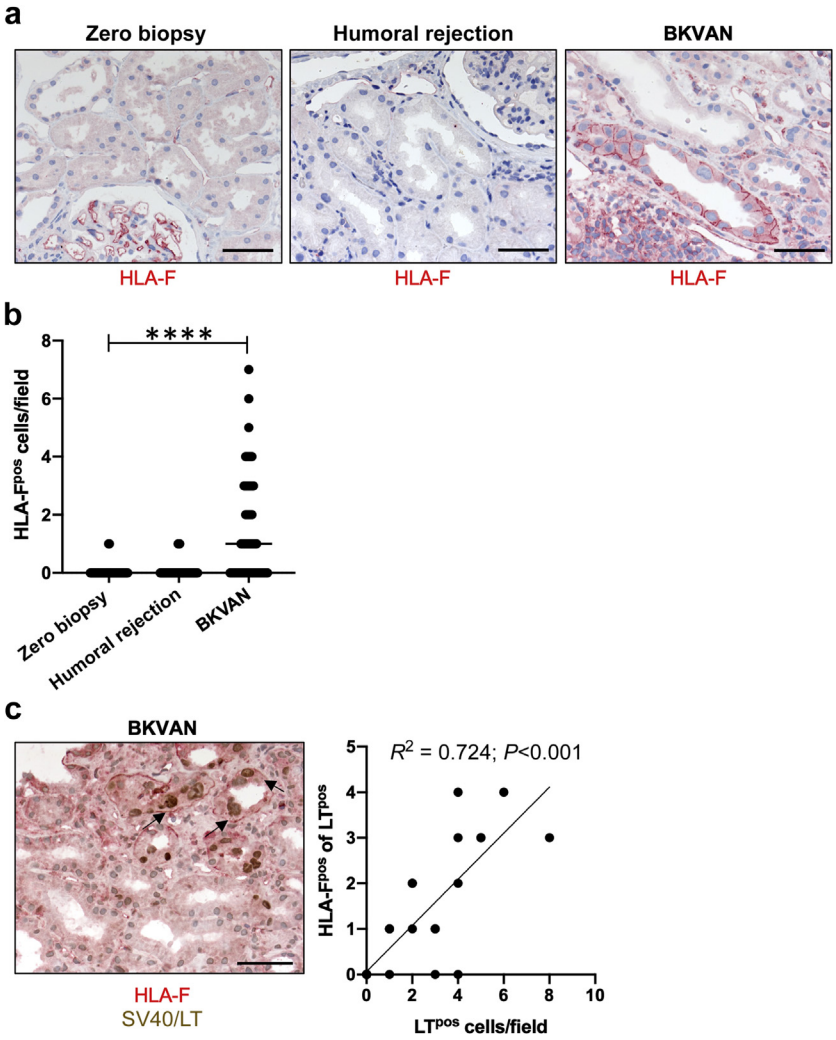

Figure 4 | Increased human leukocyte antigen class F (HLA-F) expression in paraffin-embedded kidney biopsies on large T (LT)/SV40^{pos} tubule cells in patients with BK polyomavirus–associated nephropathy (BKVAN). (**a**) Representative immunohistochemistry of zero biopsies, biopsies of humoral rejection, and biopsies from patients suffering from BKVAN. (**b**) Quantification of human leukocyte antigen class F–positive (HLA-F^{pos}) tubule cells per field (16 fields per picture, 3 biopsies per condition) reveals significantly more HLA-F^{pos} tubule cells in biopsies of patients suffering from BKVAN than in a zero biopsy or humoral rejection. Statistical testing was performed using the Kruskal-Wallis test. ****$P < 0.0001$. Bar = 50 μm. (**c**) Costaining of HLA-F and SV40/LT shows positive correlation between SV40/LT antigen–positive (LT^{pos}) cells and HLA-F^{pos} cells (of SV40/LT^{pos} cells). The diagram shows the number of SV40/LT^{pos} cells (*x* axis) and the number of HLA-F^{pos} cells of this SV40/LT^{pos} cells. $R^2 = 0.72$, $P < 0.001$. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

DISCUSSION

Viral infections are sensed by cell intrinsic receptors that can recognize pathogen-associated molecular patterns, resulting in the induction of cellular defense mechanisms and the production of proinflammatory cytokines.[15] Downstream consequences of sensing of viral infections include the upregulation of HLA-I and stress molecules on the cell surface of infected cells, enabling effector cells of the innate and adaptive immune system to recognize and kill infected cells.[16,17] Although the mechanisms leading to immune recognition and evasion from immune recognition have been extensively studied in herpes virus and retrovirus

Figure 3 | (Continued) Statistical testing was performed using the Mann-Whitney test. ***$P < 0.001$. (**d**) Coculture of uninfected (left) or infected (middle) RPTEC/TERT1 cells with KIR3DS1-CD3ζ Jurkat reporter cells. Blocking of the KIR3DS1/HLA-F interaction using anti–HLA-F antibodies (right). Red bars indicate coculturing with BKPyV-infected RPTEC/TERT1 cells. $n = 10$ technical replicates from 2 independent experiments. Statistical testing was performed using the Mann-Whitney test. ***$P < 0.001$. (**e**) CD107a NK cell degranulation assay using fluorescence-activated cell sorting–sorted KIR3DS^{pos} (left) or KIR3DS-negative (KIR3DS^{neg}; right) primary NK cells after 5 hours of coculture with infected RPTEC/TERT1 cells. *x*-fold upregulation in CD107 positivity is shown compared to coculturing of KIR3DS1^{neg} and KIR3DS1^{pos} NK cells, respectively, with mock-infected RPTEC/TERT1 cells. $n = 10$ technical replicates from 2 *KIR3DS1/L1* heterozygous donors. FSC, forward scatter. **$P < 0.01$.

infections,[18,19] very little is known about the mechanisms that enable the immune system to recognize BKPyV-infected cells.[1,20] Using samples from patients with BKVAN and *in vitro* models of BKPyV infection of renal tubular cells, we demonstrated that BKPyV infection led to the upregulation of HLA-F expression on infected cells, resulting in binding of the activating NK cell receptor KIR3DS1 and activation of KIR3DS1^{pos} NK cells. These studies provide a mechanistic correlate for the described protective effect of *KIR3DS1* in BKVAN and a rationale to harness HLA-F/KIR3DS1 interactions for immunotherapy in BKPyV infections.

BKPyV reactivation after kidney transplantation and immune suppression represents an increasing health problem, as numbers of patients receiving renal transplants or bone marrow transplants are increasing.[21–23] Given the lack of antiviral drugs with activity against BKPyV, there is an emerging need for alternative treatments, including immunotherapies. Some studies have suggested that virus-specific T cells play a role in the pathogenesis of BKVAN. BKPyV-specific T cells have been described in healthy individuals[9] and in individuals with increasing or persisting BKPyV viremia.[10] An important role of NK cells in the control of BKPyV replication and the prevention of BKVAN has furthermore been suggested by studies in patient cohorts demonstrating that the presence of the *KIR3DS1* allele in the *KIR3DL1/DS1* locus is associated with a decreased risk to develop BKVAN after kidney transplantation.[13] The importance of interactions between KIR3DS1 and its cellular ligand HLA-F in innate immunity against virus-infected cells has been demonstrated for other viral infections, including infections with HIV-1 and hepatitis C virus. Genetic population studies showed that individuals encoding for *KIR3DS1* experienced significantly slower progression to AIDS[24,25] and better clearance of hepatitis C virus infection.[26] Subsequent functional studies demonstrated that KIR3DS1^{pos} NK cells are superior to KIR3DS1-negative NK cells in inhibiting the replication of HIV-1 and hepatitis C virus *in vitro*[25–27] and that the recognition of infected cells is mediated through KIR3DS1 binding to HLA-F.[12,26,28]

Here, we described for the first time that BKPyV infection can result in a significant increase of HLA-F surface expression, which leads to the degranulation of KIR3DS1^{pos} primary NK cells. Even though HLA-F can bind to other KIRs than KIRD3S1 and also to leukocyte immunoglobulin–like receptors,[12,29–31] HLA-F binding affinity to KIR3DL2 is rather low and binding to KIR2DS4 remains controversial.[12,30] Our studies demonstrating HLA-F expression on BKPyV-infected cells both *in vivo* using kidney biopsies from patients with BKVAN and *in vitro* on renal tubular cells emphasize the important role that HLA-F expression on virus-infected cells plays in recognition by human NK cells, and potentially T cells, and provide a functional correlate for the described protective effect of *KIR3DS1*.[13] Further studies are required to determine the factors that induce HLA-F expression on BKPyV-infected cells, including soluble factors potentially produced by infected cells, and whether HLA-F expression is regulated on the transcriptional level or on the level of protein trafficking to the cell surface.

A number of clinical studies have demonstrated that adoptive transfer of antiviral effector cells can mediate control of viral infections for which no antiviral agents are available or when antiviral treatments are failing, such as cytomegalovirus– and Epstein-Barr virus–specific T cells.[32–35] Furthermore, more recent studies have suggested that chimeric antigen receptor (CAR)–engineered NK cells might have some clinical benefits over T cells, because of fewer toxic effects, a more simple manufacture, and no need for HLA matching.[36] The protective role of KIR3DS1 in BKVAN and our mechanistic data demonstrating that KIR3DS1^{pos} NK cells can sense the BKPyV-induced expression of HLA-F on infected cells provide a rationale to develop clinical interventions aimed at harnessing the antiviral activity of KIR3DS1^{pos} effector cells in BKVAN, given the lack of alternative antiviral strategies.

METHODS

Cell culture of RPTEC/TERT1 cells

RPTEC/TERT1 cells were obtained from ATCC (Manassas, VA) (CRL-4031) and cultured in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 2% (v/v) fetal calf serum, penicillin/streptomycin (1%), glutamax (1×), insulin-transferrin-selenium (1×), epidermal growth factor (10 ng/ml), hydrocortisone (36 ng/ml), and G418 (0.1 mg/ml) at 37 °C with 5% CO_2 in a humidified incubator. Cells were used up to passage 12.

Immunofluorescence

For immunofluorescence, RPTEC/TERT1 cells were infected with BKPyV (Dunlop strain) reseeded to coverslips and fixed 5 days postinfection with 4% paraformaldehyde for 30 minutes at room temperature. After permeabilization with 1% Triton-X-100 (Sigma Aldrich, St. Louis, MO) for 10 minutes at room temperature and incubation in blocking solution (1% Triton-X, 0.5% Tween 20, and 3% bovine serum albumin [all by Sigma Aldrich]), cells were incubated with ab16879 (PAb416, anti-SV40 T-antigen mouse monoclonal antibody, 1:50, abcam, Cambridge, UK) overnight at 4 °C followed by the secondary antibody goat anti-mouse Alexa Fluor 488 (1:1000, Thermo Fisher, Waltham, MA) for 1 hour at room temperature.

Flow cytometry

Flow cytometric analysis of NK cell ligand expression was performed on BKPyV-infected and mock-infected RPTEC/TERT1 cells. Cells were infected with BKPyV (Dunlop strain) at a multiplicity of infection of 1. Single cells were obtained using TrypLE Express (Gibco by Life Technologies, Carlsbad, CA) and filtering. To assess the expression of HLA-ABC (W6/32, ebioscience, San Diego, CA), HLA-F (3D11, BioLegend, San Diego, CA), and MIC/A (159227, R&D Systems, Minneapolis, MN), cells were stained with a monoclonal anti-human antibodies for 20 minutes at room temperature followed by live-dead staining (LIVE/DEAD Fixable Near-IR Stain Kit, Invitrogen, Carlsbad, CA), fixation, permeabilization, and intracellular staining with labeled SV40/LT antibody (PAb416, abcam). Fixation and permeabilization was performed with the ebioscience intracellular staining kit (Thermo Fisher) according to manufacturer's instructions. Labeling of unlabeled SV40/LT antibody

was performed according to manufacturer's instructions using the AF-647 antibody labeling kit (Thermo Fisher). Gating strategy for the identification of LT^{pos} and LT^{neg} cells is shown in Figure 1b. All antibodies used are listed in Supplementary Table S1.

KIR3DS1-Fc binding assay

KIR3DS1-Fc binding assays were performed as previously described.[12] In short, Killer Immunoglobulin-Like Receptor (KIR) binding was analyzed on BKPyV-infected and mock-infected RPTEC/TERT1 cells using a KIR3DS1-IgG Fcs (R&D Systems) for 1 hour on ice and secondary staining with goat anti-human IgG (Fc) F(ab')2 (Thermo Fisher) was performed for 30 minutes on ice. Afterward, cells were fixed (1% paraformaldehyde in phosphate-buffered saline) and analyzed using flow cytometry. Acquisition was performed on an LSRFortessa (BD Bioscience, San Jose, CA). All antibodies used are listed in Supplementary Table S1.

Virus growth and titration

BKPyV-Dunlop supernatants obtained from WI-38 cells (ATCC) were used to infect RPTEC/TERT1 cells for 2 hours. After 2 hours, growth medium was added. Virus was titrated by infecting RPTEC/TERT1 cells followed by staining with 4',6-diamidino-2-phenylindole and immunostaining for LT antigen as described above and counting of fluorescent cells per well.

KIRζ Jurkat reporter cell assay

KIRζ3DS1 Jurkat reporter cells were incubated with target cells at a reporter/target cell ratio of 5:1 at 37 °C with 5% CO_2 for 5 hours. After coincubation, cells were stained with anti-human CD3-PerCP/Cy5.5 (UCHT1, BioLegend) and anti-human CD69-BV421 (FN50, BioLegend), followed by fixation and sample acquisition. The frequency of $CD69^+$ reporter cells was assessed by flow cytometry and used as a measure of reporter cell activity. Anti-KIR3DS1/3DL1 (Z27.3.7, Beckman Coulter, Brea, CA) or anti–HLA-F (3D11, BioLegend) were used in a final concentration of 25 μg/ml to block the interaction of the reporter cells with the infected target cells. Acquisition was performed on an LSRFortessa (BD Bioscience). All antibodies used are listed in Supplementary Table S1.

CD107a degranulation assay

Primary NK cells ($CD45^+$, $CD3^-$, $CD14^-$, $CD19^-$, and $CD56^+$) were sorted using fluorescence-activated cell sorting depending on their KIR3DS/DL1 expression. Cells were stained with KIR3DS/L1-PE (Z27.3.7, Beckman Coulter) and KIR3DL1-APC (DX9, BioLegend). NK cells positive for PE-KIR3DS/L1-PE and negative for KIR3DL1-APC cells were considered KIR3DS1 positive. RPTEC/TERT1 cells were seeded in a 96-well plate and infected at a multiplicity of infection of 1. Ten days after (mock) infection, NK cells were added in an effector-to-target ratio (E:T) of 2:1 and incubated in the presence of CD107a-BV510 antibody for 5 hours. After 5 hours, cells were stained for CD3, CD14, CD16, CD19, and CD56 and fixed with 1% (v/v) paraformaldehyde. Acquisition was performed on an LSRFortessa (BD Bioscience). All antibodies used are listed in Supplementary Table S1.

Immunohistochemistry of paraffin-embedded kidney biopsies

Formalin-fixed paraffin-embedded human kidney biopsy specimens were cut into 1-μm sections. Slides were deparaffinized and pretreated in ethylenediamine tetraacetic acid buffer (pH 8.5) in a microwave. Slides were blocked with normal horse serum (S2000, Vector, Burlingame, CA). For single stain results, SV40/LT antigen (PAb416, ab16879, abcam) or HLA-F (14670-1-AP, Proteintech, Manchester, UK) antibodies were, respectively, applied for 16 hours overnight at 4 °C and detected via secondary antibodies (ZytoChem Plus AP Polymer Kit, POLAP, Zytomed Systems, Bargteheide, Germany) and new fuchsin/naphthol AS-BI phosphate substrate. Isotype control (rabbit IgG, ab37415, abcam) for HLA-F staining is shown in Figure 1b. For costaining on the same section, first SV40/LT antigen antibodies were applied for 16 hours overnight at 4 °C and visualized using the ZytoChem Plus HRP Polymer System (POLHRP, Zytomed Systems) and benzidine substrate and then HLA-F was visualized as described above for single staining. Three individuals of each group were studied and included in the article.

Data acquisition, analysis, and statistics

Flow cytometry was performed on an LSRFortessa (BD Bioscience) and analyzed using FlowJo software v10 (Tree Star, Inc., Ashland, OR). Figures were designed and statistical analysis was performed using GraphPad Prism version 8 (GraphPad Software, Inc., San Diego, CA). In general, if not stated otherwise, bar graphs indicate the median and error bars indicate the interquartile range. Because of the sample size and because data were assumed to be of non-Gaussian distribution, the nonparametric Mann-Whitney test was used to assess statistical differences between 2 groups. Comparisons between multiple groups were performed using the Kruskal-Wallis test.

DISCLOSURE

TFK reports grants from Deutsches Zentrum für Infektionsforschung during the conduct of the study. TBH reports grants from Fresenius Medical Care/Unicyte, Amicus Therapeutics, and Genzyme/Sanofi and personal fees from Fresenius Medical Care/Unicyte Boehringer Ingelheim, Goldfinch Bio, Novartis Pharma, DaVita Deutschland AG, and Bayer Vital during the conduct of the study. All the other authors declared no competing interests.

DATA AVAILABILITY

The data sets generated during the present study are available from the corresponding author on request.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. (**A**) CD107a degranulation assay of KIR3DS1 positive (left) and KIR3DS1 negative (right) primary NK cells after coincubation with BKPyV infected RPTEC/TERT1. Gates show percentage of NK cells positive for CD107a. (**B**) Isotype control for HLA-F staining in immunohistochemistry of kidney biopsies from patients suffering from BKVAN.

Table S1. Antibodies for flow cytometry.

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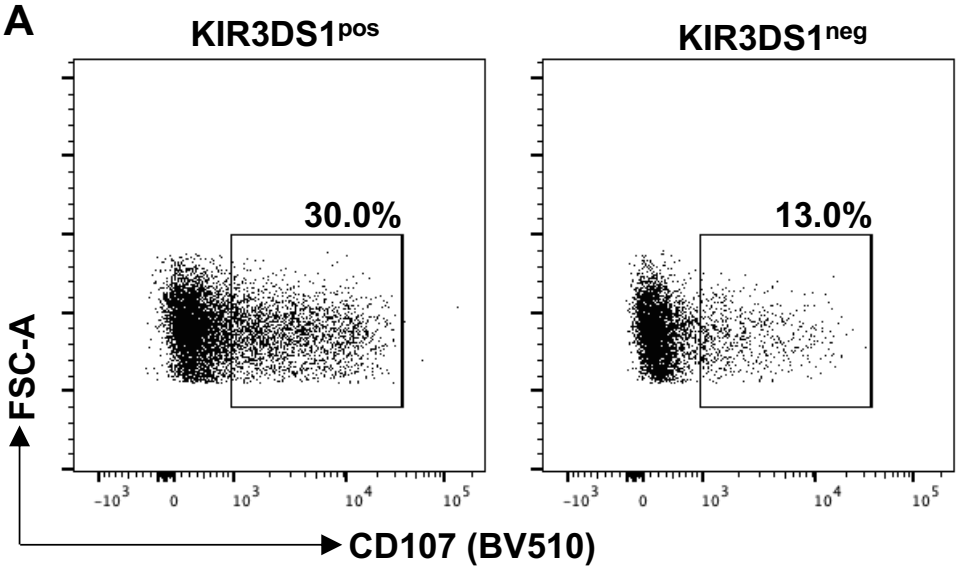
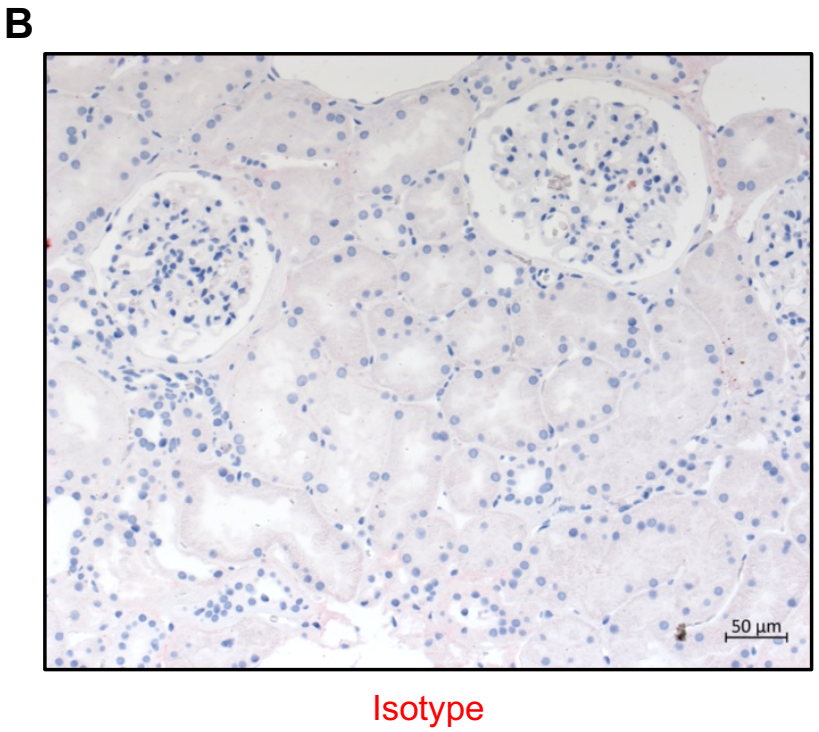
Supplementary Fig. 1: CD107a degranulation assay of primary NK cells and isotype control for HLA-F immunohistochemistry





Isotype

Supplementary Table 1 Antibodies for flow cytometry

Epitope	Manufacturer	Clone	Fluorochrome	LOT No.
CD3	Biolegend	UCTH1	PerCP-Cy5.5	B278331
CD14	Biolegend	HCD14	APC-Cy7	B289555
CD16	Biolegend	3G8	BV785	B259007
CD19	Biolegend	HIB19	APC-Cy7	B279663
CD45	Biolegend	HI30	FITC	B206649
CD56	BD Bioscience	NCAM16.2	BUV395	0007566
CD69	Biolegend	FN50	BV421	B261486
CD158e1 (KIR3DL1)	Biolegend	DX9	APC	B290375
CD158e1/e2 (KIR3DS1/DL1)	Miltenyi Biotec	REA168	PE	5200205080
Anti-SV40 T-antigen	Abcam	PAb416	Labelled with Thermofisher AF647-antibody labelling kit	GR3212700-3
HLA-ABC	ebioscience	W6/32	PE	E11463-1634
HLA-F	Biolegend (flow cytometry, IF) Proteintec (IHC)	3D11	PE	B263486
MIC/A	R&D Systems, Minneapolis, MN	159227	PE	LQT0613081

F(ab')2-Goat anti-Human IgG Fc Secondary Antibody	Invitrogen ThermoFisher Scientific	Polyclonal	PE	2045251A
KIR3DS1–immunoglobulin G fusion constructs	R&D Systems, Minneapolis, MN	---	---	PQG0817122
α-KIR3DL1/S1	Beckmann Coulter	Z27.3.7	---	200034