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A licence to kill:

Interaction of alphaherpesviruses with Natural Killer (NK) cells

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About the cover

The cover shows the actin cytoskeleton of mock-transfected and PRV US3-transfected ST (swine testicle) cells stained with fluorescently labeled phalloidin, as recorded by confocal microscope. This composition was made and modified by Belgian artist Quinten Ingelaere, a dear friend.

Quinten Ingelaere (°1985, lives and works in Antwerp) investigates the way classic themes and archetypes of art history installed themselves in our collective memory, how these allegories influence our expectations towards, and the way we perceive contemporary art. He appropriates strategies of 17th century old masters, manipulates them and tries to reduce them to their essence. Quinten Ingelaere is represented by Dauwens & Beernaert gallery.

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LIST OF ABBREVIATIONS

APC:	antigen-presenting cell
BAC:	bacterial artificial chromosome
BoHV/BHV:	bovine herpesvirus
bp:	base pairs
cAMP:	cyclic adenosine monophosphate
CDx:	cluster of differentiation (where x can be any number)
ChHV:	chimpanzee herpes virus
CHV:	canine herpesvirus
CNS:	central nervous system
CTLs:	cytotoxic T lymphocytes
DAP10/12:	DNAX activating protein of 10/12 kDa
DC:	dendritic cells
DDR:	DNA damage response
DNAM-1:	DNAX accessory molecule-1
EBV:	epstein-barr virus
EHV:	equine herpesvirus
eIF2α:	eukaryotic translation initiation factor 2α
ER:	endoplasmatic reticulum
FHV:	feline herpesvirus
gX:	glycoprotein (where X can be any letter)
HA:	hemagglutinin
HCMV:	human cytomegalovirus
HCV:	hepatitis C virus
HDAC:	histone deacetylase
HIV:	human immunodeficiency virus
HLA:	human leukocyte antigen
HSV:	herpes simplex virus
HVEM:	herpesvirus entry mediator
ICAD:	inhibitor of caspase-activated DNAse
ICPx:	infected cell protein (where x can be any number)
ICTV:	International Committee on Taxonomy of Viruses

IE/E/L:	immediate early, early or late
IFN:	interferon
IFNGR:	interferon gamma receptor
Ig:	immunoglobulin
INM:	inner nuclear membrane
IP3:	inositol-3,4,5-triphosphate
IRS:	internal repeat sequence
ITAM:	immunoreceptor tyrosine-based activation motif
ITIM:	immunoreceptor tyrosine-based inhibition motif
KIR:	human killer cell immunoglobulin-like receptor
LLT:	large latency transcript
MCMV:	mouse cytomegalovirus
MCP:	major capsid protein
MDV:	Marek's disease virus
MFG-E8:	milk fat globule-EGF factor 8, lactadherin
MFIR:	mean fluorescence intensity ratio
MHC-I/II:	major histocompatibility complex I / II
MIC:	MHC class I polypeptide-related chains
MTOC:	microtubule organizing center
MyD88:	myeloid differentiation primary response gene 88
NCR:	natural cytotoxicity receptor
NecL:	nectin-like molecule
NF$\kappa\beta$:	nuclear factor $\kappa\beta$
NK cells:	natural killer cells
NMHC-II:	non-muscle myosin heave chain II
NPC:	nuclear pore complex
oHSV-1:	oncolytic HSV-1 virus
ONM:	outer nuclear membrane
ORF:	open reading frame
PAK:	p21-activated kinase
PAMPs:	pathogen-associated molecular patterns
PANP:	PILR-associated neural protein

PARP:	poly-ADP-ribose polymerase
PBMC:	peripheral blood mononuclear cell
PE:	phosphatidylethanolamine
PILR:	paired immunoglobuline-like type 2 receptor
PK A/C/R:	protein kinase A / C / R
PML-NB:	promyelocytic leukemia protein nuclear bodies
PRV:	pseudorabies virus
PS:	phosphatidylserine
SEM:	standard error of the mean
SMAC:	supramolecular activation cluster
ss/ds:	single/double stranded
TAP:	transporter associated with antigen processing
TGN:	trans-Golgi network
TIGIT:	T cell immunoreceptor with Ig and ITIM domains
TLR:	toll-like receptor
TNFα:	tumor necrosis factor α
TNFR:	tumor necrosis factor receptor
TRAIL:	tumor necrosis factor-related apoptosis-inducing ligand
TRS:	terminal repeat sequence
UL/US:	unique long/short region
VSV:	vesicular stomatitis virus
VZV:	varicella zoster virus
WT:	wild type

Chapter 1: Introduction

1. Pseudorabies virus, an alphaherpesvirus
   a. Alphaherpesvirus taxonomy and phylogenetics

According to the International Committee on Taxonomy of Viruses (ICTV, 2015), the order of the *Herpesvirales*, a large group of double stranded (ds) DNA viruses, can be subdivided in three families: the *Herpesviridae*, the *Alloherpesviridae* and the *Malacoherpesviridae*. The *Malacoherpesviridae* contain only three viruses infecting Japanese oysters, scallops and mollusks. The *Alloherpesviridae* contain several viruses infecting fish and amphibians. The *Herpesviridae* family is subdivided in the (largest) *Alphaherpesvirinae* subfamily and two other subfamilies, the *Betaherpesvirinae* and the *Gammaherpesvirinae*. These viruses infect a broad range of hosts, among which several mammals, birds and reptiles. Viruses belonging to the *Herpesviridae* family are classified into one of the three subfamilies based on host range, genomic features, the cell type in which they can establish latency (a dormant type of persistent infection) and the duration of their replication cycle. The *Alphaherpesvirinae* have the broadest host range, shortest replication cycle and typically establish latency in neurons of the sensory ganglia. The *Betaherpesvirinae* have a rather narrow host range, the slowest replication cycle, often cause cytomegaly, an enlargement of the infected cell, and can induce latency in several tissues, among which certain secretory glands, kidneys and lymphoreticular tissue. *Gammaherpesvirinea* infect T and B lymphoblasts and can induce latency in lymph tissue.

There are three human alphaherpesviruses: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2) and varicella zoster virus (VZV). In addition, the alpaherpesvirus subfamily contains several animal pathogens, such as porcine pseudorabies virus (PRV), chimpanzee herpes virus (ChHV), equine herpesvirus 1 and 4 (EHV-1 and EHV-4), bovine herpesvirus 1 and 5 (BoHV-1 and BoHV-5), Marek's disease virus (MDV), canine herpesvirus 1 (CHV-1) and feline herpesvirus 1 (FHV-1). A recent evolutionary study favored a scenario in which HSV-1 may have resulted from ancient codivergence with the human host whereas HSV-2 may have arisen from a cross-species transmission event from the ancestor of modern chimpanzees to an extinct Homo precursor of modern humans around 1.6 million years ago (1). These new insights and the fact that the herpesvirus family possesses a set of 43 homologue 'core' genes that is present in each herpesvirus support the hypothesis that the herpesvirus family may have a common ancestor from which different viruses originated that further co-evolved with their hosts.

b. History and pathogenesis of pseudorabies virus

Swine are the natural host and reservoir of PRV. PRV causes pseudorabies or Aujezsky's disease, a serious illness in swine that is accountable for significant economic losses in the swine industry, causing severe neuropathogenesis and mortality in young piglets, and mainly respiratory and reproductive symptoms in older pigs (2-5). In addition, PRV can infect a variety of non-natural hosts although higher primates (including man) are not susceptible to PRV. Acute infection with PRV is generally lethal, except in adult pigs (6). The oldest reference of Aujezsky's disease was found in an agricultural magazine "Cultivator", which was published in the USA (1844). It was described as the 'Mad Itch' disease, resembling rabies due to the neurological disorders associated with the disease. Only in 1902, Aladár Aujeszky, a Hungarian veterinarian, described the infectious nature of the disease and isolated PRV from a dog, an ox and a cat. In 1910, ultrafiltration experiments indicated the viral origin of the infectious agent (7-10). Due to the severe economic losses caused by the weight loss and high mortality associated with PRV in livestock, several Western countries have established vaccination and eradication programs against PRV. The Belgian livestock was declared PRV free in 2011 by the European Commission, but PRV still circulates in feral swine in Belgium. Therefore, despite the fact that wild boar PRV strains are typically attenuated in domestic pigs, the threat of PRV to our domestic herds is still present and the need for constant vigilance remains crucial (11).

PRV enters the host's body through the nasal and oral cavities, followed by viral replication in the epithelia of the upper respiratory tract. After primary replication, PRV spreads to the lungs, tonsils and other lymphatic tissues where it infects numerous leukocytes. PRV then spreads through the entire body either as cell-free virus or in infected leukocytes (2). PRV also infects the trigeminal nerve, enabling the virus to establish lifelong latent infections in the trigeminal ganglia of its natural host, the adult pig. Besides the trigeminal nerve, PRV is also able to infect the olfactory nerve, allowing it to reach the central nervous system (CNS) in young piglets and non-natural hosts. This infection causes a non-suppurative meningitis characterized by severe central nervous disorders and ultimately death in these animals (12). The severity of Aujeszky's disease and the clinical symptoms in swine are age dependent. CNS symptoms are particularly apparent in piglets and usually lead to fatal meningoencephalitis. Besides CNS symptoms, piglets typically show hyperthermia, anorexia and listlessness. From an age of two months, the mortality of an acute infection is reduced to 1-2 %. From that age onwards, pigs typically display respiratory disorders and viral spread to the CNS occurs far less frequent. The clinical symptoms at that age, besides reproductive disorders, are anorexia and listlessness, coughing,

conjunctivitis, rhinitis and dyspnea. Adults pigs, at the age of 5 months, still may show similar symptoms, although symptoms are often less pronounced and sometimes even absent (2, 6). However, a highly virulent PRV isolate has been reported during an PRV outbreak in Northern China in 2011 to cause systemic neurological symptoms and mortality of 10 to 30% in growing and adult pigs (13, 14).

c. PRV as an alphaherpesvirus model organism

PRV is an excellent model to study alphaherpesvirus biology for various reasons. It allows to perform experiments with primary cells of the porcine natural host, thus providing important information on virus-host interactions in natural host cells. In addition, the virus has a broad host range and can be studied in a broad range of laboratory animals. PRV does not infect man or other higher primates, which ensures lab worker safety (6). Also, PRV grows very well in cell culture, the entire PRV genome has been sequenced and bacterial artificial chromosome (BAC) constructs encompassing the PRV genome have been generated (15, 16). The latter and the fact that the naked viral genome is infectious when delivered in the nucleus of cells, facilitates the study of the contribution of individual viral proteins to several processes by site-directed mutagenesis. Importantly, of the 70 proteins encoded by PRV, none is unique to this virus, all genes have homologs in other alphaherpesviruses (6, 17). All these features led to the widespread use of PRV to study general and largely conserved aspects of alphaherpesvirus biology, including replication, spread and interaction with the immune system. Also in the current thesis, PRV was used as an alphaherpesvirus model organism.

d. PRV virion structure

PRV, like all herpesviruses, is an enveloped double stranded DNA virus and the virion contains four morphological distinct structural components (Figure 1): the genome, capsid, tegument and the outer envelope, a lipid membrane containing several viral glycoproteins. Nearly half of all PRV gene products are highly conserved structural components of the mature virion (17, 18). The different components of the PRV virion will be briefly summarized below.
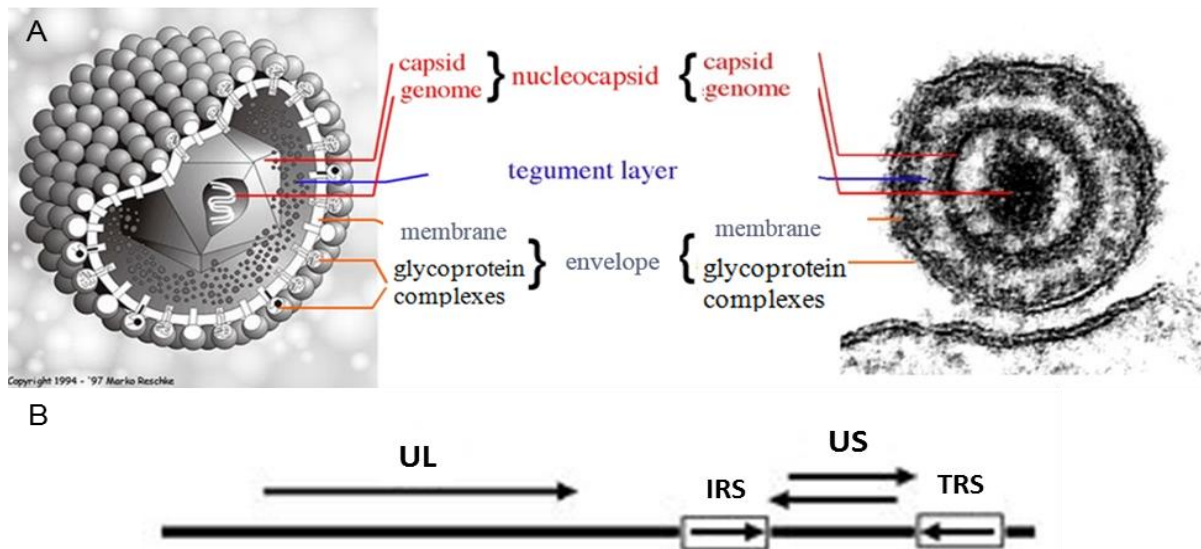
Figure 1. Structure of a PRV virion and its genome organization. (A) Schematic representation (left panel) and transmission electron microscope image of a PRV virion (Figure adapted from (19)). (B) Organization of the PRV genome, a typical class D herpesvirus genome (Figure adapted from (20)).

Genome

The complete annotated sequence of the PRV genome has been determined and consists of a single double stranded DNA strand containing 143700 bp. The genome consists of a large latency transcript (LLT) and 72 open reading frames (ORF), which encode 70 different proteins (17), and is organized in a unique long region (UL) and a unique short region (US). The US region is bordered by the internal repeat sequence (IRS) and terminal repeat sequence (TRS), which are two large inverted repeats. Since the UL region is only at one side flanked by inverted repeats, the PRV genome exhibits the typical class D herpesvirus genome, similar to VZV. Recombination between these inverted repeats, flanking both ends of the US region, can produce two possible isomers of the genome, differing in the orientation of the US region (6). The PRV genome is largely colinear with that of HSV-1 and other alphaherpesviruses, except for a large internal inversion in the UL region situated between the UL46 gene and UL26.5 gene (17).

Capsid

The PRV genome is encapsulated in an icosahedral capsid. Most information about alphaherpesvirus capsid structure and composition is derived from ultrastructural studies on HSV-1 virions. Since capsid features are highly conserved throughout the herpesviruses, most of this information very likely also holds true for PRV. The capsid is mainly assembled by the major capsid protein (MCP or VP5) encoded by UL19 and is organized into 162 capsomers, 150 hexavalent capsomers (hexons) and 12 pentavalent capsomers (pentons). The pentons are

located at the peaks and the hexons form the faces and edges of the capsid. The capsomers are connected in groups of three by triplexes composed of heterotrimers of the UL38 gene product and two UL18-encoded proteins. The hexons are formed by six proteins encoded by UL19 and six proteins encoded by UL35, while all but one of the pentons are pentamers of the UL19 gene product. The remaining penton is formed by twelve proteins encoded by UL6 and forms a cylindrical channel allowing one copy of the viral genome to be packaged in newly formed capsids. Furthermore, during HSV-1 infection the portal complex also acts as initiator of the capsid formation (21).

Tegument

The tegument layer is a protein matrix that fills the space between the capsid and the envelope membrane of PRV particles and can be sub-divided in two layers, an inner and outer layer. The inner layer shows an icosahedral symmetry, due to its association with the capsid, while the outer layer forms an asymmetrical layer surrounding the inner tegument layer. For PRV, the tegument is composed of at least 20 different viral tegument proteins and cellular proteins, such as actin. These proteins interact with each other as well as with capsid and envelope proteins. Besides playing an important structural role in virion morphogenesis, tegument proteins may have different regulatory functions, including activation of viral gene expression, modulation of the host cell for viral replication and mediating post-translational modification of proteins (6, 19, 22, 23). For example, the PRV US3 protein kinase and HSV-1 ICP0 ubiquitin ligase are both present in the tegument of their respective mature virions and can directly modify cellular proteins by either phosphorylation (PRV US3) or ubiquitin ligation (HSV-1 ICP0) (24, 25).

Envelope

The mature PRV virion acquires its envelope by budding through the intracellular membranes of vesicles from the Trans-Golgi Network (TGN). The viral envelope has been reported to contain 14 different viral membrane proteins. Ten of these viral membrane proteins are modified by N- and/or O-linked sugars and are therefore designated as glycoproteins (glycoprotein B or gB, gC, gD, gE, gH, gI, gK, gL, gM and gN) and four additional transmembrane proteins are not glycosylated (UL20, UL43, US9, and possibly UL24). Viral glycoproteins are expressed in the viral envelope as well as in different membranes of the infected host cell, including the plasma membrane, and may be involved in several processes including virus entry and egress, cell to cell spread and modulation of the host's immune response (6, 18).

e. Viral replication cycle

The PRV replication cycle in a host cell is illustrated in Figure 2 and can be subdivided in 5 stages: (1) virion attachment and entry, (2) cytoplasmic transport and entry into the nucleus, (3) transcription and replication of the PRV genome, (4) capsid and nucleocapsid formation and (5) egress.
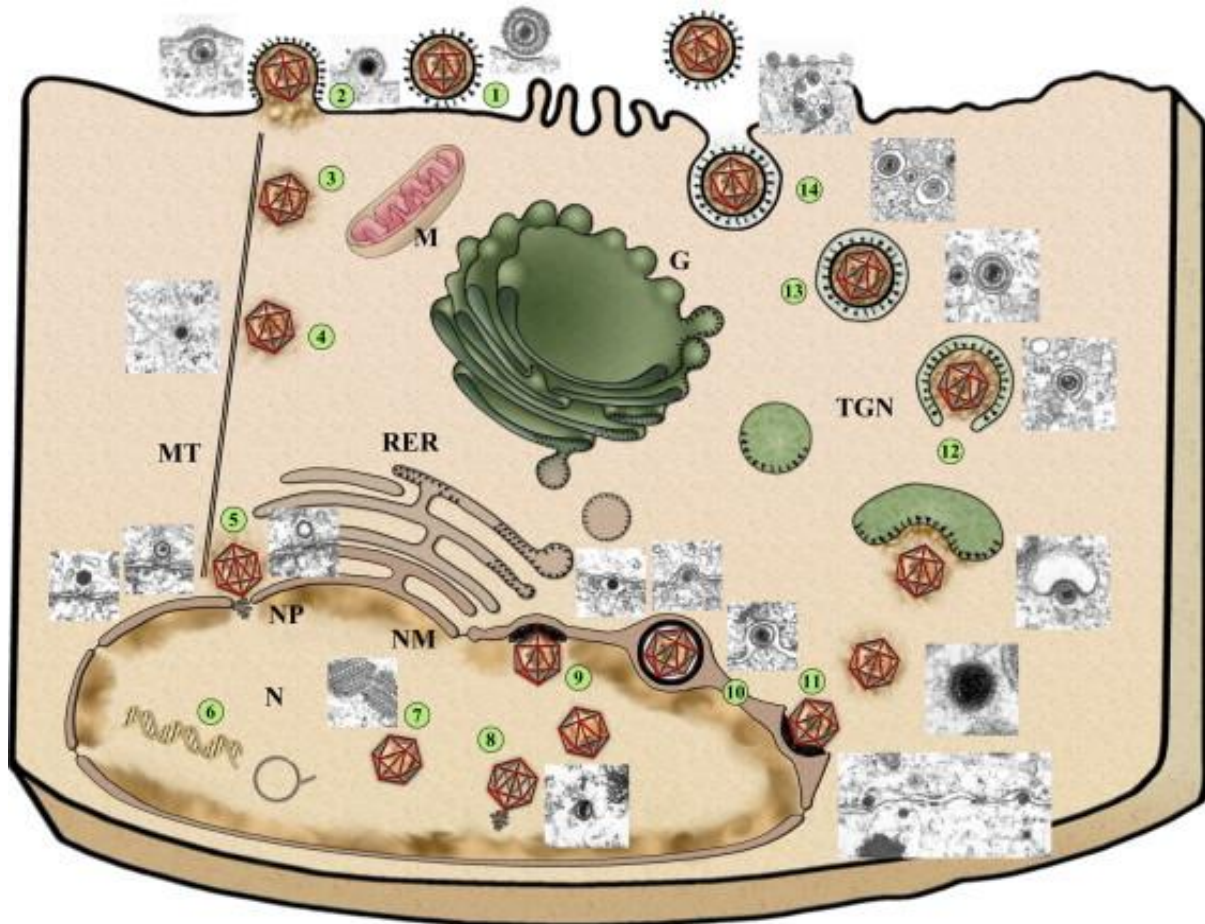

Figure 2. Schematic overview of the PRV replication cycle. (1) The virion forms loose connections between its envelope, containing viral glycoproteins, and glycosylated structures on the outer cellular membrane (2) This is followed by more sturdy protein interactions where particular viral glycoproteins bind with cellular membrane proteins, referred to as entry receptors, which (3) trigger a fusion process between the viral envelope and the plasma membrane for the capsid to enter the cell (in particular cell types, fusion may take place after endocytic uptake of the virion in the cytoplasm). Alphaherpesviruses have a dsDNA genome and to overtake the host cell, the viral genome has to (4) reach and (5) enter the nucleus, where the virus makes use of the cellular DNA transcription and replication machinery (6) to transcribe the viral genes and replicate the viral genome. (7-8) During assembly of progeny virions, newly produced viral capsid proteins and replicated genome form new nucleocapsids that (9-11) bud through the inner nuclear membrane, entering the perinuclear space and subsequently fuse with the outer nuclear membrane to release the nucleocapsid in the cytoplasm. The nucleocapsid will be covered with tegument proteins and is transported to the Trans-Golgi Network (TGN). (12) The mature virion is formed by budding of the tegumented nucleocapsid in vesicles from the TGN, which contain the viral membrane proteins. (13-14) Upon transport of the vesicles to the cellular membrane and exocytosis, mature virions are released in the extracellular space (Figure adapted from: (19)).

Virion attachment and entry

The PRV virion first initiates weak interactions between the viral envelope and the cellular membrane, mainly by gC and, to a lesser extent, gB binding with heparin sulphate moieties on cell surface proteoglycans (6). These weak interactions facilitate the binding of gD to cellular entry receptors. For PRV, three gD receptors have been identified: CD111 (nectin-1), CD112 (nectin-2) and CD155 (poliovirus receptor) (26, 27). This stable gD binding activates the gB and gH/gL fusion complex (28). This fusion mechanism is not fully clarified, but appears to rely on the interaction of gB with cellular entry receptors. For gB of HSV-1, different cellular entry receptors have been identified recently: paired immunoglobulin-like type 2 receptor alpha (PILRα), non-muscle myosin heavy chain IIA (NMHC-IIA) and NMHC-IIB (29-31). PILRα has also been reported to also serve as an entry receptor for PRV (32). Besides entry via direct fusion at the plasma membrane, the PRV virion can also be endocytosed followed by fusion of the envelope with the endosomal membrane. Fusion upon endocytic uptake relies on the same viral glycoproteins. Both types of fusion result in release of the virus particle in the cytoplasm (6).

Transport to and entry in the nucleus

Upon entry in the cytoplasm, tegument proteins of the outer layer (UL11, UL41, UL46, UL47, UL48 and UL49) quickly dissociate from the capsid. The cytoplasm is a serious barrier to cross. The passive diffusion of viral particles based on Brownian movement, the random movement of particles, makes this translocation possible in theory. However this movement is random and slow, therefore translocation based on Brownian movement alone would be extremely inefficient. Hence, the capsid associates with microtubules using dynein, a microtubule-dependent motor protein, to be transported towards the nucleus (33). At the nucleus, for HSV-1, UL6 initiates docking at the nuclear pore (NPC) followed by injection of the viral genome in the nucleus via the NPC (21). The capsid and genome size of PRV and HSV-1 are comparable. A recent study on HSV-1 looked at the pressure of the packed viral genome in the capsid. By slowly dissolving the portal protein in the viral capsid and measuring the force by which the viral genome is released, it was found that the viral genome is packed in the capsid at 10 times atmospheric pressure (34). At such pressure and at the temperature of infection (37°C), it was shown that the HSV-1 genome in the capsid is in a fluid-like state, which facilitates ejecting it through the nuclear pore (34, 35).

Transcription and replication

Transcription and replication of the herpesvirus genome are strictly temporally regulated and occur in a cascade-like fashion. Based on the time point in infection when they are transcribed, the viral genes are divided in three classes: immediate early (IE), early (E) and late (L) genes. The single IE gene of PRV, IE180, is rapidly expressed once the PRV genome has arrived in the nucleus. The IE180 gene is a transactivator responsible for activating several cellular and five viral promotors, inducing expression of US4, UL12, UL22, UL23 and UL41, which are presumed to assist overtaking DNA synthesis (36). Subsequently, different early genes are expressed, like EP0, US3, UL54 and UL48. Several of these early genes, similar to IE180, act as transactivators to induce expression of late genes, which mainly encode structural proteins. The late genes can be subdivided into early-late and true-late genes, with true-late gene expression initiating only after replication of the viral genome has started (6). Several viral proteins contribute to viral DNA replication, including UL5 (helicase), UL8 (helicase/primase), UL9 (ORF-binding protein), UL29 (major DNA binding protein), UL42 (polymerase associated protein) and UL52 (primase) together with several host proteins and enzymes (37). Viral DNA replication occurs through a rolling circle mechanism which leads to the formation of concatameric DNA strands that contain several viral genomes (6).

Capsid and nucleocapsid formation

Capsid assembly occurs in the nucleus of the cell and requires several viral proteins, such as capsid structural components (UL38, UL35, UL19, UL18 and UL6) and scaffolding proteins (UL26 and UL26.5) that participate in capsid formation but are not found in the mature virion. Based on HSV-1 data, the viral DNA of PRV is believed to be synthesized, cleaved and encapsidated in the nucleus with the help of the UL15/UL28 terminase complex and UL6 (21). UL6, encoding the portal protein, can initiate the capsid formation process (38). The concatameric DNA is then cleaved into genome-length DNA fragments upon insertion into the preformed capsids, which then leave the nucleus through passage through the nuclear membrane, passing through the perinuclear space (6).

Egress

Based on data obtained mainly on HSV-1 and PRV, it is believed that UL31 and UL34 of PRV, located on both leaflets of the nuclear membrane of infected cells, can recruit cellular proteins (such as protein kinase C) and the viral US3 kinase to the inner nuclear membrane (INM), which trigger disruption of the nuclear lamina network to enable budding of the virus particle

at the INM (39-42). By budding through the INM, virus particles gain a primary envelope and migrate into the perinuclear space between the INM and the outer nuclear membrane (ONM). Subsequent fusion of this primary envelope with the ONM releases the nucleocapsids in the cytoplasm, which is followed by the addition of inner layer tegument proteins (6).

During early tegumentation, the UL19 and UL25 capsid proteins are presumed to interact with the UL36 tegument protein, which on its turn binds UL37. UL37 is believed to be responsible for the addition of several other tegument proteins and the recruitment of microtubule associated transport molecules. The protein-protein interactions within the tegument and between tegument and envelope proteins are likely to drive the secondary and final envelopment process and may be regulated by phosphorylation (43). This secondary envelopment is most commonly believed to occur in vesicles derived from the TGN (44). Several tegument proteins (UL11, UL31, UL34, UL37, UL47, UL48 and UL51) and envelope proteins (such as gM and gE) have an important role to fulfill since deletion of these proteins results in an accumulation of unenveloped cytoplasmatic capsids (45, 46). Finally, after exocytosis the particles remain tightly confined on the outer cell surface. Current knowledge of alphaherpesvirus egress indicate a model that links together the intracellular transport pathways and exocytosis mechanisms to mediate viral egress (47).

For the current thesis, three viral proteins are of particular importance: two glycoproteins (gB and gD) and a tegument protein (US3). In the next paragraphs, the function of these three proteins will be discussed in more detail.

f.   Glycoprotein B: an overview

Structure and processing

PRV glycoprotein gB, formerly known as gII, is a 110 kDa glycoprotein, abundantly expressed in the viral envelope and ER, trans-Golgi network (TGN) and plasma membranes of infected cells. Viral glycoprotein gB consists of an extracellular domain, a transmembrane region and a rather long carboxy-terminal domain, is synthesized as a monomer in the ER and is subsequently assembled to a homodimer, linked by disulfide bonds. The protein then traffics to the TGN, where the protein receives post-translational sugar modifications, referred to as glycosolylation, and is cleaved by furin, a cellular endoprotease, to form the mature gB protein. The cytoplasmic domain of gB contains a endocytosis motif that can associate with the cellular clathrin-associated AP-2 adaptor complex, enabling gB to be internalized from the

plasma membrane (48). PRV gB can induce a humoral response through several B cell epitopes, even though this is not sufficient to protect against a challenge infection (49).

Role during infection

PRV gB is essential for cell entry and direct cell-to-cell spread and, therefore, is a crucial element of an infectious virion (50). All herpesviruses utilize the gB and gH/gL glycoproteins for membrane fusion, of which gB is the most conserved. Together with the G protein of vesicular stomatitis virus, gB is a member of the Class III fusion proteins. Unlike VSV G, gB only functions when partnered with gH/gL. However, gH/gL does not resemble any known viral fusion protein and there is evidence that it functions by triggering the fusogenic activity of gB. For several alphaherpesviruses, including HSV-1/2 and PRV, binding of the gD glycoprotein with one of its receptors activates the gB- and gH/gL-dependent fusion machinery. The necessity of PRV gB to bind with a cellular entry receptor increases in the absence of gD binding with its entry receptors (51, 52).

gB entry receptors

Several gB entry receptors for PRV and HSV-1 have been identified. Besides binding to heparan sulphate glycosaminoglycans during primary attachment of the virion with the host cell, non-muscle myosin IIA/B (NMHC-IIA/B) and paired immunoglobulin-like type 2 receptor α (PILRα) have recently been identified as gB receptors that mediate HSV-1 entry (29-31). NMHC-IIA/B are ubiquitously expressed in various human tissues and cell types and are involved in membrane repair and form a part of the contractile system of nonmuscle cells (53). Both proteins are typically located intracellularly, but can be found exposed at the cellular membrane during their function as gB receptor. PILRα is predominantly present on immune cells. PILR family members will be discussed further in the section covering natural killer (NK) cell receptors (section 2.d.v). HSV-1 gB has also been reported to bind and thereby activate TLR-2, a pattern recognition receptor that is present on diverse immune cells. However, TLR-2 has not been reported to contribute to gB-mediated virus entry in host cells (54). The PRV virus has been shown to bind to and use the human homologue of PILRα for virus entry in host cells (32). Whether PRV gB is able to interact with the porcine homologues of PILRα, TLR-2 and/or NMHC-IIA/B has not been reported yet.

g. Glycoprotein D: an overview

Structure and processing

PRV glycoprotein gD, formerly known as gp50, is a type I membrane glycoprotein and is synthesized as a 44.3 kDa precursor late in infection. In the TGN, the gD precursor is converted to an O- but not N-glycosylated 50 to 60 kDa mature form (55). The protein consists of an extracellular domain, a transmembrane region and a short carboxy-terminal domain. The latter contains an endocytosis motif, similar to PRV gB (56).

Role during infection

The gD protein is well conserved throughout most alphaherpesviruses, but is not present in beta- or gammaherpesviruses. As previously mentioned, gD plays an important role during host cell entry for many alphaherpesviruses, although some alphaherpesviruses encode a gD homologue that is not required for entry (MDV) or do not encode a gD protein at all (VZV). Upon binding with its cellular entry receptor, gD mediates entry through activation of the gB/gH/gL fusion complex. In contrast to gB, gD does not display similarity with any known fusion protein and likely does not have fusogenic activity (57). For several alphaherpesviruses, gD is essential for both virus entry in host cells and direct cell-to-cell spread of the virus. In PRV, gD is required for virus entry but dispensable for viral cell-to-cell spread. Nevertheless, by triggering the formation of varicosities (synaptic boutons) along the axons of infected neurons, PRV gD may still enhance cell-to-cell spread by creating possible virus exit sites (58). For HSV-1, gD has been reported to display anti-apoptotic activity in two independent studies. One study reported that the interaction of HSV-1 gD with its receptor Herpesvirus Entry Mediator (HVEM) prevents apoptosis during several apoptotic stimuli in a NF-κβ-dependent manner (59). The other study focused on the binding of HSV-1 gD to the mannose 6-phosphate receptor, which is involved in regulation of lysosomal targeting. This study hypothesizes that HSV-1 gD blocks the influx of lysosomal enzymes into the endosomal compartment, thereby disturbing and reducing the efficiency of the cellular autophagic machinery and apoptotic consequences of a viral infection (60). Potentially both pathways can work cooperative in HSV-1 infected cells expressing HVEM. Currently, it is unknown whether gD homologs of other alphaherpesviruses also display anti-apoptotic properties.

gD entry receptors

For HSV-1, HSV-2 and/or PRV, different gD entry receptors have been identified and include a variety of related and unrelated membrane proteins: Herpesvirus Entry Mediator (HVEM), CD111 (Nectin-1), CD112 (Nectin-2), CD155 (Poliovirus Receptor) and modified heparan sulphate (3-O-sulphated heparan sulphate) on proteoglycans. HVEM is a member of the tumor necrosis factor receptor (TNFR) family. The receptor is expressed in different tissues, but abundantly expressed in lymphoid organs and different immune cells (Montgomery, 1996). The human and mouse homologue of HVEM has been reported to serve as an entry receptor for HSV-1 and HSV-2 gD, but not PRV gD. Whether PRV gD binds to the porcine HVEM homologue remains to be studied (61). CD111 and CD112 are Nectins while CD155 is a Nectin-like molecule (Necl). All three proteins are members of the Immunoglobulin (Ig) superfamily and have a similar cell adhesion function. CD111, CD112 and CD155 are abundantly expressed in various cell types, such as epithelial and endothelial cells, fibroblasts and neurons (62). The ectodomains of CD111 and CD112 are composed of three Ig-like domains, one domain with V-like structure and two domains with a C-type structure. This domain with V-like structure of CD111 is important for HSV-1 gD binding. CD111 is known to mediate entry for many alphaherpesviruses, including HSV-1, HSV-2, and PRV. Binding of HSV-1 gD to CD111 has been reported to abolish its cell adhesion functions (63). CD112, on the other hand, was shown to mediate gD-dependent entry of PRV and HSV-2, but typically not HSV-1, although certain HSV-1 isolates originating from patients with HSV-1 encephalitis have been reported to interact with CD112 (64). Research has shown that the affinity of HSV-1 gD for CD112 can be increased substantially by specific point mutations, which are also found in these clinical isolates (65). PRV and BoHV-1 can also use CD155 as gD entry receptor. The affinity of gD for the different entry receptors varies throughout the alphaherpesviruses. The wide range of alphaherpesvirus gD entry receptors possibly contributes to the broad range of host cells that these viruses can infect.

h. US3 serine/threonine kinase: an overview

Structure and processing

The US3 gene is conserved throughout all alphaherpesvirus subfamily members. It encodes a serine/threonine kinase that contains the catalytic domain in the C-terminal region of the protein (66). The PRV US3 gene contains two open reading frames (ORF) which results in the expression of two US3 isoforms: an abundantly expressed short isoform of 41 kDa and a long

form of 53 kDa (67). The long isoform of the PRV US3 is a protein of approximately 390 amino acids and shares 39% amino acid identity with the HSV-1 and HSV-2 US3 kinase, while HSV-1 and HSV-2 US3 share 75% amino acid identity. Both PRV US3 isoforms are catalytically active and both contain a 101 amino acid carboxy-terminal membrane/vesicular localization domain and an N-terminal 102 amino acid nuclear localization domain. Both isoforms differ by a functional 51 amino acid N-terminally located mitochondrial localization sequence in the long isoform of US3 (68). Most other alphaherpesviruses do not appear to encode two isoforms of the US3 protein, except for HSV-1 which expresses, besides the complete US3 protein, also the C-terminal domain of US3 as a truncated form, designated as US3.5 (69, 70).

Role during infection

The US3 kinase is a tegument protein and is the only tegument protein identified to date that is both present in primary virions (present in the perinuclear space) and mature virions (71, 72). The kinase nature of the US3 protein was identified based on homologies with protein kinase gene families of eukaryotes and retroviruses (73). *In vitro* biochemical studies characterized the consensus sequence of US3 phosphorylation sites for PRV, HSV-1 and other alphaherpesviruses as $(R)_n$-X-(S/T)-Y-Y, where $n \geq 2$, S/T is the target site where either serine or threonine is phosphorylated, X can be absent or any amino acid, but preferably arginine, alanine, valine, proline or serine, and Y can be any amino acid except proline or an acidic residue (74-77). This consensus sequence is similar to that of protein kinase A (PKA), a cyclic adenosine monophosphate (cAMP)-dependent protein kinase (78-80). However, work on HSV-1 has shown that US3 is a promiscuous kinase and is able to phosphorylate targets that do not contain the typical consensus sequence (81). Up to date, no function of US3 has been found that did not depend on its kinase activity. PRV US3 has been reported to affect the phosphorylation status of several cellular proteins, including p21-activated kinases (PAK), cofilin and Bad, although only PAK has been confirmed to represent a direct phosphorylation target of PRV US3 (24, 82, 83). The US3 protein has been extensively investigated for several alphaherpesviruses and displays several functions, several of which are illustrated in Figure 3. Seen the variety of functions associated with US3, it may come as no surprise that genetically engineered PRV or HSV strains that lack the US3 gene are severely attenuated *in vivo* (84-87). A summary of the known functions of the PRV US3 protein kinase will be discussed in the next paragraphs, which includes nuclear egress of virions, anti-apoptotic activity, cytoskeletal rearrangements, downregulation of MHC-I cell surface expression and other functions.
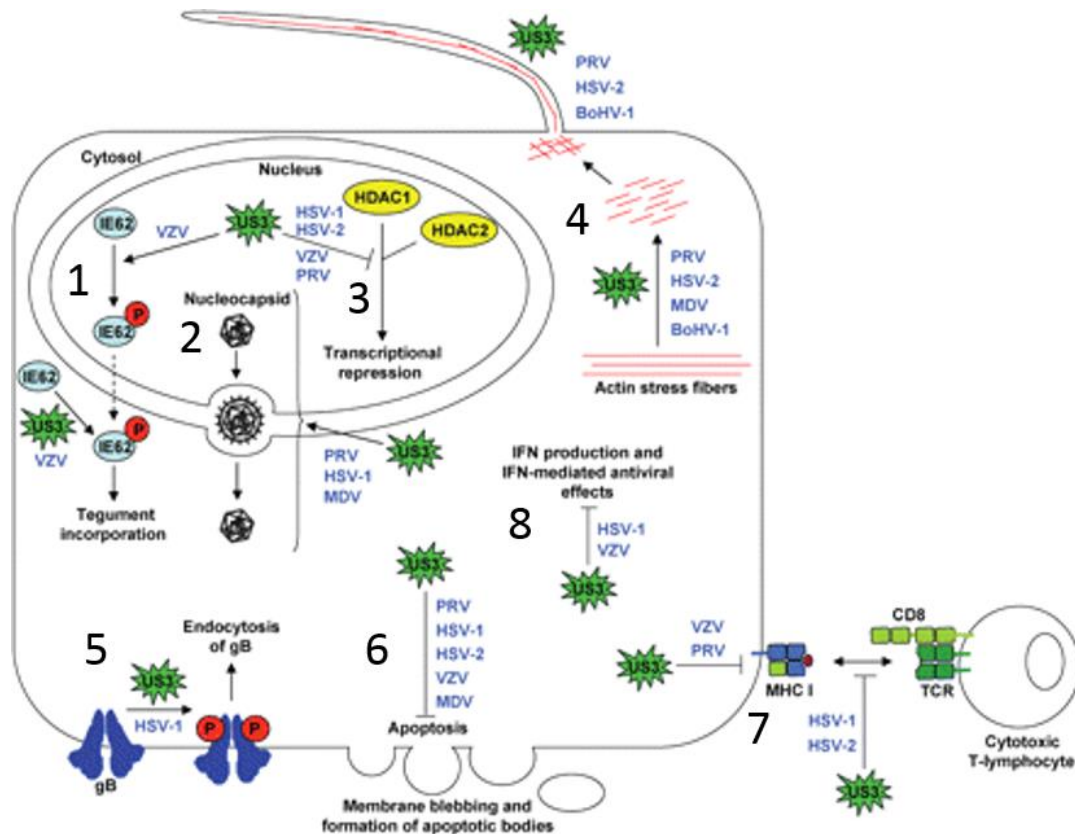
Figure 3. Schematic overview of several US3 functions. (1) Promoting nuclear egress to the cytoplasm of viral immediate early protein 62 (IE62) of VZV, where it is incorporated in the tegument of progeny virus; (2) Promoting nuclear egress of the virion of several alphaherpesviruses and disrupting the lamina network in the nucleus. (3) Promoting viral gene expression by phosphorylation of cellular gene expression regulators; like histone deacetylases (HDACs) for several alphaherpesviruses. (4) Promoting cytoskeletal rearrangements for many alphaherpesviruses (also including BoHV-5): disruption of actin stress fibers and formation of long actin projections, enhancing intercellular spread. (5) Promoting phosphorylation and endocytosis of gB for HSV-1. (6) Preventing apoptosis mediated by various stimuli for many alphaherpesviruses (including BoHV-5), such as infection itself, staurosporine and overexpression of pro-apoptotic proteins. (7) Contributing to reduced cell surface expression of Major Histocompatibility Complex – I (MHC-I), a central factor involved in the cytotoxic T lymphocyte (CTL) response, for VZV and PRV and interference with CTL-mediated lysis of infected cells for HSV-1/-2 and (8) counteracting the production and antiviral effects of type I interferon for HSV-1 and VZV (Figure adapted from: (88)).

PRV US3-mediated nuclear egress of virions

Upon formation of nucleocapsids in the nucleus, these nucleocapsids gain a first tegument layer and bud through the inner nuclear membrane (INM) to obtain a primary envelope, thereby entering the perinuclear space. Subsequently, the primary envelope is lost upon de-envelopment by fusion of the primary envelope with the outer nuclear membrane (ONM), by which the nucleocapsid reaches the cytoplasm. Budding at the INM requires the viral UL31/34 complex (which is conserved in all herpesvirus subfamilies) to be present at the INM (89-92). US3 is not essential for budding at the INM, although US3 does induce phosphorylation of UL31 and

regulates the homogenous localization of the UL31/34 complex across the INM, thereby contributing to budding efficiency (77, 93, 94). Besides its involvement in primary envelopment, absence of US3 during an infection leads to an accumulation of primary virions in the perinuclear space, suggesting that US3 contributes to fusion of the primary envelope with the ONM via an unknown mechanism (93).

PRV US3-induced cytoskeletal rearrangements

The ability of US3 to cause profound cytoskeletal rearrangements is very well conserved across alphaherpesviruses, and has been reported for PRV, HSV-2, MDV, BoHV-1 and BoHV-5 (88, 95). These cytoskeletal rearrangements have been best characterized for PRV US3 and consist of disassembly of actin stress fibers, leading to cell contraction, and the formation of long actin- and microtubule-containing cell projections (68, 96). PRV US3-induced actin rearrangements have been reported to enhance intercellular virus spread, with virions migrating within the cell projections from one cell to another (96). The ability of PRV US3 to trigger actin rearrangements relies on US3-mediated phosphorylation and activation of the group I p21-activated kinases (PAK) PAK1 and PAK2, central regulators in RhoGTPase signaling. PAK1 appears to be involved in US3-mediated cell projection formation, while PAK-2 is mainly involved in actin stress fiber break down (24). Through its ability to activate PAKs, PRV US3 also leads to dephosphorylation and activation of cofilin, a member of the actin depolymerizing factor family and central player in actin dynamics, which contributes to the US3-mediated actin rearrangements (82).

PRV US3-mediated modulation of MHC-I cell surface expression

MHC-I molecules play a central role in presenting endogenous and viral peptides to $CD8^+$ cytotoxic T lymphocytes (CTLs) which allows these CTLs to trigger apoptosis of virus-infected cells. Besides their crucial role in activating CTLs, MHC-I molecules are also of central importance in providing inhibitory signals to natural killer (NK) cells, which will be discussed further on. PRV US3 has been reported to contribute to some extent to the downregulation of MHC-I, a widespread strategy of herpesviruses to suppress CTL-mediated killing of infected cells. However, effects of PRV US3 on MHC-I are limited, highly cell type dependent, rely on additional viral proteins and occur via a yet unidentified mechanism (97). Effects of US3 on MHC-I cell surface levels have also been reported for HSV-1 and VZV (98-101). For HSV-1, US3 was reported to provide protection to infected cells against cytotoxic T cells and to activate

NK cells (102). Whether this protection is solely mediated by a reduction of MHC-I molecules on the infected cellular surface, remains to be studied.

Anti-apoptotic activity of PRV US3

Both virus infection itself and particular immune cells (e.g. CTLs, NK cells) can trigger virus-infected cells into apoptosis. Hence, it may come as no surprise that many viruses have developed anti-apoptotic proteins. The anti-apoptotic function of US3 is highly conserved throughout the alphaherpesviruses, as such activity has been documented for PRV, HSV-1, HSV-2, MDV and BoHV-5 (88, 95). Both during infection and transient transfection, PRV US3 increases resistance of cells to certain apoptotic stimuli, such as treatment with sorbitol or staurosporin or overexpression of the pro-apoptotic protein Bax (103, 104). Transient transfection of the long isoform of US3 results in a more efficient anti-apoptotic activity, compared to the short isoform (103, 105). The latter may be explained by the mitochondrial localization of the long US3 isoform and how apoptotic signals are processed. There are three main apoptotic pathways: an ER apoptosis pathway, an extrinsic death receptor pathway and an intrinsic mitochondrial apoptosis pathway. All these pathways result in the activation of the apoptosis effector proteins, the caspases. The intrinsic pathway is regulated by the Bcl-2 family, which contains both anti- and pro-apoptotic members. This protein family regulates mitochondrial membrane permeability. Activity of Bcl-2 family proteins depends on their phosphorylation and oligomerization status. PRV US3 has been reported to trigger phosphorylation and thereby inactivation of the Bcl-2 family protein Bad, a pro-apoptotic protein (83). Phosphorylation of Bad prevents its binding to anti-apoptotic proteins and oligomerization with pro-apoptotic proteins, and thus suppresses apoptosis (106). The anti-apoptotic activity of PRV US3 has also been reported to depend to some extent on the above mentioned cellular substrate of PRV US3, group I PAKs. Specifically, PAK1 shows a significant but limited involvement in the anti-apoptotic effect of US3, while PAK2 does not appear to play a role. PAK-1 has been reported previously to phosphorylate Bad *in vitro* (107), but it is unclear at this stage whether this contributes to the role of PAK1 in the anti-apoptotic effect of PRV US3. Interestingly, PRV US3 was also found to increase the expression of anti-apoptotic signaling molecules, including Akt and NF-κβ, in PRV infected trigeminal ganglia of pigs *in vivo* (105). Whether PRV US3 prevents apoptosis independent of the mitochondrial/intrinsic pathway remains to be investigated.

Other functions

PRV US3 has also been reported to play a role in disruption of promyelocytic leukemia protein nuclear bodies (PML-NBs), hyperphosphorylation of histone deacetylase 2 (HDAC2) and axonal transport. The disruption of PML-NBs is caused by a US3-dependent degradation of their main structural component, PML, via the proteasome. These PML-NBs are discrete dynamic nuclear bodies that play a key role in host cell antiviral defense and their disruption appears to be crucial for herpesviruses, as representative members of all three herpesvirus subfamilies encode proteins that disrupt PML-NBs (108-110). The PRV US3 also leads to hyperphosphorylation of HDAC2 to reduce viral genome silencing and allow efficient viral replication (111). A study using US3null PRV virus mutants in a rat eye infection model indicated that US3null mutants have a delay in axonal spread (112). This finding was partly confirmed by a study that investigated the role of viral kinases in axonal transport. The viral kinases US3 and UL13 were found to act together to sustain long distance transport of egressing viral particles to the distal axon by preventing membrane dissociation from capsids and directing the capsids away from the nucleus (113).

2. Natural Killer cells
    a. Discovery and lineage

Natural Killer (NK) cells were discovered during a study of T cell responses against leukemia cells. Spleen cells of athymic mice, thus devoid of T cell maturation, were found to display *in vitro* cytolytic activity against tumor-derived cells (114, 115). Such cytolytic activity was previously solely attributed to cytotoxic CD8+ T cells and, in contrast to CD8+ T cells, these newly discovered cells could lyse tumor cells without being previously sensitized to them. This was initially referred to as natural reactivity and was found to be related to a previously unknown, separate functional group of lymphocytes, the NK cells (116).

NK cells are a member of the innate immunity and form an important first immunological barrier against tumor cells and cells infected with viruses or other intracellular pathogens. NK cells are present in lymphoid and non-lymphoid tissue and represent a relatively minor fraction of total lymphocytes in the blood (from 2 to 18% in human blood) (117). NK cells originate from a lymphoid progenitor in the bone marrow, which they share with the lymphoid cells of the adaptive immunity, T and B cells. Somewhat similar to B and T cells, NK cells undergo an education process to prevent them from being autoreactive (inducing a response against healthy cells and tissues). Importantly, NK cells are considered innate lymphocytes because of their

rapid response and their ability to develop without receptor gene rearrangements, essential for T and B cell-associated antigen receptors (118). Instead of these antigen receptors, NK cells express a high variety of germline-encoded receptors. Some of these stimulate NK cells to mediate cytotoxicity or to produce cytokines, while others inhibit NK cells activation. The wide distribution of most germ-line encoded activating receptors on virtually all NK cells suggests that, unlike for T and B cells, no NK cells endowed with a narrow Ag specificity need to be expanded to reach an effective response against cells expressing virus-induced or stress-derived antigens. Therefore, NK cells can respond quickly upon engagement, but lack the ability to be trained against recurring antigens (adaptive memory). However, increasing evidence indicates that NK cells may possess a form of memory or can be trained in recognition of particular pathogens (119, 120), which will be discussed further (section 3.C).

b. NK cells: functions

i. Introduction

The innate immunity is necessary to form a first barrier against bacteria, parasites, viruses and cancer cells and to trigger an adequate adaptive immune response. NK cells contribute to these important functions via cytolytic activity against various malignant cells and the production of immunomodulatory cytokines, particularly interferon γ (IFNγ).
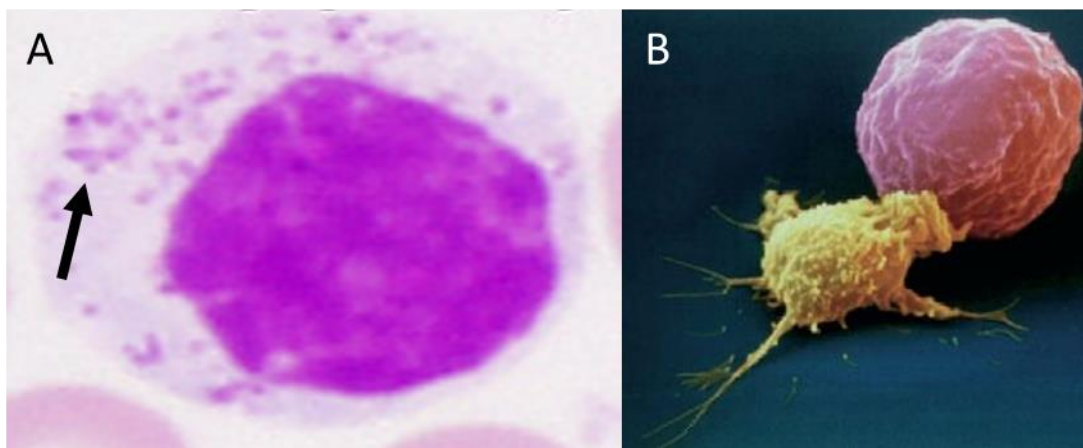
Figure 4. Morphological structure of an NK cell. (A) Hematoxiline-eosine staining of NK cell. The cytolytic granules of the NK cell are indicated by an arrow (Figure adapted from: (121)). (B) Electron microscope image of an NK cell (yellow) recognizing a tumor cell (red) (Figure adapted from: (122)).

The morphological features of NK cells already elude information about their main effector functions. As shown in Figure 4, NK cells display obvious cytoplasmic cytolytic granules, a critical element in their cytolytic activity. These NK cells also have a large endoplasmic reticulum (ER) to produce large amounts of cytokines (123-127). Most substantial information on NK cells is derived from studies investigating human and murine NK cells. Unfortunately,

NK cells of other species, including pig, are poorly characterized. Therefore, in the following sections, most information is derived from studies on human NK cells. Relevant *in vivo* studies in mice and available information on porcine NK cells will be explicitly mentioned.

<div align="center">ii. Cytolytic activity</div>

The cytolytic activity of NK cells is mainly executed by their cytotoxic granules, which are released upon activating receptor engagement (128). Intriguingly, this mechanism leads to killing of the target cell, without damaging other cells. This is mainly due to the formation of an immunological synapse, a dense interaction site of NK cells with target cells formed by adhesion molecules/receptors on the NK cells binding to their ligands on the target cell (as described in Figure 5).
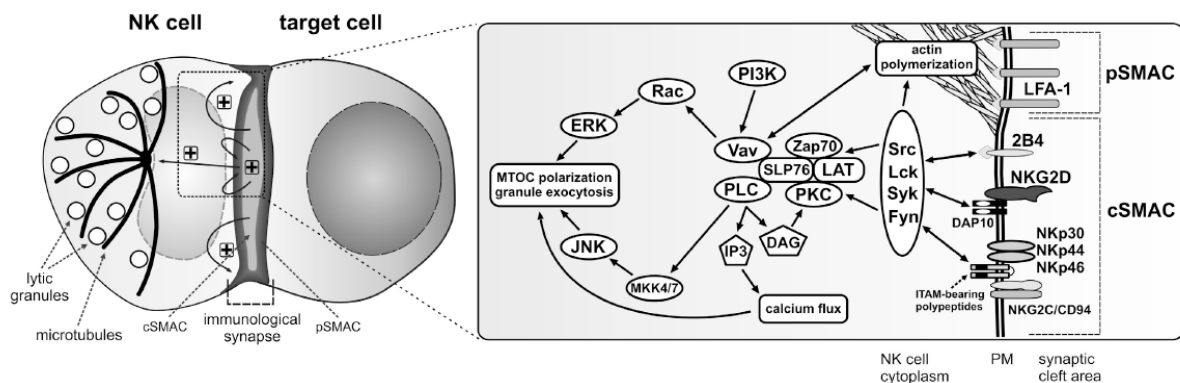
Figure 5. The immunological synapse and lytic polarization in NK cells. The encounter between an NK cell and a susceptible target cell results in the formation of an immunological synapse. Adhesion molecules, such as LFA-1, segregate into the outer region of the synapse, the peripheral supramolecular activation cluster (pSMAC), while NK cell receptors localize into the central area of the synapse (cSMAC). Upon NK cell activation, phosphorylation of membrane signaling molecules and positive feedback loops cause signal amplification to stimulate actin polymerization at the synapse periphery and polarization of the microtubule organizing center (MTOC) and lytic granules to the immunological synapse (Figure adapted from: (129)).

Once the immune synapse is formed, the lytic granules move along the microtubules toward the microtubule organizing center (MTOC) in a dynein-dynactin complex-dependent manner. The MTOC and the granules then polarize toward the immune synapse, where the granules switch from microtubules to the filamentous actin network at the synapse and navigate through the cortical filamentous actin meshwork by association with the actin motor protein myosin IIA. This allows the lytic granules to get in close proximity with the plasma membrane at the immunological synapse. The final step of lytic granule release consists of the fusion of secretory lysosomes with the plasma membrane, which is mediated by the SNARE protein family. Consequently, SNARE proteins, the actin cytoskeleton and microtubule are essential for

degranulation of NK cells (129-131). Remarkably, a novel study found that the structural integrity of the actin cytoskeleton in target cells is also crucial in the lytic response by NK cells (132).

The cytotoxic granules contain several proteins, among which perforin, several granzymes and calreticulin, that mediate and regulate target cell destruction (133). In the cytotoxic granule, these proteins are complexed with chondroitin sulfate-rich proteoglycans (134).

Perforin is constitutively expressed in NK cells, is synthesized as a 65 kDa precursor in the ER and exposure to NK-activating cytokines can increase its expression (135). The N-terminus of perforin contains a signal peptide and the membrane attack complex/perforin domain. The latter is known to have lytic activity (136). Perforin, secreted in very low quantities estimated to be below 10 nM and fewer than 500 molecules per cell, can make large pores in the plasma membrane. Perforin pores facilitate entry of the granzymes and can, cooperatively with granzymes, induce apoptosis of the target cell. Even if they are cleared by the target cells, the pores can facilitate entry of granzymes into the targets (131). Granzymes are serine proteases. Once inside the target cells, several of the granzymes can mediate intracellular damage. However, the exact mechanism of perforin and the role of granzymes in biological responses have been debated extensively (137). Compared to perforins, higher concentrations (~1 μM) of granzymes are needed to kill cells (138). NK cells from perforin-/- knockout mice, lacking the perforin gene, show a strongly impaired cytotoxicity response (139). In contrast, the cytotoxicity response of granzyme A/B double knockout mice was not impaired (140). As mice express 10 different granzymes, this may implicate a form of redundancy between different granzymes (140). Granzymes can cleave both extracellular and intracellular proteins of target cells. These extracellular substrates are yet to be defined, but some granzymes have been found to mediate detachment of cells from their substrate in vitro (141). Intracellularly, all granzymes can induce apoptosis by caspase dependent and independent pathways. Caspases are central executioner/effector proteins of apoptotic cell death and are expressed as inactive pro-caspases, which are activated upon processing by cleavage. Upon activation, caspases can subsequently process other caspases and cleave other cellular proteins, ultimately inducing apoptosis. Human NK cells express five granzymes: granzyme A, B, H, K and M. An overview of these granzymes, their mode of apoptosis induction and their substrates is listed in Table 1 (129). Information concerning porcine granzymes is very limited, but porcine homologues of all human granzymes are found in the porcine genome and expression of granzyme A and B in porcine PBMCs has been confirmed by mRNA analsysis (142). Remarkably, mice, unlike

human and pigs, do not encode granulysin, an antimicrobial protein from the saponin-like family. This cytotoxic granule constituent has been reported to induce ER-mediated apoptosis and might compensate the lack of granzyme diversity in humans and pigs, compared to murine NK cells (143, 144).

Table 1. Human granzymes and their substrates (Table adapted from: (129)) .

Name	Induction of apoptosis	Substrates	References
Granzyme A	Caspase-independent	Mitochondrial respiratory complex I protein	(145) (146) (147)
		SET complex	(148) (149) (150, 151)
		Poly-ADP-ribose polymerase (PARP)	(152)
		Ku70	(153)
		Lamins	(154)
		Histones	(155)
Granzyme B	Caspase-dependent and -independent	Caspase-3, -7,-8 and 10	(156) (157) (158)
		Bid	(159) (160) (161)
		Tubulin-α	(162)
		ROCK kinase II	(163)
		Lamin B	(154)
		Inhibitor of caspase-activated DNAse (ICAD)	(164)
		DNA-dependent kinase catalytic subunit	(165)
		PARP	(166)
Granzyme H	Caspase-dependent and -independent	Caspase-3 (indirect)	(167)
		Adenoviral 100K assembly protein	(164)
		Does not cleave ICAD or Bid	(168)
Granzyme K	Caspase-independent	Bid	(169)
		SET complex	(170)
		p53	(171)
Granzyme M	Caspase-dependent and -independent	Nucleolar protein nucleophosmin	(172)
		Fas-associated protein with death domain	(172)
		Survivin/BIRC5	
		Heat shock protein TRAP1	(173)
		ICAD	(174)
		PARP	(175)
		Ezrin	(176)
		Tubulin-α	(175)
		Serpin PI-9/B6	(177) (176)

The role of the other cytotoxic granule constituent, calreticulin, is not fully clarified. Calreticulin is a Ca^{2+} binding protein and has been shown to inactivate perforin and prevent perforin-mediated lysis, but calreticulin is also a target for the protease activity of the

granzymes. The latter inactivates calreticulin and promotes perforin-dependent lysis. Therefore calreticulin is believed to have a form of control function, controlling perforin-mediated lysis and granzyme function as additional competition-substrate (178).

Besides killing of target cells by cytotoxic granules, NK cells can also induce apoptosis by engagement of death receptors on the target cells via FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expressed on the surface of NK cells and through secretion of tumor necrosis factor α (TNFα) (179). Engagement of these death receptors on the target cells results in their oligomerization, which attracts several adaptor proteins. These adaptor proteins subsequently process and activate caspases to initiate apoptosis. This pathway does not involve mitochondrial membrane integrity and can independently of this induce apoptosis. However, the caspases activated by the death receptor pathway can also initiate the mitochondrial apoptotic pathway. Apoptosis induced by FasL also relies on cleavage and activation of Bid, which subsequently is transported to the mitochondria as truncated Bid and compromises mitochondrial membrane integrity (180, 181). TNFα induces apoptosis by both the death receptor and mitochondrial signaling pathways and also induces the expression of the Fas death receptor on target cells, thereby rendering these cells more susceptible to Fas-mediated lysis (182).

iii. Production of Interferon gamma

Cytokine secretion is distinct from secretion of cytotoxic granules in NK cells. The secretory pathway for the release of cytokines in NK cells is still unclear, but the localization of IFNγ trafficking compartments and vesicles does not overlap with any late endosome granule markers and perforin (183). IFNγ production represents a crucial element in shaping the innate and adaptive immunity, and contributes to a substantial extent to the importance of NK cells (184). Initially, the production of IFNγ was exclusively contributed to T cells and NK cells (185). However, several studies report that other cells, such a NKT cells, B cells and antigen-presenting cells (APCs), can secrete IFNγ as well (186). Currently, there is evidence that IFNγ produced by NK cells and APCs is important in the early host response. This IFNγ production is then gradually taken over by T lymphocytes, which then become the main source of IFNγ (187, 188). IFNγ belongs to the family of interferons, which are closely related by their ability to protect cells from viral infections. Based on several criteria, the IFN molecules have been subdivided into three distinct classes. Type I IFN includes IFNα and IFNβ and additional minor IFN species, type II IFN is composed solely of IFNγ, and the more recently discovered Type III IFN currently comprises four members: IL-29, IL-28A, IL28B and IFNL. All IFNs share an

α-helical bundle structure and function in a similar manner, however significant differences exist. Both type I and type III IFNs are broadly produced upon activation of pattern recognition receptors, such as the TLR receptors, whilst type II IFN is produced by specialized immune cells (NK cells and T cells). Type I and type III IFN have been reported to share many biological activities, in particular direct antiviral effects (189).

The dimeric IFNγ receptor (IFNGR) consists of two ligand binding chains (IFNGR1) and two signal transducing chains (IFNGR2). The receptor belongs to the class II cytokine receptor family and binds IFNγ by the small groove formed by the two Ig-like folds of the two IFNGR1 chains which comprise most of the extracellular domain of the receptor. Upon interaction between the IFNGR1 chains with IFNγ, the small extracellular domain of IFNGR2 associates with the IFNGR1 chains, followed by activation of the Janus kinases JAK1 and JAK2, and phosphorylation of the tyrosine residue in the intracellular domain of IFNGR1. This leads to recruitment and phosphorylation of STAT1 (signal transducers and activators of transcription 1), which is thereafter translocated to the nucleus to activate a wide array of IFNγ-responsive genes (190, 191). Although IFNγ displays most of the biologic activities that have been ascribed to other IFN, it has a lower direct antiviral activity, but more immunomodulatory properties than the type I Interferons (192).

The direct antiviral effect of IFNγ consists of establishing an antiviral state in host cells by eliciting he expression of several key antiviral cellular proteins. One example is the antiviral protein kinase R (PKR), a serine/threonine kinase that is produced in an inactive form, but upon activation by viral dsRNA, it can phosphorylate eukaryotic translation initiation factor 2 alpha (eIF2α). The latter is important during initiation of translation and PKR-mediated phosphorylation of eIF2α inhibits both viral or cellular protein synthesis, thereby preventing viral spread (193, 194). Next to this direct antiviral effect, IFNγ can modulate both innate and adaptive immunity.

Through the production of IFNγ, NK cells can activate several members of the innate immunity, such as DCs and macrophages, and improve antigen presentation towards T cells by inducing an enhanced MHC class I presentation by nucleated cells and MHC class II presentation by APCs, such as macrophages and DCs. MHC I molecules present peptides derived from the intracellular cytoplasm, while MHC II molecules present peptides derived from the extracellular environment. However, certain cells, mainly APCs, are endowed with the ability to present peptides derived from the extracellular environment through MHC class I presentation. This exception is defined as cross-presentation (195, 196). Enhancement of MHC

I antigen presentation by IFNγ occurs at two levels: by increased MHC I expression levels and by the expression of an immuno-proteasome. The proteasome processes and cleaves the proteins to peptides and can increase the quantity, quality and repertoire for MHC I loading of peptides (197, 198). IFNγ triggers upregulation of MHC II levels in APCs, but can also induce MHC II expression on cells that do not constitutionally express MHC II, the so-called non-professional APCs. As for MHC I, IFNγ also enhances the processing of proteins for MHC II loading by increasing the expression of lysosomal enzymes responsible for the degradation of proteins to peptides (199). Besides stimulating the innate immunity and enhancing antigen presentation, IFNγ also has an important direct effect on the adaptive immunity. T cell polarizations towards a certain T cell effector phenotype largely depends on the cytokine environment at the time of T cell receptor engagement. Both IFNγ and IL12 are the prototypic cytokines directing the differentiation of naïeve T cells towards a T helper 1 phenotype. This influence of IFNγ can even be more relevant *in vivo*, as IFNγ induces IL12 production in phagocytes and inhibits IL4 secretion, a cytokine important in the Th2 differentiation, further contributing to a Th1 polarization of the adaptive immune response (200, 201).

iv.   Functional heterogeneity

In different species, including man, mouse and swine, NK cells have been found to display functional heterogeneity.

In humans, natural killer cells can be roughly subdivided into 3 different populations based on the relative expression of the surface markers CD56 an CD16: the $CD56^{high}$ $CD16^{dim/-}$, $CD56^{dim}$ $CD16^{high}$ and $CD56^{-}$ $CD16^{high}$ NK cells (202). The expression of CD56 plays a major role in defining NK cell functional heterogeneity. The majority of NK cells have a low surface density of CD56 and are referred to as $CD56^{dim}$ NK cells. This NK cell subpopulation produces a relative low amount of cytokines, but can efficiently lyse susceptible target cells (203). $CD56^{high}$ NK cells are less capable of killing target cells and correspondingly express lower amount of activating receptors, but express higher amounts of cytokines. This subpopulation can interact with DCs in lymph nodes. $CD56^{high}$ NK cells can also be considered as an immature population that can differentiate to the $CD56^{dim}$ phenotype (202).

In mice, four subsets of circulating NK cells can be found: the immature $CD27^{+}$ $CD11b^{-}$ NK cells, $CD27^{+}$ $CD11b^{+}$ and $CD27^{+}$ $CD11^{-}$ intermediate cells, and $CD27^{-}$ $CD11b^{+}$ mature NK cells. The expression levels of CD27 also discriminates functional NK subsets. The $CD27^{high}$ NK subset mainly responds by cytokine production, while $CD27^{low}$ NK subsets rather engage

in cytolytic activity (204, 205). Interestingly, CD27b$^+$ NK cells that express the chemokine receptor CXCR3 display, compared to CD27b$^+$ CXCR3$^-$ NK cells, stronger cytolytic capabilities, profilic expansion and more robust IFNγ production, corresponding to the human CD56^{high} NK cell subpopulation (206).

The phenotype of porcine NK cells was recently described as perforin$^+$ CD2$^+$ CD3$^-$ CD4$^-$ CD5$^-$ CD6$^-$ CD8α$^+$ CD8β$^-$ CD11b$^+$ CD16$^+$ (207, 208). In porcine blood, two NK cell populations are found that differ in their expression of NKp46, an activating NK cell receptor. Both subsets, NKp46$^+$ and NKp46$^-$ NK cells, are present in the blood in roughly equal amounts and have comparable cytotoxic responses, although the NKp46$^+$ NK subset produces several folds more IFNγ (209). Besides these two subpopulations found in blood, a third subpopulation of NKp46^{high} CD8^{dim} was found in the liver. This NKp46^{high} CD8^{dim} NK cell subset is thought to be in an increased active state, as these cells produce several folds more IFNγ and have an increased degranulation response upon activation (210). The existence of this NKp46$^-$ NK subpopulation in swine is very peculiar, as NKp46 was thought to be a marker for NK cells throughout mammalian species. Indeed, except for swine, NKp46 expression was found in all NK cells of tested mammals, including human, non-human primates, mouse, rat and cow (211).

v. Cross-talk between NK cells and other immune cells

To fully understand the immune response mediated by NK cells, it is important to understand the interaction of these NK cells with other innate and adaptive immunity immune cells. There is particular crosstalk between NK cells and dendritic cells (DC) and macrophages, important antigen-presenting cells that drive the adaptive immune response. Macrophages are present in various strategic tissues throughout the body and derived from monocytes, present in the blood, which can enter the site of infection by passing through the endothelium of the blood vessel. The inflamed tissue then induces the differentiation of monocytes towards macrophages or dendritic cells to assist in the immune response. DCs are located mostly in tissues in contact with the external environment and in an immature state in the blood and tissues. Upon activation, DCs migrate to the lymph nodes where they interact with T and B cells (123-127).

There are several reports indicating that NK cells and other immune cells mutually influence each other, both at the periphery and in secondary lymphoid organs. In man, due to a specific subset of adhesion molecules (e.g. CD62L) and chemokine receptors (CCR7 and CXCR3), the CD56^{bright} NK subpopulation can enter lymph nodes where they are in close proximity to other immune cells like dendritic cells (DCs), enabling mutual activation and maturation (202). NK

cell-mediated effects on DCs and the T cell response are provided in Table 2. Through the production of IFNγ, NK cells can activate DCs and macrophages, inducing an enhanced MHC class I and II antigen presentation. Vice versa, activated DCs and macrophages mainly promote the development of NK cells through secretion of IL15, but also IL12 and IL18 (212). Interestingly, NK cells possess activating receptors (e.g. NKp44) that recognize and lyse immature DCs, activated macrophages and activated T cells, contributing to the regulation of the immune response. Hence, NK cells display a regulatory function by contributing to the homeostasis of immune cells and by editing the immune response (212, 213).

Table 2. Overview of NK cell effects on DC and T cell immune response (Table adapted from: (214)).

DC maturation	T cell priming	Cytotoxicity	Immunological memory
• Mediate 'DC editing' and maturation by killing immature myeloid-DCs and selecting for the 'most-fit' DC to cause T cell priming	• IFN-γ produced by NK cells influences Th1 priming	• Kill virus-infected, stressed or neoplastic cells	• Mediate memory-like responses which can be specific for antigen or can be induced non-specifically and provide enhanced protection against pathogens
• Can also enhance IFN-α production from plasmacytoid DCs	• Enhance CD8+ T cell responses	• Mediate killing of activated T cells, APCs and endothelial cells	
• Mature DCs can activate NK cells by secretion of cytokines, eg. IL-12, IL-18	• Influence DC-induced polarisation of naïve T cells		

c. NK cell regulation

i. Introduction

NK cells are able to directly recognize and respond to target cells by cytolytic activity and/or cytokine production, in principle without the need for priming. Nevertheless, priming of NK cells by IFNs, IL2, IL12, IL15, IL18 and/or other cytokines *in vitro* and *in vivo* by APCs or other immune cells greatly increases the cytolytic activity and IFNγ production upon target cell recognition (215). This was also reported for mouse and porcine NK cells (142, 216, 217). Recognition of and response to target cells is regulated by a variety of germ-line encoded NK cell receptors. Through these receptors, NK cells are able to interact with several cellular, tumor and viral proteins and structures. NK cells express a diverse set of Toll-like receptors (TLRs), which mainly contribute to NK cell cytokine production, and a variety of so-called activating/inhibitory NK cell receptors, which will be discussed below.

ii. *NK cell regulation by Toll-like receptors*

NK cells express several TLRs, such as TLR2, TLR3, TLR7, TLR8 and TLR9, independent of their activation status. These receptors are, in adults, constitutively expressed in various other immune cells and cells exposed to the external environment. TLRs are type I transmembrane proteins characterized by an extracellular domain containing leucine-rich repeats and a cytoplasmic tail containing a conserved region, called the Toll/IL-1 receptor (TIR) domain. TLRs recognize several conserved features of micro-organisms/pathogens, the so-called microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs) (218). An overview of TLRs expressed on NK cells and their ligands are given in table 3.

Table 3. An overview of TLR receptors expressed on NK cells with corresponding ligands (Table adapted from (218)).

TLR	Ligand(s) type	Ligand(s) expression	Reference
TLR2	Bacterial lipoprotein HSV-1 glycoproteins	Bacteria Viruses	(219) (220) (54)
TLR3	dsRNA	Viruses	(221)
TLR5	Flagellin	Bacteria	(222)
TLR7/8	ssRNA	Viruses	(223) (224)
TLR9	CpG DNA motifs	Bacteria/viruses	(221)

TLRs signal via MyD88 (myeloid differentiation primary response gene 88), although MyD88-independent signaling has been reported for TLR3 and TLR4. Triggering of TLRs on NK cells results in a minimal cytolytic response, but increased levels of IFNγ (222, 225). TLR triggering on NK cells tends to synergizes with chemokine- or cytokine-mediated signals to activate NK cell functions. This appears to be well conserved in humans, mice and swine (142, 226-228). The decision of the NK cell to engage a full cytolytic and IFNγ response appears to be mainly determined by the activating/inhibitory NK receptors described below. Nevertheless, TLR receptors expressed by NK cells contribute to the innate sentinel function of NK cells by triggering cytokine production and elevating the NK cell activation status (229, 230). Besides directly affecting NK cells, TLR signaling may also indirectly modulate NK cell activity and function, since TLRs on different other cells, including macrophages and DCs, may trigger these cells to secrete various cytokines that in turn can attract and prime NK cells and other immune cells (218, 231).

iii.   NK cell regulation by activating/inhibitory NK receptors

NK cells express a high variety of inhibitory and activating receptors, which are specialized in differentiating healthy from malignant or infected cells. These NK cell receptors differ greatly in their extracellular domains to recognize a broad array of cellular and pathogen-derived proteins on the outer surface of the target cell. Healthy cells constitutively express a range of ligands for activating but particularly inhibitory NK cell receptors. These inhibitory NK cell receptors commonly possess a canonical immunoreceptor tyrosine-based inhibition motif (ITIM), defined by the sequence (I/L/V/S)XYXX(L/V) (where X represents any amino acid), in their cytoplasmic region to transduce an inhibitory signal upon engagement of their ligands. Upon engagement, the tyrosine (Y) residue in the ITIM domain is phosphorylated by a Src family kinase, resulting in the recruitment of the lipid phosphatase SHIP-1 or the tyrosine phosphatases SHP-1 and SHP-2 (232). These tyrosine phosphatases dephosphorylate the protein substrates linked to the signal transduction cascade of activating receptors. Engagement of an activating NK receptor on the other hand is followed in most cases by the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) by Src family kinases. Such ITAM motifs are defined by the following sequence: (D/E)XXYXX(L/I)X6–8YXX(L/I), where X6–8 denotes any 6 to 8 amino acids between the two YXX(L/I) elements, and are either located in the cytoplasmic domain of the activating NK cell receptors or in the cytoplasmic domain of adaptor proteins associated with these activating receptors. These adaptor proteins, such as FcεRI-γ, CD3-ζ, DAP10 and DAP12, are type I transmembrane-anchored proteins that exist as either disulfide-bonded homodimers or, in the case of FcεRI-γ and CD3-ζ, as disulfide-bonded heterodimers. All have minimal extracellular regions comprising only a few amino acids, principally the cysteine residues through which they dimerize. Phosphorylation of conventional ITAMs, as several ITAM domains are reported to contain an ITIM domain as well (233), leads to the recruitment of Syk, a Src family kinase, and ZAP70 to the engaged receptor in the immunological synapse. Depending on which adaptor protein is phosphorylated, several key cellular signaling nodes, such as ERK and Akt, NF-κB and inositol-3,4,5-trisphosphate (IP3)-dependent Ca^{2+} release, are activated. Recent studies reveal that degranulation of NK cells appears to be linked to Ca^{2+} release, while cytokines are mainly produced as a response to the activation of NF-κB (234, 235).

Due to the broad variety of cellular signaling pathways that activating NK receptors trigger, the phosphatases SHIP-1, SHP-1 and SHP-2, recruited by the inhibitory receptors can potentially differentially act upon these pathways. As a consequence, NK degranulation or IFNγ could be

partly separately regulated. Interestingly, up to now, only the activating NK cell receptor CD16 was found to induce a cytotoxic response without affecting the cytokine response. Hence, NK cells are regulated by engagement of both activating and inhibitory receptors and it is the total sum of interactions that determines the particular NK cell response, as shown in Figure 6. Activation of NK cells by target cells can occur as a consequence of an increased expression of ligands for activating NK cell receptors and/or by a decreased expression of ligands for inhibitory NK cell receptors these on target cells. These events are transient and do not interfere with the ability of the same NK cell to recognize and discriminate healthy and malignant/infected cells later (236).
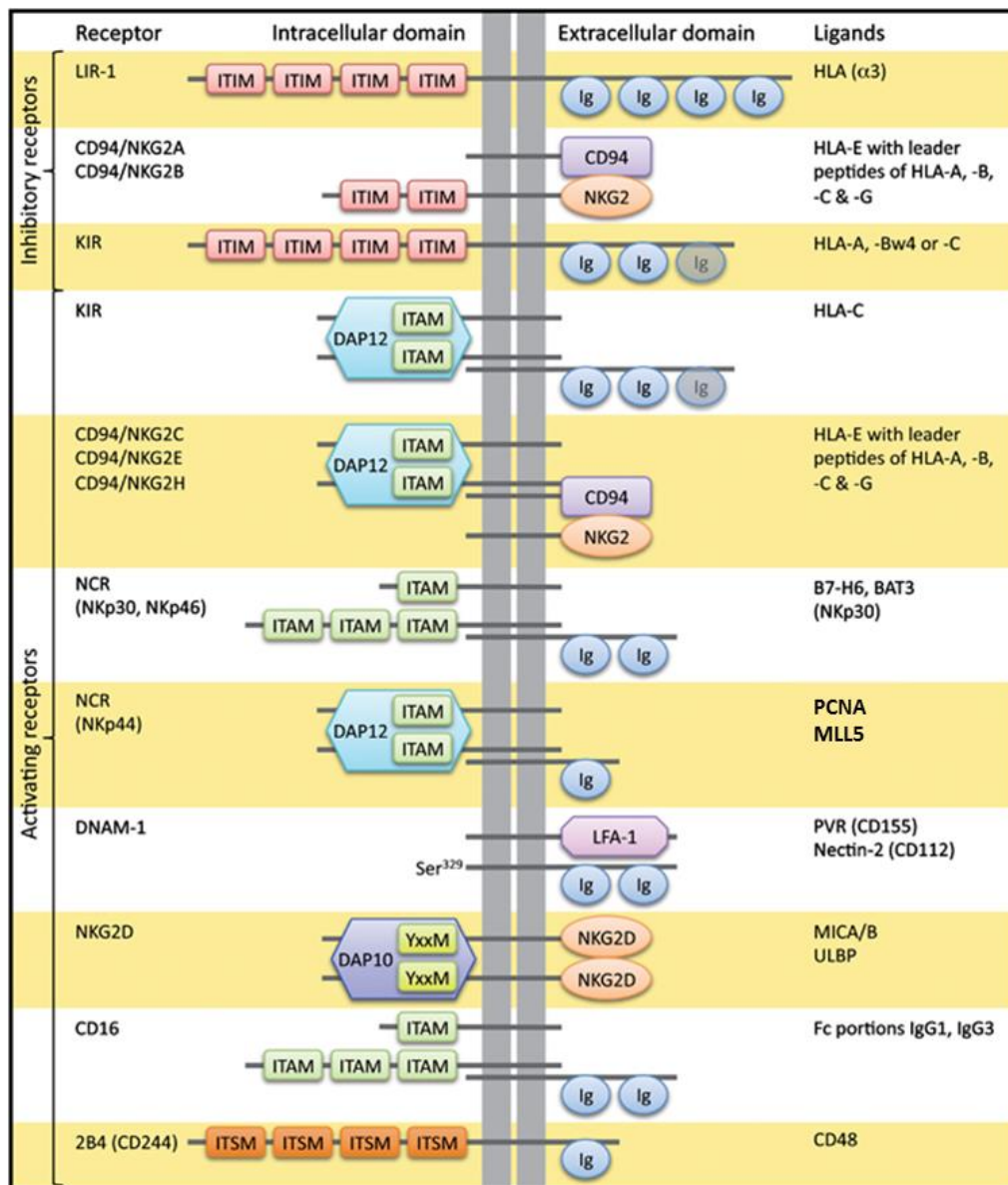

Figure 6. NK cells are regulated by multiple activating and inhibitory NK cell receptors recognizing their ligands (Figure adapted from: (237)).

    d. NK receptors

        i. KIR receptors

The human killer cell immunoglobulin (Ig)-like receptor (KIR) family contains both inhibitory and activating NK receptors. The activating members of this family usually have a short cytoplasmic tail and are associated with the ITAM bearing adaptor protein DNAX activating protein of 12 kDa (DAP12). In contrast, the inhibitory receptors have a rather long cytoplasmic domain, containing one or more ITIMs. The different receptors are categorized by the number of Ig-like domains (KIR2D/KIR3D) and the length of the cytoplasmic tail, it being either short (S) or long (L) which typically reflects their activating/inhibitory function. For example, KIR-3DS2 is an activating receptor that contains three Ig-like domains and a short cytoplasmic tail. Importantly, in mouse, activating and inhibitory KIR homologues have been described and are referred to as Ly49 receptors (238). Porcine homologues for both activating and inhibitory KIR receptors have been predicted, but currently only a porcine KIR2DL1 homologue has been identified to be expressed on mRNA level (142, 238, 239).

In man, the known ligands for the inhibitory KIR receptors consist of the three main classes of MHC class I molecules or more commonly referred to as human leukocyte antigens (HLA)-A, HLA-B and HLA-C. Due to the high abundancy of these ligands expressed on all healthy cells, the KIRs represent a significant inhibitory signal to prevent NK cells from lysing healthy cells. As cytotoxic T cells (CTLs) depend on presence of peptide-MHC I complexes to recognize and kill virus-infected and tumor cells. Certain cancers and viruses have evolved mechanisms to lower the cell surface availability of MHC-I molecules to prevent or minimize elimination by CTLs (240, 241). The KIR receptors provide an important mechanism to recognize (and ultimately kill) such cells by screening for MHC I molecule homeostasis on the cell surface.

Both activating and inhibitory KIR receptors are clonally distributed throughout the human NK cells, although roughly more inhibitory NK cell receptors are expressed and a small population of blood NK cells does not detectably express any inhibitory KIR receptors. The latter NK subpopulation is hypo-responsive to various stimuli, including MHC I deficient cells (242). As MHC I molecules are abundantly expressed, the KIR receptors are of obvious importance for recognition of healthy cells. The lack of sufficient MHC I/KIR-mediated inhibitory signals and consequent killing of target cells by NK cells is commonly referred to as 'missing-self' (243). The only KIR receptor found to respond to another ligand than MHC I molecules, is KIR2DL4. This KIR receptor is directly modulated by heparan sulphate, but its function remains unclear (244).

ii. CD300a receptor

The human CD300 protein family contains 7 members, CD300a to CD300g, that are present on various immune cells, including NK cells, and recognize several lipids on the surface of target cells, such as ceramide, phosphatidylserine (PS) and phosphatidylethanolamine (PE) (245). In mice, functional homologues of CD300a and CD300f have been reported (245). The CD300a, also referred to as Irp60, is a 60 kDA glycoprotein containing four ITIMs in its cytoplasmic domain, which create a docking site for SHP-1 and SHP-2 tyrosine phosphatases upon phosphorylation (246, 247). The extracellular domain of CD300a possesses a single V-like Ig domain that share approx. 80% amino acid similarity to the CD300c receptor. The latter is an activating receptor, as it has a short cytoplasmic domain, lacking any ITIMs, and is believed to be associated with an ITAM containing adaptor protein (246, 248). Until recently, commercially available antibodies could not discriminate between CD300a and CD300c (246). Therefore, reliable protein expression an functional analyses of these CD300 family members in immune cells has only recently been possible. CD300a is reported to be widely expressed and functional in immune cells, including in NK cells, monocytes, mast cells, T cells and eosinophils (246, 249-252), whilst CD300c is reported to be expressed and functional on monocytes and mast cells (248, 253).

Although the extracellular domains of CD300a and CD300c show substantial homology, both proteins differ in their ligand recognition (253). Two independent groups identified PS as a ligand for the CD300a receptor, while one group also identified PE as a ligand for the CD300a receptor. Both ligands are also found to interact with CD300c, but with a lesser affinity (253, 254). PS, a constitutively expressed membrane phospholipid, is asymmetrically distributed in the plasma membrane, with most PS normally being present on the inner leaflet of the membrane (255). Several biological processes trigger expression of PS at or redistribution of PS to the outer leaflet of the plasma membrane, such as platelet activation, early stages of apoptosis and micro-environmental stress on certain tumors (256-258). PS externalization represents a signal by which apoptotic cells are recognized and subsequently engulfed by phagocytes (257). This interaction of CD300a with phospholipids appears highly conserved, as a chicken CD300a homologue also interacts with these phospholipids and these phospholipids are even found in the membrane of yeast and bacteria (255, 259).

The CD300a receptor may transduce an inhibitory signal to NK cells upon recognition of PS-exposing cells. Indeed, recently, certain tumor cells that express PS on the outer leaflet of the cellular membrane have been reported to directly inhibit NK cell activation, which was

hypothesized to occur via CD300a. Whether CD300a was directly responsible was not investigated due to the lack of antibodies capable of inhibiting CD300a (260). CD300a-mediated inhibition of NK cells could be even more significant than estimated by PS expression alone, since certain tumors also have an increased exposure of PE, another CD300a ligand, on the outer leaflet of their plasma membrane (261).

iii.   DNAX accessory molecule-1 (DNAM-1)

The DNAX accessory molecule-1 (DNAM-1) or CD226 is a member of the Ig-like family and has a molecular weight of 65 kDa. The DNAM-1 receptor is expressed in a variety of cells, among which NK cells, T cells, platelets and mast cells (262, 263). Several species have been found to express DNAM-1 functions as an activating receptor and as an adhesion molecule, promoting intercellular adhesion (264-266). DNAM-1 is composed of an extracellular domain, containing two Ig-like domains, a transmembrane domain and a cytoplasmic domain, containing several putative tyrosine and serine residues that play an important role in DNAM-1 mediated activating signaling. Protein kinase C has been reported to phosphorylate serine 329 in DNAM-1, contributing to intracellular signaling. In contrast to most activating NK cell receptors, DNAM-1 does not associate with an ITAM containing adaptor protein (264).

Ligand recognition and cell signaling depend on the first extracellular Ig-like domain of DNAM-1 (267). For human and mouse DNAM-1, two ligands have been described: CD112 and CD155, both members of the nectin and nectin-like family (268). Recognition of CD112 or CD155 by DNAM-1 triggers a cytolytic response towards a target cell, and, as a consequence, target cell lysis can be triggered via upregulation of CD112 or CD155 on the cell surface (269, 270). By recognizing these ligands, and possibly other unknown ligands, DNAM-1 is involved in cytolytic activity against a broad variety of primary tumor cells, among which neuroblastoma cells, colon carcinoma cells, ovarian carcinoma cells and hematopoietic malignancies (271-274). In line with this, low expression of CD112 by human tumors is usually associated with a reduced tumor clearance (275, 276). As several viruses, including HIV (human immunodeficiency virus), HCMV (human cytomegalovirus), HCV (hepatitis C virus) and EBV (Epstein-Barr virus), have been reported to trigger a cellular response that stimulates DNAM-1 ligand expression on the infected cell surface, DNAM-1 could also be involved in the recognition of virus-infected cells (277-281). Although the underlying mechanism is not entirely clear, for some viruses, including HIV, DNAM-1 ligand upregulation likely occurs via activation of the cellular DNA damage response (DDR) (277, 279). The DDR is a stress-induced pathway that detects DNA damage, maintains genome integrity and prevents mutated

DNA duplication (282). The DDR pathway upregulates various stress molecules, among which CD112 and CD155 (277). Upregulation of DNAM-1 ligands appears to represent a serious threat to viruses, since different viruses, including HIV and HCMV, have evolved mechanisms to ultimately reduce their cell surface availability of these DNAM-1 ligands (268, 281, 283, 284).

Besides DNAM-1, other 'paired' NK cell receptors can also bind to CD112 or CD155. Receptors that compete for the same ligands are referred to as 'paired' receptors. For instance, CD96 (also referred to as T cell-activated increased late expression or tactile) and TIGIT (T cell immunoreceptor with Ig and ITIM domains) have CD155 as a common ligand. In addition, TIGIT also binds CD112. TIGIT is an inhibitory NK cell receptor, which contributes to the inhibition and control of the NK response (285). CD96, on the other hand, mediates binding with the target cell and triggers a low level NK cell activation, compared to other activating NK cell receptors (286). In contrast to this finding, a study using CD96 knock-out mice revealed that CD96, instead of having an activating function, rather suppressed NK cell function independently of DNAM-1 (287).

iv.   Natural cytotoxicity receptors

The natural cytotoxicity receptors (NCRs) are activating NK cell receptors and members of the immunoglobulin superfamily. This group of receptors consists of three important members, referred to according to their mass (kDa): NKp30, NKp44 and NKp46. Both NKp30 and NKp46 are associated with the adaptor protein CD3ζ which contains 3 ITAM in its cytoplasmic domain, while NKp44 is associated with the adaptor protein DAP12 which contains 1 ITAM in its cytoplasmic domain. The NCRs are mainly expressed by NK cells (288). NKp44 is not expressed on resting human NK cells, but is found on activated NK cells (289, 290). Engagement of only one of the NCRs in NK cells is sufficient to initiate signaling of the other NCRs as well. This feature was shown to be specific and, currently, unique for the NCR receptors (291). Remarkably, mouse NK cells only express one functional NCR, homologous to NKp46 and referred to as NCR1 (292). In swine, an NKp46 homologue was recently characterized and serve as an activating NK receptor. Surprisingly, about half of the porcine blood NK cells do not express detectable NKp46 protein levels, while in humans, NKp46 is considered a general NK cell marker (209). Besides a NKp46 homologue, expression of NKp30 and NKp44 homologues has also been reported in porcine NK cells (142). The functionality of these NCR homologues in porcine NK cells still needs to be studied. As shown in Table 4, all NCRs appear to have ligands expressed on tumor cells or virus infected cells, although the

identity of these ligands remains largely elusive (293). Besides particular peptide sequences, the NCR recognize different microdomains on heparan sulphate moieties with different affinities (294).

Table 4: overview of NCR ligands (Table adapted from: (293)).

NCR	Ligand	Signal	References
NKp30	HA of the ectromelia and vaccinia virus	Inhibition	(295)
	Released pp65 of the human cytomegalovirus	Inhibition	(296)
	PfEMP1 of *Plasmodium falciparum*	Activation	(297)
	Heparin and heparan sulphates	Activation	(294) (298) (299)
	BAT3 on tumour cells, stressed cells and DC	Activation	(300)
	B7-H6 on tumour cells	Activation	(301) (302)
NKp44	HA and HN of the influenza virus, Sendai virus and Newcastle disease virus	Activation	(303) (304) (305)
	Envelope glycoprotein of the Dengue and West Nile viruses	Activation	(306)
	Unknown ligand of Mycobacterium tuberculosis, *M. bovis, Nocardia farcinica* and *Pseudomonoas aeruginosa*	Unknown	(220) (307)
	Heparin and heparan sulphates	Activation	(294)
	PCNA expressed on tumour cells	Inhibition	(308) (309)
	NKp44L (i.e. MLL5 isoform) expressed on tumour cells and bystander CD4+ cell during HIV infection	Activation	(310) (311) (312) (313)
NKp46	HA and HN of the influenza virus, Sendai virus, Newcastle disease virus, ectromelia virus and vaccine virus	Activation	(314) (303) (305) (295)
	Vimentin expressed on *M. tuberculosis*-infected cells	Activation	(315)
	Unknown ligand of *Fusobacterium nucleatum*	Activation	(316)
	PfEMP1 of *Plasmodium falciparum*	Activation	(297)
	Heparin and heparan sulphates	Activation	(294) (299)
	Unknown ligand on pancreatic β Langerhans cells	Activation	(317) (318)

Abbreviations table 4: BAT3, HLA-B-associated transcript 3; HA, hemagglutinins; HN, hemagglutinin neuraminidase; PCNA, proliferating cell nuclear antigen; PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein-1.

Remarkably, the interaction of NCRs with viral hemagglutinin (HA) in the NK cell mediated immune response against influenza virus is well conserved in human and swine (319, 320). A NCR1 knock-out mouse model showed that this receptor is important *in vivo* to control influenza infection (321, 322). This interaction appears important enough that influenza viruses

evolved a strategy to evade NCR-mediated recognition by NK cells. Influenza uses its neuraminidase protein to remove sialic acid structures necessary for recognition of infected cells by NKp44 and NKp46 (323).

v.   Paired immunoglobulin-like type 2 receptors

The paired immunoglobulin-like type 2 receptor (PILR) family consists of two members, PILRα and PILRβ (324). PILRα is an inhibitory receptor with two ITIMs in its cytoplasmic domain, which recruit SHP-1 and SHP-2 to deliver an inhibitory signal. Quite the contrary, PILRβ has a short cytoplasmic domain and is associated with the ITAM-bearing adaptor protein DAP12 for signal transduction (325). Both receptors are widely expressed among immune cells, and particularly on monocytes, DCs and macrophages. Interestingly, human NK cells only express the activating PILRβ receptor (326, 327). Mouse NK cells also express the activating PILRβ receptor, but whether PILRα is also expressed remains somewhat elusive (324).

PILRs are referred to as paired receptors, as they share CD99 as a common ligand (324). CD99 is expressed on lymphocytes, endothelial cells, fibroblasts and epithelial cells (328). In Ewing's sarcoma tumors and granulosa cell tumors, increased CD99 expression has been reported (329). PILRβ on NK cells can therefore be involved in contributing to an anti-tumor response. The latter still needs to be investigated, as *in vivo* studies are currently lacking. Next to CD99, two more ligands have been identified for the inhibitory receptor PILRα, PILR-associated neural protein (PANP) and HSV-1 gB (330, 331). The PANP protein is mainly expressed in neural tissue (330), while HSV-1 gB is, as mentioned above, a viral envelope protein expressed on the mature virion as well as on the cell surface of infected cells (332).

O-glycan structures on the PILR ligands have been reported to be essential for their recognition by PILRs (333). A recent elaborate study has provided more mechanistic insight in the recognition of different ligands by the PILR family (334). This study indicated that the PILR receptors show homology to the siglec family, which are sialic-acid binding immunoglobulin-type lectins. Although the extracellular domain of PILRα and PILRβ are very similar, PILRβ lacks a critical amino acid (W108) required for the interaction with sialic acids on PILR ligands and therefore does not bind these ligands (334, 335). Both activating and inhibitory mouse homologues of PILR have been identified and have been found to interact with their corresponding homologous ligands (324, 331).

vi. NKG2 receptors family

The human NKG2 family of C-type lectin type II transmembrane molecules consists of 7 members, NKG2A, B, C, D, E, F, and H, and contains both activating and inhibitory NK cell receptors. As indicated in Table 5, these NK cell receptors can either associate with ITAM-bearing DAP12 or contain an ITIM in their cytoplasmic domain, depending on whether they are activating or inhibitory receptors. Five members have been found to dimerize with CD94, a lectin that virtually lacks a cytoplasmic domain. NKG2A and NKG2B form obligate disulfide-bonded heterodimers with CD94 for their stable cell surface expression (336, 337). In mice, five NKG2 family members have been found, NKG2A-E, with similar characteristics as the human homologues (338, 339). A homologue of CD94 was also identified in mice (340). In swine, NKG2A and NKG2D mRNA was found to be expressed in NK cells (142).

The human CD94-associating NKG2 family members have been found to bind the non-classical MHC I molecule HLA-E (341, 342). Although HLA-E typically is expressed at lower cell surface levels compared to other MHC I molecules, it was shown to be involved in the NK cell-mediated antiviral response, and, as it is upregulated in various cancers, also in the anti-tumor response (343, 344).

Table 5: overview of human NKG2 receptor family with related functionality.

Name	Adaptormolecules and signaling motifs	Functionality	Reference
NKG2A	-associates with CD94 -contains ITIM in cytoplasmatic domain	inhibitory	(345) (346)
NKG2B	-associates with CD94 -contains ITIM in cytoplasmatic domain	inhibitory	(341)
NKG2C	-associates with CD94 and DAP12	activating	(347) (345)
NKG2D	-associates with DAP10 -NKG2D has splice variant lacking the ligand-binding domain, competing for DAP10	activating	(348) (349)
NKG2E	-associates with CD94 and DAP12 -reported to form an intracytoplasmatic complex	activating	(350)
NKG2F	-associates with DAP12 -reported to locate in intracellular compartments	unknown	(351) (352)
NKG2H	-associates with CD94 and DAP12	activating	(353)

The activating NK cell receptor NKG2D interacts with MHC I polypeptide-related chains (MIC) MICA and MICB (354, 355). These NKG2D ligands are structurally similar to MHC I

and are expressed at low levels in many normal cells. Their expression is increased on stressed cells by infection or by malignant transformation. As is the case for DNAM-1 ligands, MICA/B have been reported to be upregulated by the DDR pathway during stress responses, including viral infection (277). Besides MICA/B, NGK2D also recognizes the ULBP1-6 proteins, which are structurally similar to MICB (356). As these proteins are also found to be upregulated in stressed and malignant cells, NKG2D appears to be mainly involved in recognizing cells stressed by various conditions (357-359). In general, the NKG2 family potentiates the NK cells to sense and react to infected, stressed or malignant cells (360).

3. Alphaherpesvirus interactions with Natural Killer cells
   a. Introduction

NK cells are crucial to fend off an alphaherpesvirus infection. In adults, alphaherpesviruses usually cause only relatively minor disease symptoms, such as herpetic lesions. Several studies have shown a correlation between severe alphaherpesvirus symptoms, particularly HSV-1 and VZV related encephalitis, and impaired NK cell function in these patients (361-363). In support of this, mouse models have confirmed that NK cells are crucial to protect against lethal HSV-1 infection (364). While being crucial to protect a host from severe alphaherpesvirus disease, the activity of NK cells may also limit the efficacy of alphaherpesvirus-based therapeutic vectors. Indeed, mouse models have shown that genetically modified HSV-1 vectors that are designed to specifically replicate in and kill tumor cells, particularly glioblastoma cancers, are prematurely cleared by NK cells, preventing effective spread of the therapeutic virus in the tumor (365). Despite the importance of the interaction between NK cells and alphaherpesviruses in virus pathogenesis and potential therapeutic applications, there is only very little information available on the mechanisms that NK cells use to recognize alphaherpesvirus-infected cells and on potential immune evasion strategies that alphaherpesviruses have developed against NK cells.

   b. Alphaherpesvirus-mediated downregulation of MHC I molecules

Cytotoxic T lymphocytes (CTL) recognize virus-infected and tumor cells via antigens presented by MHC I (366). MHC I loading with antigens is controlled by the transport of proteasome-generated peptides from the cytosol to the ER lumen, which is regulated by the antigen processing (TAP) complex that consists of two membrane-spanning subunits TAP1 and TAP2 (367). MHC I-mediated viral antigen presentation to CTL and consequent CTL-mediated lysis of virus-infected cells obviously represents a serious threat to viruses. Hence, several viruses

have evolved mechanisms to interfere with antigen presentation in infected cells, which often results in downregulation of MHC I cell surface expression. An impressive diversity of alphaherpesviruses have been reported to display several distinct and conserved mechanisms that suppress MHC class I molecule levels on the surface of infected cells, including HSV-1, HSV-2, VZV, PRV, BoHV-1, MDV, EHV-1 and EHV-4 (100, 368-372).

The ICP47 protein of both HSV-1 and HSV-2, which is expressed early in infection, binds TAP, preventing transport of peptides to the ER lumen (368, 369, 373). As a result, ICP47 inhibits peptide loading on MHC I molecules, and thereby also blocks MHC I molecule transport to the cell surface, resulting in gradual downregulation of MHC I cell surface levels (241). Other alphaherpesviruses do not encode an ICP47 homolog. Several members of the varicella genus of alphaherpesviruses encode a conserved protein that also reduces cell surface expression of MHC class I molecules, the UL49.5 protein (372). UL49.5 prevents the conformational change of TAP, necessary to translocate peptides into the ER lumen. The latter function of UL49.5 appears to be conserved throughout the varicella genus, with the exception of VZV. VZV UL49.5 does bind TAP, but does not prevent loading of MHC I molecules. In addition to binding TAP, BoHV-1 UL49.5 also induces degradation of TAP1 and TAP2 and EHV-1 and EHV-4 UL49.5 prevent ATP binding to TAP further inhibiting translocation of peptides into the ER lumen (370, 374, 375). Besides UL49.5 and ICP47, the US3 protein of some alphaherpesviruses, including HSV-1, PRV and VZV, has been reported to affect MHC I cell surface expression by retaining or delaying the transport of the loaded MHC I molecule from the ER to the Golgi apparatus. For some viruses, particularly PRV, this US3-mediated effect however appears to be highly cell type dependent (97, 98, 100, 102, 376).

The reduced cell surface expression of MHC I provides a protection against cytotoxic T cells, but the effect of this MHC class I reduction on NK cells remains somewhat unclear (102). MHC I molecules are ligands for inhibitory NK cell receptors, KIR and NKG2A/CD94, and thus alphaherpesvirus-mediated reduction of these ligands likely increases the cytolytic activity of NK cells towards these infected cells (341, 377). Interestingly, HSV-1 US3 was found to reduce MHC-I cell surface levels and, accordingly, to contribute in the NK cell-mediated IFNγ response raised against HSV-1-infected mouse embryonic fibroblasts (MEFs). This activating effect of HSV-1 US3 on NK cells was not displayed in HSV-1-infected MEFs lacking MHC-I (102). This is further in line with another study, reporting HSV-1 US3 to have no NK modulatory properties in HSV-1-infected lymphoblasts, a cell line in which HSV-1 US3 does not mediate MHC-I downregulation (101). However, the specific NK cell receptors involved in

this US3-dependent NK cell activation remain to be investigated and *in vivo* studies are required. Future studies to investigate the consequences of alphaherpesvirus-mediated reduction of MHC-I molecules on the infected cell surface towards NK cell responses appear warranted.

c. Effect of alphaherpesviruses on recognition by activating NK cell receptors

Only a handful of studies have reported on the effect of alphaherpesvirus infection on the recognition of host cells by NK cell receptors, and these are limited to HSV-1 thus far (305, 378). For HSV-1 and HSV-2, virus particles are recognized by NK cells through TLR2 (379). In this study, UV-inactivated HSV-1/2 was found to bind the TLR2 receptor and directly activate NK cells, as determined by the early activation marker CD69. Interestingly, this TLR2 activation by HSV-1/2 virions was found to induce increased expression of HLA-DR and HLA-DQ on blood NK cells, increasing the potential to interact with CD4$^+$ T cells (379). The HSV-1 glycoproteins gB and gH/gL have been described to bind TLR2, but their potential role in NK cell activation was not investigated in the previous study(54).

Human foreskin fibroblasts (HFF) infected with HSV-1 rapidly express increased cell surface ligands for the activating NCR receptors NKp30, NKp44 and NKp46 (305). Upregulated expression of NKp30 and NKp46 ligands has been confirmed in glioblastoma cells infected with an HSV-1-derived oncolytic viral vector (365). These authors demonstrated that NCR-dependent NK cell cytotoxicity is responsible for premature NK cell-mediated clearance of these viral vectors in mouse models of glioblastoma (365). Increased cell surface expression of NCR ligands in HFF partly depends on expression of the viral immediate early protein ICP0 (305). This protein is an E3 ubiquitin ligase and is required for an efficient lytic viral replication cycle. Furthermore, it regulates the switch between lytic and latent states of HSV-1 infection, directs proteasomal degradation of several cellular ligands and has been shown to influence gene expression (380). Expression of this protein alone is sufficient to trigger an NCR-mediated cytolytic NK cell response, but deletion of the protein from the virus does not completely abrogate the NCR-mediated cytolytic NK response towards infected cells (305). ICP0 homologues exist in other members of the alphaherpesvirus family, but sequence similarity between these proteins is very limited except for the RING finger domain near the N-terminus. This RING finger domain is essential for most of its functions (25, 380, 381). The putative involvement of ICP0 homologues, their RING finger domain in particular, and NCR towards NK cell-mediated killing of cells infected with other alphaherpesviruses has yet to be unraveled.

A recent study in HeLa cells investigated the modulation of ligands for the activating NK cell receptor NKG2D by an HSV-1 and VZV infection. Both HSV-1 and VZV were found to modulate expression of NKG2D ligands MICA, ULBP2 and ULBP3 expression on infected cells, whilst HSV-1 additionally modulated NKG2D ligand ULBP1. VZV infection upregulated MICA while reducing ULBP2 and ULBP3 expression on the surface of infected cells. Despite being closely related to VZV, infection with HSV-1 resulted in a remarkably different effect on NKG2D ligand expression. This study found an HSV-1 infection to reduce MICA, ULBP1, ULBP2 and ULBP3 cell surface levels (382). The reduction of MICA by HSV-1 was previously described and was contributed to a yet unidentified late viral protein (378). The underlying viral mechanism of both HSV-1 and VZV to modulate NGK2D ligands and whether this affects NK cell recognition of HSV-1/VZV-infected cells remains to be investigated. However in HFF, mock-infected and HSV-1-infected cells did not display differences in NKG2D-dependent NK cell-mediated killing (305).

d. Memory NK cells

Until recently, immunological memory was considered to be exclusively present in adaptive immune cells, the T and B lymphocytes. However, increasing evidence indicates that NK cells can also display memory features against pathogens (viruses) and haptens (383). With regard to viruses, NK cell memory has been particularly well documented for the mouse betaherpesvirus murine cytomegalovirus (MCMV) (384), but also for other viruses including influenza virus, HIV, vesicular stomatitis virus (VSV), HCMV, Hantavirus and Chikungunya virus (385-389). Interestingly, a recent study in mice indicates that memory-like NK cells may also be triggered upon infection with HSV-2, an alphaherpesvirus (390). NK cells of mice that were exposed to HSV-2 30 days earlier showed increased IFNγ production upon contact with HSV-2 antigens, compared to NK cells from naïve mice. More importantly, this increased production of IFNγ in NK cells was independent of B- and T- lymphocytes, was specific for the HSV-2 antigens and provided protection against HSV-2 mediated morbidity and mortality upon re-exposure (390). Collectively, these data suggest that NK cells may remember to some extent a prior encounter with HSV-2 independent of B- and T- lymphocytes. Although the mechanisms driving memory-like features in NK cells are still poorly understood, and more research is needed to estimate the magnitude and importance of NK cell memory and to investigate its potential conservation in other animal species, this research domain may hold promise for possible future vaccination strategies.

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Chapter 2: Aims

Herpesviruses have developed complex and often subtly fine-tuned interactions with the immune system of their host. As a result, these viruses typically cause relatively mild, although economically important and socially discomforting disease symptoms and are able to persist lifelong in a latent state in their host.

For alphaherpesviruses in particular, in humans, the presence of functional NK cells is crucial in suppressing these viruses from causing substantially more severe symptoms such as encephalitis (1-3). Herpes simplex virus (HSV)-based oncotherapy is currently also limited by premature clearance of the virus by NK cells (4). This indicates that NK cells are able to recognize and kill alphaherpesvirus-infected cells. Indeed, previous studies have shown that cells infected with human HSV-1 or swine PRV activate NK cell-mediated cytotoxicity, although the underlying mechanisms are unclear (5-9). On the other hand, seen the importance of NK cells in alphaherpesvirus biology, these viruses must be under substantial evolutionary pressure to develop mechanisms to suppress NK cell activity. Remarkably, however, only one such alphaherpesvirus NK cell evasion mechanism had been described to date (6), whereas a multitude of such evasion mechanisms have been described for beta- and gammaherpesviruses (10-22).

The general aim of the current thesis was to unravel novel aspects of the interaction between NK cells and alphaherpesviruses, with the purpose of identifying viral proteins that activate or suppress NK cell activity. We particularly focused on the PRV virus which is commonly used to assess general aspects of alphaherpesvirus biology, including interactions with the host immune system (23). Using PRV virus mutants lacking certain viral genes, we identified three viral proteins that modulate NK cell activity and investigated the NK cell receptors and ligands involved:

1) CD112 (nectin-2) is a known ligand for the activating NK cell receptor DNAM-1 and also functions as a receptor for the gD glycoprotein of alphaherpesviruses like PRV and HSV-2 (24-26). In Chapter 3, we investigated the effect of PRV and/or HSV-2 gD on (DNAM-1-dependent) NK cell cytotoxicity, cell surface expression of CD112 and binding of recombinant DNAM-1.

2) In Chapter 4, we investigated the effect of the PRV US3 viral protein kinase on NK cell cytotoxicity, and whether such effects may be attributed to alterations in binding of the inhibitory NK cell receptor CD300a and cell surface exposure of its ligands, such as phosphatidylserine.

3) For both HSV-1 and PRV, early studies indicated that expression of viral glycoproteins gB and/or gC directly or indirectly triggers cytotoxicity of NK cells or NK-like cells (7-9). In Chapter 5, we investigated whether PRV gB expression leads to increased susceptibility of cells to NK cell-mediated cytotoxicity and whether known gB receptors, like PILR (paired immunoglobulin-like receptor) family members (27), may be involved in such an effect.

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Chapter 3: Modulation of CD112 by the alphaherpesvirus gD protein suppresses DNAM-1-dependent NK cell-mediated lysis of infected cells

ABSTRACT

Natural killer (NK) cells are key players in the innate response to viruses, including herpesviruses. In particular, the variety of viral strategies to modulate the recognition of certain herpesviruses witnesses the importance of NK cells in the control of this group of viruses. Still, NK evasion strategies have remained largely elusive for the largest herpesvirus subfamily, the alphaherpesviruses. Here, we report that gD glycoprotein of the alphaherpesviruses pseudorabies virus (PRV) and herpes simplex virus 2 (HSV-2) displays previously uncharacterized immune evasion properties towards NK cells. Expression of gD during infection or transfection led to degradation and consequent down-regulation of CD112, a ligand for the activating NK receptor DNAX accessory molecule-1 (DNAM-1) to bind to the surface of both virus-infected and gD-transfected cells. Consequently, expression of gD suppressed NK cell degranulation and NK-cell mediated lysis of PRV- or HSV-2-infected cells. These data identify an alphaherpesvirus evasion strategy from NK cells and point out that interactions between viral envelope proteins and host cell receptors have biological consequences that stretch beyond virus entry.

INTRODUCTION

Alphaherpesviruses constitute the largest subfamily of the herpesviruses, comprising closely related and important pathogens like herpes simplex virus (HSV) in man, pseudorabies virus (PRV) in pigs, and bovine herpesvirus 1 (BHV1) in cattle. Natural killer (NK) cells play a central role in the defense against viral infections and cancer development. Functional NK cells are of particular importance in preventing herpesviruses from causing aggravated disease, including life-threatening encephalitis for alphaherpesviruses like HSV and varicella-zoster virus (1-3). The significance of the NK cell response against herpesviruses is also reflected by the various mechanisms that these pathogens have evolved to evade or delay it (4). Indeed, for beta- and gammaherpesviruses, a variety of molecular mechanisms avoiding the NK-mediated anti-viral activity have been defined (4-16). Remarkably and paradoxically, such mechanisms have remained largely elusive for the alphaherpesviruses (17).

Identifying and understanding these mechanisms is of particular relevance for alphaherpesviruses also in view of the potential therapeutic applications of HSV. Indeed, a limiting factor in HSV vector-based oncotherapy is the premature clearance of the viral vector by NK cells (18). NK cell activity is regulated by an array of germline-encoded activating and inhibitory surface receptors capable of transducing signals upon engagement by their respective ligands (19, 20). The sum of these signals determines the outcome of NK cell effector responses including cytotoxicity against NK-sensitive targets (20). A variety of NK activating receptors are involved in recognition of virus-infected cells (18, 21-26). One of the important NK activating receptors is DNAX accessory molecule-1 (DNAM-1), which binds to CD112 (Nectin2) and CD155 (poliovirus receptor, PVR), whose expression can be induced in both virus infected and tumor cells (5, 26-28). Interestingly, the viral gD envelope glycoproteins of certain human and animal alphaherpesviruses, including HSV-2, PRV and BHV-1, interact with CD112 and/or CD155 to facilitate viral entry (29, 30). HSV-1 gD does not display substantial affinity for CD112, except for particular HSV-1 isolates retrieved from patients with encephalitis (31).

In the current study, we demonstrate that the significance of these virus ligand-cellular receptor interactions can stretch beyond virus entry and can influence immune recognition. We report that expression of gD of PRV and HSV-2 reduces DNAM-1-mediated cell lysis by NK cells through suppression of CD112 levels in infected and transfected cells. The gD/CD112/DNAM-

1 interplay identified here may have consequences for the development of medical applications ranging from vaccination to oncolytic virotherapy.

RESULTS

1. Expression of PRV gD reduces NK cell-mediated killing

To investigate a potential effect of alphaherpesvirus gD expression on NK-mediated lysis, the porcine PRV was used. PRV is commonly used as a model pathogen to study general aspects of alphaherpesvirus biology (32). In addition, gD of PRV has the unique capacity to bind both CD112 and CD155 (29, 30). To investigate whether expression of PRV gD affects the susceptibility of cells to NK-mediated lysis, 293T cells were infected with wild type (wt) PRV or an isogenic gDnull virus, co-incubated with IL2-primed human NK cells and subsequently assessed for viability by flow cytometry. Figure 1A shows that cells infected with wt PRV display lower susceptibility to NK cell-mediated lysis as compared to cells infected with gDnull PRV. This difference was not due to possible differences in virus replication efficiency or MHC class I cell surface levels, as expression levels of other viral proteins (e.g. gB and gC) and MHC class I were similar for both viruses (Suppl. Figures 1A & 2A).
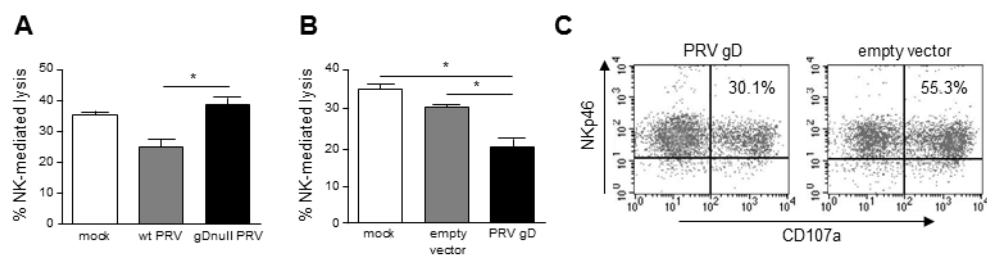

Figure 1. Expression of PRV gD during infection or transfection reduces NK cell degranulation and NK-mediated cell lysis. (A-B) 293T cells were mock-infected or infected with wt or gDnull PRV for 6h (A) or mock-transfected or transfected with PRV gD or empty vector for 48h (B) and subsequently incubated with IL2-primed NK cells for 4h at a target:effector ratio of 1:1. Viability of target cells was assessed by propidium iodide and flow cytometry. Data represent mean + SEM of three independent repeats (* p<0.05). (C) Cell surface expression of CD107a on NK cells incubated for 4h with 293T cells that were mock-transfected or transfected for 48h with PRV gD or empty vector. Histograms show CD107a cell surface signal, with marker to discriminate positive cells. Bar graphs represent mean + SD of three independent repeats (** p<0.01).

To confirm the inhibitory effect of gD expression on NK-mediated cell lysis, 293T cells were transfected with a gD-encoding vector or an empty vector and assayed for NK cell-mediated lysis. Again, expression of gD resulted in reduced susceptibility of cells to NK cell-mediated lysis (Figure 1B). We next investigated whether this diminished susceptibility to NK cell-mediated cell lysis is reflected by a decreased ability of target cells to trigger NK cell degranulation. NK cells were co-incubated with transfected 293T cells and then analysed for surface expression of CD107a (i.e. a marker of degranulation) by flow cytometry (33), which showed that gD expression resulted in substantially

reduced degranulation of NK cells (Figure 1C). In conclusion, the above data indicate that gD has the previously uncharacterized ability to interfere with NK-mediated cytotoxicity.

2. Expression of PRV gD reduces cell surface levels of CD112 and leads to reduced binding of DNAM-1 and reduced DNAM-1-dependent NK-mediated cell lysis

Given the ability of PRV gD to bind CD112 and CD155 (29, 30), we investigated whether expression of PRV gD compromises normal cell surface levels of CD112 and CD155 and, consequently, binding of the activating NK receptor DNAM-1. Figure 2A shows that cell surface levels of CD112 are significantly reduced in cells infected with wt PRV but not with gDnull PRV. On the other hand, CD155 cell surface levels were not significantly reduced. To determine whether reduced cell surface levels of CD112 affects binding of DNAM-1 to the cell surface, binding of recombinant DNAM-1Fc was assessed by flow cytometry and was found to be significantly reduced on wt PRV-infected cells as compared to cells infected with gDnull PRV (Figure 2A). We also consistently noticed a reproducible, yet statistically non-significant, trend of increased DNAM-1Fc binding in cells infected with gDnull PRV as compared to mock-infected cells (Figure 2A). As shown in Figure 2A, left panel, gD protein was not completely absent from the surface of gDnull PRV-infected cells. Since the gD envelope protein is essential for virus entry, gDnull virus stocks were grown on gD-expressing cells to allow incorporation of gD in the viral envelope and render progeny virions infectious. Hence, these viruses do not carry the gD gene in their genome but do carry the gD protein in their envelope. Upon fusion of the viral envelope with the host plasma membrane during virus entry, gDnull PRV leaves the gD envelope proteins in the host cell membrane, thereby yielding the weak gD cell surface signal shown in Figure 2A. In line with this, the gD signal observed in gDnull PRV-infected cells was already observed at 2 hpi (upon virus entry) and did not increase at later time points of infection. This also indicates that comparing wt PRV-infected cells with gDnull PRV-infected cells may underestimate any gD-mediated effects.

To be able to better address the magnitude of the gD-mediated effects, and to confirm our results, transfection assays were performed, which confirmed that expression of gD leads to substantially reduced levels of CD112 on the cell surface and reduced binding of DNAM-1Fc (Figure 2B). In these assays, gD expression also led to significantly reduced cell surface levels of CD155, although the effect on CD155 was substantially less pronounced compared to the effect on CD112 (Figure 2B). To assess whether reduced binding of DNAM-1Fc also results in reduced susceptibility of cells to DNAM-1-dependent NK-mediated cell lysis, NK cytotoxicity assays were performed in the presence or absence of the DNAM-1 blocking antibody F5 and DNAM-1-dependent NK-mediated cell lysis was calculated. As shown in Figure 2C (left and middle panels), cells infected with wt PRV or transfected with PRV gD showed lower susceptibility to DNAM-1-dependent NK-mediated cell lysis as compared to cells infected with gDnull PRV or transfected with empty vector, respectively. In line with this, DNAM-1-

dependent degranulation of NK cells was reduced when co-incubated with PRV gD-transfected cells as compared to cells transfected with empty vector (Figure 2C, right panel).

In conclusion, expression of PRV gD leads to substantially reduced cell surface expression of CD112, reduced binding of DNAM-1Fc and reduced susceptibility of the cells to DNAM-1-dependent NK cell degranulation and NK-mediated cell lysis.
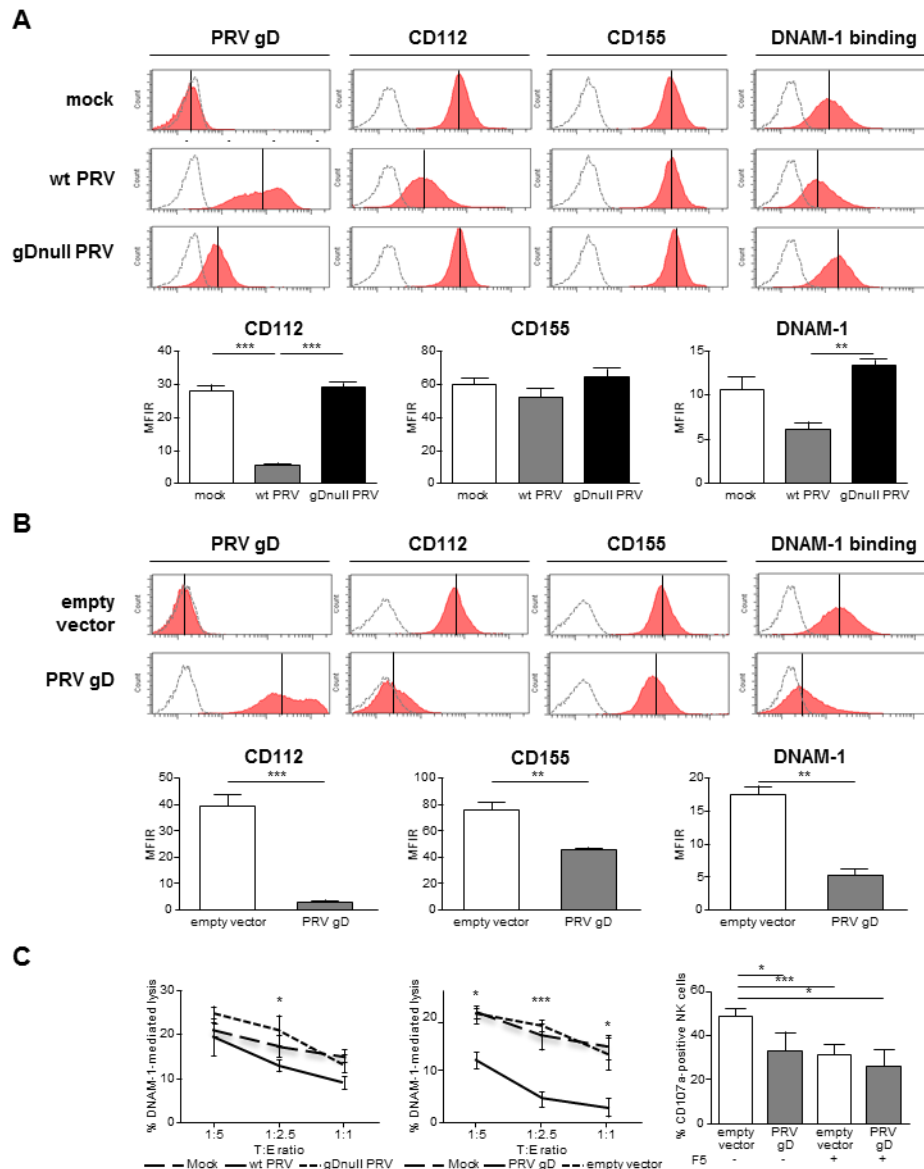
Figure 2. Expression of PRV gD reduces cell surface availability of CD112, DNAM-1Fc binding and DNAM-1-dependent NK degranulation and cell lysis. (A-B) 293T cells were infected with wt or gDnull PRV for 6h (A) or transfected with PRV gD or empty vector for 48h (B) and subsequently assessed by flow cytometry for cell surface expression of gD, CD112 and CD155 or for binding of recombinant DNAM-1Fc. Histograms show relative fluorescence intensities, with vertical lines indicating median fluorescence intensity. Dotted line histograms represent isotype matched antibody control. Bar graphs represent mean fluorescence intensity ratios (MFIR). Data represent mean + SEM of three independent repeats (** $p < 0.01$, *** $p < 0.001$). (C) Cells infected or transfected as in (A) and (B) were incubated for 4h with IL2-primed NK cells at the indicated target:effector (T:E) ratios, in the absence or in the presence of the DNAM-1-blocking antibody F5. Viability of target cells was assessed by propidium iodide and flow cytometry, and percentage of DNAM-1-dependent NK-mediated killing was calculated.

3. Expression of PRV gD leads to degradation of CD112

To further assess the effect of PRV gD expression on CD112, total cell lysates were analyzed for CD112 protein levels by Western blotting. Blots were probed with antibodies specific for CD112, CD155 and gD as well as for gB and gC (infection controls) and tubulin (loading control). As shown in Figure 3A, wt PRV-infected cells showed substantially reduced CD112 protein levels as compared to mock-infected cells or cells infected with gDnull PRV, while CD155 protein levels were only marginally reduced. To determine whether CD112 degradation occurs via an acidification-dependent pathway, experiments were repeated in the presence of the acidification inhibitor BFLA-1 (Figure 3B). This restored CD112 levels in wt PRV-infected cells to levels of mock-infected and gDnull PRV-infected cells. Furthermore, experiments performed in the presence of the proteasome inhibitor MG132 also, albeit partially, restored CD112 levels of wt PRV-infected cells (Figure 3C). Degradation of CD112 was confirmed in gD transfection assays (Figure 3D). These results indicate that expression of PRV gD leads to an acidification-dependent and partly proteasome-mediated degradation of CD112.
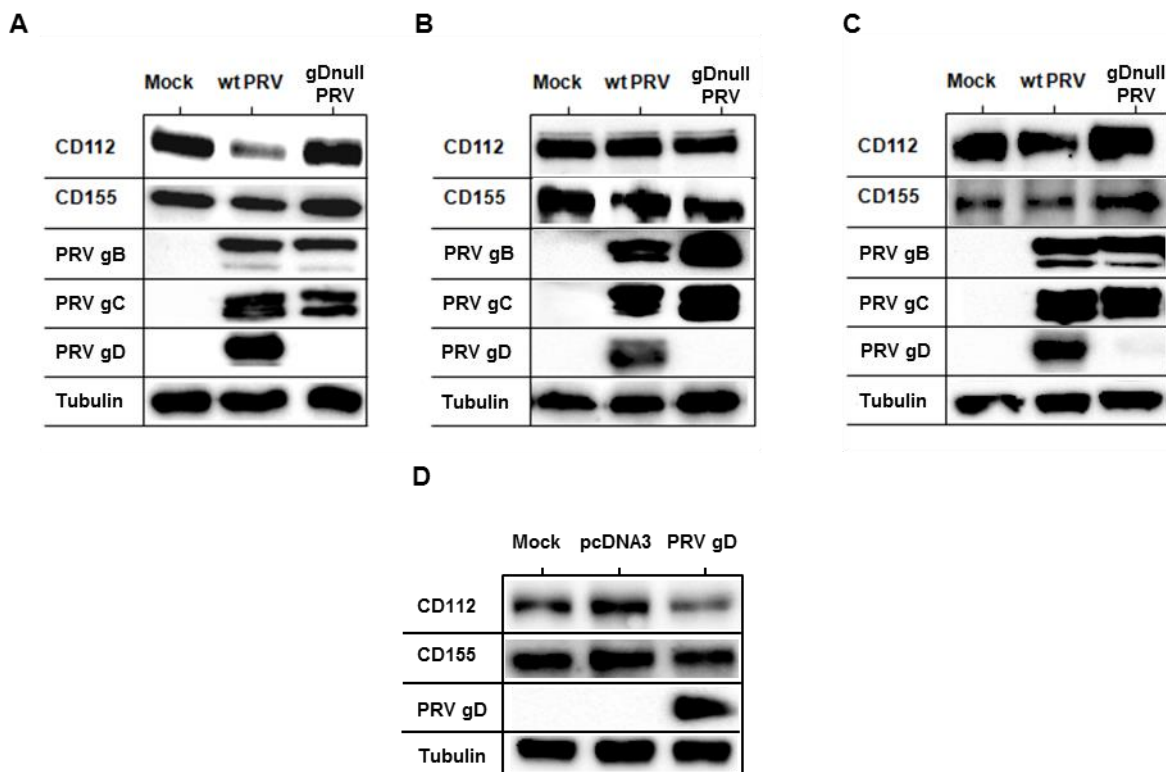
Figure 3. Expression of PRV gD leads to CD112 degradation that can be reversed using the acidification inhibitor BFLA-1 and proteasome inhibitor MG132. 293T cells were mock-infected or infected with wt or gDnull PRV for 6h in the absence (A) or in the presence (B) of the acidification inhibitor BFLA-1 (bafilomycin A1) or (C) the proteasome inhibitor MG132 or (D) transfected with PRV gD or control plasmid and assessed by Western blotting for the expression of CD112, CD155, gB, gC, gD and tubulin.

4. Expression of PRV gD leads to degradation of CD112 in porcine cells and reduced cell lysis by porcine NK cells

The natural host of PRV is the pig. To better assess the potential biological significance of our findings, we investigated whether the gD-mediated effects that we observed also occur in porcine cells. As a first step, we confirmed the expression of CD112, CD155 and DNAM-1 by RT-PCR on mRNA isolated from the porcine SK cell line (CD112 and CD155) or from primary porcine NK cells (DNAM-1) (Figure 4A). All PCR products were confirmed by sequencing (see Suppl. Materials and Methods). We then assessed whether expression of PRV gD in porcine cells reduced CD112 protein levels. Figure 4B shows that SK cells infected with wt PRV virtually lack CD112 protein as compared to mock-infected cells or cells infected with gDnull PRV. CD155 protein levels could not be assessed due to the lack of antibodies crossreacting with porcine CD155. Protein levels of gB and gC served as infection controls and tubulin levels as loading control. These results were confirmed in primary porcine epithelial cells (Figure 4C).
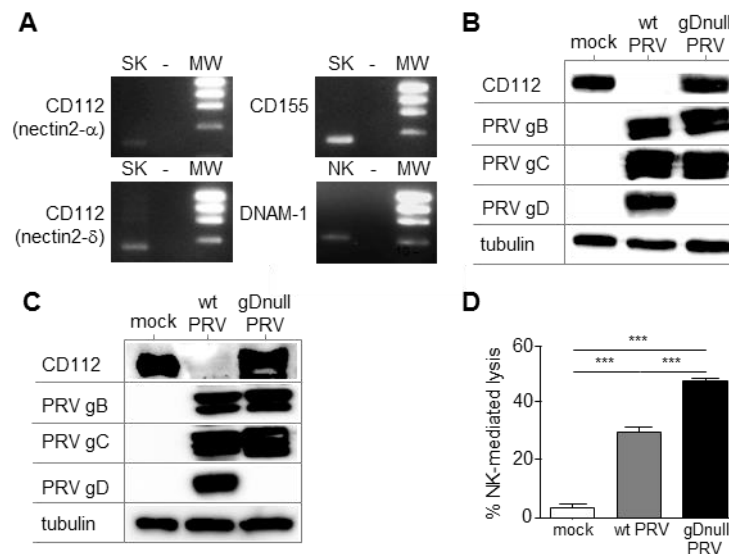
Figure 4. Expression of PRV gD in porcine cells leads to CD112 down-regulation and reduced killing by porcine primary NK cells. (A) mRNA expression, assessed by RT-PCR, of CD112 and CD155 in porcine SK cells and of DNAM-1 in primary porcine NK cells. (B and C) Porcine SK cells (B) or primary porcine epithelial cells (C) were mock infected or infected for 12h with wt or gDnull PRV, were analyzed by Western blotting for expression of CD112, gB, gC, gD and tubulin. (D) SK cells were infected with wt or gDnull PRV for 12h, and subsequently incubated with primary porcine NK cells at a target:effector ratio of 1:25 for 4h. Viability was assessed by flow cytometry using propidium iodide. Data represent mean + SEM of three independent repeats (*** p<0.001).

Finally, we investigated whether expression of PRV gD in porcine cells alters their susceptibility to lysis by primary porcine NK cells. As shown in Figure 4D, SK cells infected with wt PRV showed significantly reduced susceptibility to lysis by primary porcine NK cells as compared to cells infected with gDnull PRV. Hence, also in the porcine system, PRV infection leads to gD-dependent degradation of CD112 and reduced susceptibility to cell lysis by primary porcine NK cells.

5. Expression of HSV-2 gD leads to degradation of CD112 and reduced NK-mediated cell lysis

Although PRV gD can bind both CD112 and CD155 (29, 30), our findings suggest that its protective effect against NK-mediated cell lysis is mainly due to its ability to bind and modulate CD112. As a consequence, our findings on PRV may have a more general relevance, as the gD proteins of other alphaherpesviruses, most notably HSV-2, also interact with CD112 (30). Therefore, we investigated whether HSV-2 gD also affects cell surface levels of CD112, DNAM1-Fc binding and susceptibility of cells to NK-mediated cell lysis. Figure 5A shows that infection of 293T cells with wt HSV-2 resulted in reduced cell surface expression of CD112 and in reduced binding of DNAM-1Fc, as compared to mock-infected cells or cells infected with gDnull HSV-2. These results were also confirmed in the human U87-MG glioblastoma cell line (Figure 6). In line with these results, 293T cells infected with wt HSV-2 showed significantly reduced susceptibility to NK-mediated cell lysis as compared to cells infected with gDnull HSV-2 (Figure 5B). Like for PRV, flow cytometric analysis of viral protein gB and MHC class I confirmed that these parameters were similar for both wt and gDnull HSV-2 infected cells, thus ruling out that changes in viral replication or virus-induced modulation of HLA expression could account for the differences in susceptibility to NK cell lysis (Suppl. Figure 1B). Again, similar to PRV, we consistently noticed a trend of increased DNAM-1 ligand expression and increased DNAM-1Fc binding in cells infected with gDnull HSV-2 as compared to mock-infected cells (Figure 5A & 6B). In conclusion, also in HSV-2 infection, expression of gD results in reduced CD112 levels, reduced DNAM-1Fc binding and reduced susceptibility of cells to NK-mediated cell lysis (Figure 5B).
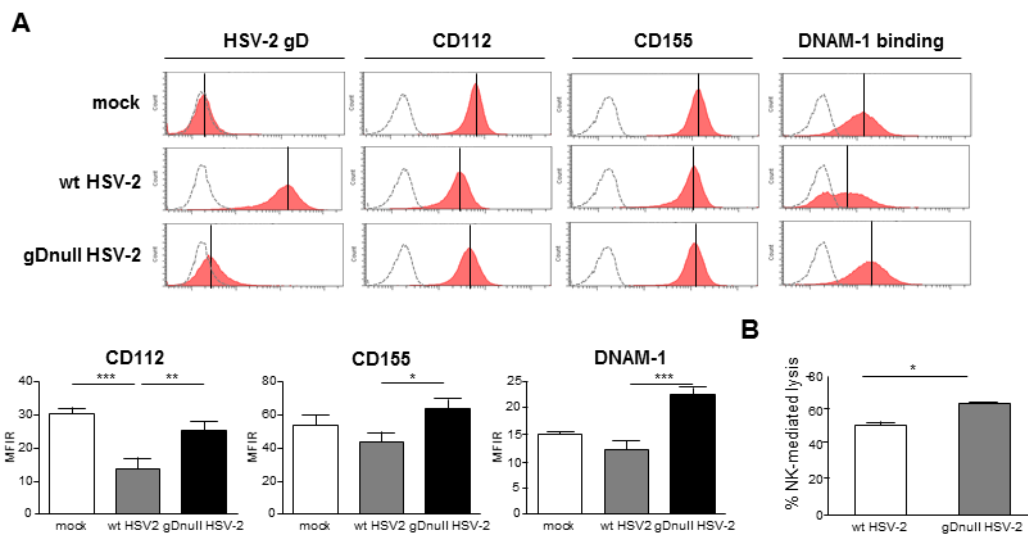
Figure 5. Expression of HSV-2 gD in 293T cells leads to CD112 down-regulation, decreased cell surface binding of DNAM-1Fc and reduced NK-mediated cell lysis. (A) 293T cells were infected with wt or gDnull HSV-2 for 28h and subsequently assessed by flow cytometry for cell surface expression of CD112 and CD155 or for binding of recombinant DNAM-1Fc. Histograms show relative fluorescence intensities, with vertical lines indicating median fluorescence intensity. Dotted line histograms represent isotype matched antibody control. Bar graphs represent mean fluorescence intensity ratios (MFIR). Data represent mean + SEM of three independent repeats (* p<0.05, ** p<0.01, *** p<0.001) (B) 293T cells infected with wt or gDnull HSV-2 were incubated for 4h with IL2-primed NK cells at a target:effector ratio of 1:2.5. Viability of target cells was assessed by propidium iodide staining and flow cytometry, and percentage of NK-mediated killing was calculated. Data represent mean + SEM of three independent repeats (* p<0.05).
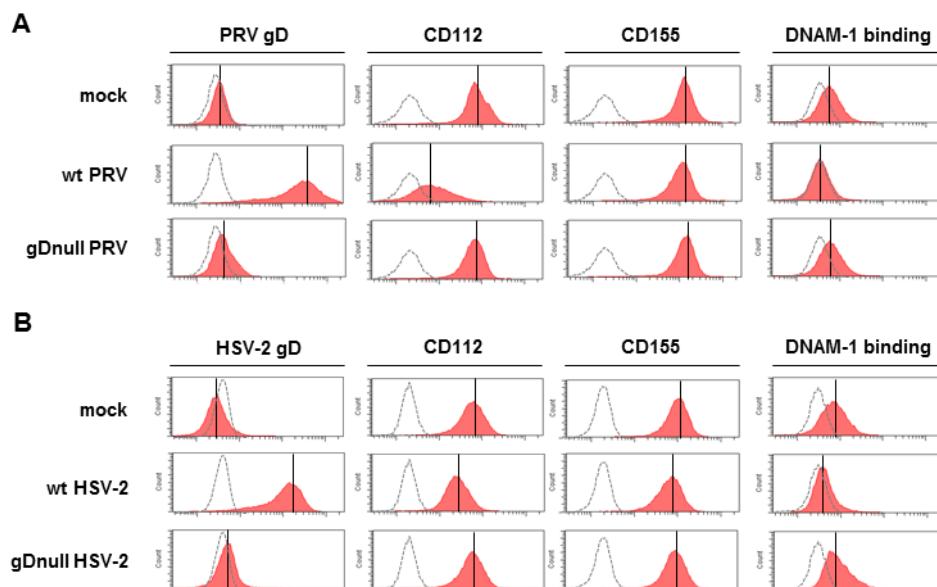
Figure 6. PRV gD and HSV-2 gD-mediated suppression of CD112 cell surface expression and DNAM-1 binding is also observed in U-87 MG glioblastoma cells. U-87 MG cells were either mock-infected or infected with (A) wt PRV or its isogenic gDnull mutant or with (B) wt HSV-2 or its isogenic gDnull mutant and assessed by flow cytometry for cell surface expression of gD, CD112 and CD155 and binding of DNAM-1Fc to the cell surface. Vertical lines indicate median fluorescence intensity. Dotted line histogram represents isotype-matched antibody control signal.

DISCUSSION

In the current report, we describe that the gD glycoprotein of PRV and HSV-2 displays previously uncharacterized immune evasion properties towards NK cells, thereby identifying an NK evasion strategy of alphaherpesviruses. Expression of gD leads to reduced cell surface availability and degradation of its cellular ligand CD112 (Nectin-2), thereby reducing binding of the activating NK cell receptor DNAM-1 and, consequently, decreasing NK-mediated lysis of gD-expressing cells.

Interestingly, the UL141 protein of the betaherpesvirus human cytomegalovirus (HCMV) also targets DNAM-1 ligands to protect infected cells from NK cell-mediated lysis. UL141 sequesters CD155 in the ER, and, similar to what we observe for PRV and HSV-2 gD, also leads to CD112 degradation (8, 34). However, unlike what we observe for gD, transfection of UL141 does not affect CD112, implicating an additional, not yet identified viral protein in this process (34). UL141 of HCMV does not display amino acid sequence similarity to gD of alphaherpesviruses. This points to convergent evolution in the herpesvirus family, with alpha- and betaherpesviruses that have developed evolutionary distinct methods to interfere with DNAM-1 ligand expression. This underscores the recent view that the activating NK receptor DNAM-1 represents a serious threat that different viruses need to circumvent (26). Further in line with this view, our data and data by others indicate that cells may trigger increased cell surface expression of DNAM-1 ligands as a response to virus infection, which is subsequently counteracted by viral proteins like gD of PRV/HSV-2, UL141 of HCMV and Nef/Vpu of HIV. Indeed, mutant viruses that lack these respective viral immune evasion proteins all show higher cell surface expression of DNAM-1 ligands/higher DNAM-1 binding, as compared to mock-infected cells (8, 35) [current study: Figure 2A & 4A]. Also, productive infection of cells with the gammaherpesvirus EBV is associated with upregulation of the DNAM-1 ligand CD112 (25). Although the underlying mechanism of such virus-induced cellular response remains to be investigated, the cellular DNA damage response (DDR) may play a role, as many viruses, including herpesviruses and retroviruses, activate the DDR, and DDR activation has been reported to trigger DNAM-1 ligand up-regulation (26, 36-38).

Our data also underscore the conservation of the DNAM-1 receptor activity over different species, as we showed that PRV gD-mediated interference with CD112 and consequent reduced killing by NK cells was also observed in porcine cells. Although the porcine genome was known to encode a DNAM-1 homologue, our RT-PCR data for the first time demonstrate the expression of DNAM-1 in porcine NK cells. Hence, this aspect of the immune system appears well conserved over different species, including humans, other primates, pigs and mice (39, 40). The biological consequences of the suppressive effect of gD on DNAM-1 function may not be limited to NK cells. DNAM-1 is also expressed on a variety of other immune cells, including T cells, monocytes/macrophages, platelets, and a subset of B lymphocytes

(24, 41). In most cells, DNAM-1 is involved in cellular activation. For instance, in cytotoxic T cells DNAM-1 contributes to the cytolytic activity against tumor cells expressing CD112 and CD155 (42).

Blocking of endosomal acidification with an inhibitor of vacuolar H^+ATPases prevented gD-mediated degradation of CD112, implicating the involvement of endosomal degradation. These results are in line with earlier reports showing HSV-1 gD-induced endosomal degradation of Nectin-1 (CD111) (43) and suggest conserved routes of internalization and subsequent degradation for different nectin members. It will be interesting to further characterize these virus-induced internalization/degradation routes of CD112 and other nectins in future research, as this may also shed new light on the physiological role and regulation of nectins. Indeed, CD112 internalization has, to our knowledge, up to now only been reported and studied during spermatogenesis (44).

Our identification of a gD-mediated alphaherpesvirus NK cell evasion mechanism may have consequences for the design of future vaccines. For betaherpesviruses, indeed, a mutant MCMV with increased sensitivity to NK cells displayed improved vaccine properties (45). At the same time, our findings may have consequences for the design of herpes-based therapeutic vectors. Specifically, the use of attenuated HSV-1-based oncolytic vectors for glioblastoma therapy is limited by premature clearance of the viral vector by NK cells of the recipient (18). We observed that expression of PRV gD or HSV-2 gD in the U87-MG glioblastoma cell line reduces cell surface CD112. However, unlike PRV gD and HSV-2 gD, HSV-1 gD does not display significant affinity for CD112 (30). Thus, chimeric HSV-1 oncolytic vectors, expressing HSV-2/PRV gD (fragments), may possibly display reduced susceptibility to NK cell-mediated clearance. In support of the potential of such an approach, a vesicular stomatitis virus-based oncolytic vector expressing the DNAM-1-interfering HCMV protein UL141 displayed increased oncolytic potential through vector-mediated inhibition of NK cells (46).

In conclusion, the gD glycoprotein of different alphaherpesviruses (PRV and HSV-2) displays previously uncharacterized immune evasion properties, by reducing the susceptibility of infected cells to NK cell-mediated lysis through downregulation of the cellular DNAM-1 ligand CD112. This represents a novel aspect of the pathogenetically important interplay between alphaherpesviruses and NK cells. Interactions between virus envelope glycoproteins and cellular receptors have been predominantly studied in the context of their role during virus entry. Our current data point out that these interactions can also have profound implications for other aspects of virus biology and pathogenesis, including evasion of the host immune system.

MATERIALS AND METHODS

Viruses and cells – PRV Kaplan and HSV-2 333 wild type viruses and their isogenic gDnull mutants were kindly provided by Dr. Thomas Mettenleiter (Friedrich-Loeffler-Institute, Germany) and Dr. Patricia Spear (Northwestern University, USA), respectively (47, 48). Primary porcine epithelial cells were obtained and cultivated as described before (49).The primary glioblastoma human cell line U-87 MG was kindly provided by Dr. Dominique Schols (Rega institute, Belgium). Human embryonic kidney HEK-293T cells, human U-87 MG cells and porcine SK cells were maintained in DMEM (293T & U-87 MG) or MEM (SK) supplemented with 10% FCS, L-glutamine and antibiotics (penicillin, streptomycin and gentamycin).

Antibodies & reagents – Antibodies directed against PRV glycoproteins gB (mIgG2a, 1C11) and gD (mIgG1, 13D12) were described before (50). Antibody directed against HSV-2 glycoprotein gB (mIgG1, 10B7) was purchased from Abcam (UK). Mouse monoclonal anti-PRV gC antibody (mIgG1, 8P19) was kindly provided by A. Brun and rabbit polyclonal anti-HSV gD antibody was kindly provided by R. Eisenberg and G. Cohen (University of Pennsylvania, USA). For FACS analysis L14 antibody (mIgG2a) against Nectin2/CD112 and L95 antibody (mIgG1) against PVR/CD155 were used (28). For Western blotting, rabbit polyclonal antibodies ab87220 against nectin2/CD112 and ab123252 against PVR/CD155 were purchased from Abcam (UK). Mouse monoclonal antibodies against porcine markers CD3c (mIgG1, PPT3), CD4a (mIgG2b, 72-14-4), CD8b (mIgG2a, 11/295/33) and CD172a (IgG1, 74-22-15) were used and titered on freshly isolated porcine PBMC, and were all described earlier (51-53). Mouse monoclonal antibodies against porcine CD16 (mIgG1, G7), anti-alpha tubulin (mIgG1, DM1A), FITC labeled anti-CD107a (mIgG2a, 1D4B) and MHCI (mIgG2a, PT85A) were purchased from AbD Serotec (UK), Abcam (UK), BD Biosciences (Belgium) and VMRD$_{INC}$ (USA), respectively. DNAM-1-blocking antibody (mIgM, F5) and recombinant huFc-tagged DNAM-1 protein (DNAM-1Fc) were previously described (54, 55). For flow cytometric analysis, goat anti-human and anti-mouse R-PE labeled antibodies (Invitrogen™, UK) were used. Goat anti-mouse IgG1 R-PE, IgG2a AF647 and IgG2b FITC (Invitrogen™, UK) and goat anti-mouse IgG MACSbeads (Miltenyi Biotec, Germany) were used for cell sorting. HRP labeled polyclonal goat anti-mouse or anti-rabbit antibodies (Dako, Denmark) were used for Western blot detection. Control plasmid pcDNA3 was purchased from

Invitrogen^{TM} (UK), and the pcDNA3 plasmid encoding gD of PRV Kaplan was kindly provided by Dr. B. Klupp and Dr. T. Mettenleiter (Friedrich-Loeffler-Institute, Germany) (56).

Infections and transfections – SK cells were detached from the cell culture flask using trypsin, seeded in suspension culture flasks (Sarstedt, Germany) at $1.2x10^6$ cells/ml, inoculated at a multiplicity of infection (m.o.i.) of 10, and put on a rocking platform at 37°C basically as described before (57). 293T cells and U-87 MG cells were seeded at $1.5x10^6$ cells/ml and inoculated the next day. Infections with PRV were performed at an m.o.i. of 10, while HSV-2 infections were done at an m.o.i. of 5. PRV-infected SK cells, 293T cells and U-87 MG cells were collected at 12 h post inoculation (hpi), 6 hpi and 10 hpi, respectively. HSV-2-infected 293T cells and U-87 MG cells were collected at 28 hpi. For cytolytic assays, cells were collected 2 hours earlier. For transfections, 293T cells were seeded at $2.5x10^5$ cells/ml and transfected the next day using JetPEI transfection reagent^{TM} (Polyplus-transfection, France). Transfected cells were collected at 48 h post transfection.

Western Blot – Cell lysis was performed on a shaker at 4°C for 1 h, nuclei were removed by centrifugation (13000g, 10 min), and protein content was measured using the BCA protein assay kit (Thermo Scientific, USA). Per sample, 20 µg protein was loaded on 10% acrylamide SDS-PAGE gels and transferred to a P-Hybond membrane (GE Healthcare, UK), which was afterwards blocked using 5% milk powder diluted in PBS-T (PBS supplemented with 0.1% Tween20 (Sigma Aldrich, Germany). Incubations with primary monoclonal or HRP-labeled secondary antibodies were performed for 1 h in 5% milk powder PBS-T at room temperature. Incubations with primary polyclonal antibodies were performed overnight at 4°C. Bands were detected by chemiluminescence using the ECL Prime (GE Healthcare, UK) or WesternBright Sirius (Advansta, USA) kit and visualized using a ChemiDocMP Imager (Biorad, USA), according to the manufacturer's instructions. For some experiments, cells were treated with bafilomycin A1 (BFLA-1, Sigma Aldrich, Germany) 2 hours post infection by replacing the medium with medium supplemented with 10 µM BFLA-1 or a DMSO control (Sigma Aldrich, Germany).

Flow cytometric analysis – Cells were collected, incubated with primary mouse antibodies or recombinant DNAM1-Fc (10 µg/ml) and subsequently incubated with R-PE labeled goat anti-mouse or anti-human secondary R-PE antibodies (Invitrogen^{TM}, UK), respectively. Analysis was performed on 20,000 living cells with a FACSAria III and FACSDiva software (BD BioSciences, Belgium) using the Sytoxblue dye staining (Invitrogen^{TM}, UK).

NK cells – Human NK cells were isolated from PBMC using the RosetteSepTM NK cell Enrichment kit (Stem-Cell Technologies, Vancouver, Canada), cultured in the presence of 100 U/ml huIL2 (Chiron, USA) as described before (58) and used within 3 weeks. Primary porcine NK cells were isolated from porcine PBMC by negative MACS depletion and a FACS purification step using antibodies against porcine CD172, CD3, CD4 and CD8α, essentially as described before (59, 60) and primed for 18h with 40 U/ml recombinant huIL2 (Invitrogen™, UK). CD16 expression on sorted cells confirmed ≥98% NK purity.

Cytolytic & Degranulation assays – A flow cytometric PI/CFSE-based assay was used to quantify the NK-mediated lytic activity against infected or transfected target cells, essentially as described before (61). Viability of 5,000 target cells was evaluated by flow cytometry using propidium iodide (Invitrogen™, UK). The % NK-mediated lysis was calculated using the formula: (%dead target$_{NK}$ - %dead target$_{spont}$)/(%dead target$_{maximum}$ - %dead target$_{spont}$) (59). NK cell degranulation was assessed by flow cytometry using a FITC-labeled CD107a-specific antibody as described before (22). For DNAM-1-dependent cell killing/degranulation assays, cytotoxicity or expression of the CD107a degranulation marker was evaluated in the absence or presence of a DNAM-1-blocking IgM antibody (10 μg/ml) (54).

RT-PCR analysis – Total RNA was extracted using the RNeasy Micro or Mini Kit (Qiagen, Netherlands) from porcine SK cells and from purified porcine NK cells. Oligo(dT)-primed cDNA was prepared by standard technique using a Transcriptor First Strand cDNA Synthesis Kit (Roche diagnostic, Germany). Reverse transcription was performed at 42°C for 10 min and at 55°C for 50 min. PCR amplifications were carried out for 30 or 35 cycles with Platinum TAQ (Invitrogen™, UK) following the manufacturer's instructions. Primers used were: pNectin2 for 5' ATCTGGCTATGATGACAACTG; pNectin2 rev alpha 5' CCTGAGTTTTCGGATCATCG; pNectin2 rev delta 5' GTCTTGAGCGGGCTGTGC; pPVR for 5' TACCTCGGCCGCAGTGAG; pPVR rev 5' GCTGCAAGATCCCCATCCTT; pDNAM-1 for 5' TTCTATAGATCGGCCTCCC; pDNAM-1 rev 5' AAAATGGCCTGCAACACTGA; β-actin for 5' ACTCCATCATGAAGTGTGACG, β-actin rev 5' CATACTCCTGCTTGCTGATCC. Annealing temperatures were 58°C (β-actin), 62°C (DNAM-1) or 65°C (Nectin-2 alpha, Nectin-2 delta, PVR). PCR products (443 bp fragment for Nectin-2 alpha, 516 bp for Nectin-2 delta, 515 bp for PVR, 617 bp for DNAM-1, 249 bp for β-actin) were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. DNA sequences of all PCR products were checked using a BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems,Belgium) and compared with the published and predicted sequences of porcine CD112 (NM_001097494 and EU069826 Nectin2 alpha and delta isoforms, respectively), porcine CD155 (NM_005653283.1) and porcine DNAM-1 (XM_003480436.2).

Statistics – Statistical analysis was performed using Prism software (GraphPad, USA) based on the mean and the Standard Error of the Mean (SEM) of at least three independent replicates, using a One-way Anova test.

ACKNOWLEDGEMENTS

SUPPLEMENTAL FIGURES

Suppl. Figure 1. Cell surface expression of MHCI and gB in cells infected with wt PRV and HSV-2 and their isogenic gDnull mutant. 293T cells were either mock-infected or infected with wt PRV or its isogenic gDnull mutant (A) or wt HSV-2 or its isogenic gDnull mutant (B) and assessed by flow cytometry for cell surface expression of PRV gB (A) or HSV-2 gB (B) and MHC I. Vertical lines indicate median fluorescence intensity. Dotted line histogram represents isotype-matched antibody control signal.

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Chapter 4: Alphaherpesvirus US3 protein kinase protects infected cells from NK cell-mediated lysis via increased binding of the inhibitory NK cell receptor CD300a

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ABSTRACT

Natural killer (NK) cells are key players in the innate response to viral infections. NK cells are of particular importance during infection with alphaherpesviruses, and herpesviruses in general. Killing of target cells by NK cells is regulated by a complex sum of signals received through activating/inhibitory receptors on the NK cell surface. Unravelling the largely unstudied interactions of alphaherpesviruses with NK cells could provide essential insights in alphaherpesvirus biology. Here, we report that the protein kinase US3 of the alphaherpesvirus pseudorabies virus (PRV) displays previously uncharacterized immune evasion properties towards NK cells. Expression of PRV US3 during infection triggered binding of the inhibitory NK receptor CD300a to the infected cell surface, and, in line with this, US3 increased CD300a-mediated protection of infected cells against NK-mediated lysis. US3-mediated CD300a binding was found to depend on anionic phospholipid CD300a ligands and on group I p21-activated kinases. These data identify a novel alphaherpesvirus NK evasion strategy and demonstrate for the first time a role for CD300a in regulating NK cell activity upon contact with virus infected target cells.

INTRODUCTION

Natural killer (NK) cells are members of the innate immunity and play a central role in the defense against viral infections and cancer development (1). For herpesviruses in particular, functional NK cells are crucial to limit virus spread and disease symptoms. Indeed, impaired NK cell activity has been associated with life-threatening encephalitis for the human alphaherpesviruses herpes simplex virus 1 (HSV-1) and varicella zoster virus (VZV) (2-4). Seen the strong antiviral potential of NK cells particularly against herpesviruses, it comes as no surprise that several herpesvirus NK cell evasion strategies have been discovered (5). Interestingly and paradoxically, such evasion strategies have been reported mainly for betaherpesviruses and gammaherpesviruses (5-17), with only three reports thus far describe NK cell evasion strategies for the largest herpesvirus subfamily, the alphaherpesviruses (18-20).

NK cells display a diversity of activating and inhibiting germ-line encoded receptors on their cell surface that recognize specific ligands on the plasma membrane of potential target cells. As a consequence, NK cells can sense a wide array of alterations in the cell surface profile of target cells and respond immediately without the need to clonally expand (21, 22). Viral NK cell evasion often consists of decreased ligand exposure to activating NK cell receptors and/or increased ligand exposure to inhibitory NK cell receptors. One of the inhibitory receptors on the NK cell surface is CD300a, a 60 kDa glycoprotein belonging to the immunoglobulin (Ig) superfamily characterized by a single V-type Ig-like domain in the extracellular domain and several immunoreceptor tyrosine-based inhibition motifs (ITIMs) in the cytoplasmic domain (23, 24). CD300a recognizes cell surface exposed phosphatidylserine (PS) and phosphatidylethanolamine (PE) and suppresses the cytolytic activity of NK cells (25-27). The inhibitory CD300a receptor and its binding with these polar lipids is highly conserved across animal species, and has been described in mammals, birds and fish (28, 29). To date, to our knowledge, CD300a had not yet been associated with viral NK cell evasion strategies.

Here, we report that the US3 protein kinase of pseudorabies virus (PRV), a porcine alphaherpesvirus, contributes to NK cell evasion by inducing an increase in CD300a binding to

the infected cell surface. This novel alphaherpesvirus NK cell evasion mechanism may shed new light on the role of CD300a and its ligands in NK cell and virus biology.

RESULTS

1) <u>US3 reduces NK cell-mediated lysis of PRV-infected cells</u>

Using a variety of gene deletion mutants of pseudorabies virus (PRV), we recently discovered that the gD glycoprotein of PRV suppresses NK cell activity via downregulation of CD112, a ligand for the activating NK cell receptor DNAM-1 (19). The initial NK cell cytotoxicity assays with different PRV mutants indicated that US3 may also display NK cell evasive properties. To investigate whether PRV US3 indeed affects the susceptibility of infected cells to NK cell-mediated lysis, cytolytic assays were performed with SK cells infected with wild-type (WT) PRV, an isogenic US3null virus or an isogenic US3rescue virus. At 10 hpi, mock-infected and PRV-infected cells were coincubated with IL2-primed primary porcine NK cells for 4h and subsequently assessed for viability by flow cytometry (Figure 1A). Mock-infected SK cells did not elicit a significant cytolytic response by NK cells, as the percentage of NK cell-mediated lysis was not statistically different from zero, in line with earlier data (19). Also as reported earlier, PRV infection triggered NK-mediated killing of SK cells (19). However, cells infected with US3null PRV showed increased susceptibility towards NK-cell mediated lysis, compared to SK cells infected with WT or US3rescue PRV. The higher susceptibility of US3null PRV-infected cells to NK cell-mediated cell lysis compared to WT or US3rescue PRV-infected cells was not due to differences in viral replication (Figure 1B), nor to differences in the ability of these viruses to downregulate MHC I expression (an important ligand for inhibitory NK cell receptors) (Figure 1C).

As there is currently only a limited range of reagents and tools available to investigate NK cell activation/inhibition in the porcine model, we analyzed whether PRV US3 also generated a protective effect towards human NK cells. To this end, the cytolytic activity of human IL2

cultured NK cells was assessed against mock-infected SK cells or SK cells infected with WT PRV, US3null PRV or US3rescue PRV (Figure 1D). As observed before, IL2-cultured human NK cells lysed mock-infected SK cells, which is in line with the known human anti-porcine NK cell xenogeneic response (30-33). US3null PRV-infected cells again showed higher susceptibility to NK cell-mediated lysis compared to wild type or US3 rescued PRV-infected cells. In conclusion, PRV US3 reduces susceptibility of infected cells towards both porcine and human NK cells.
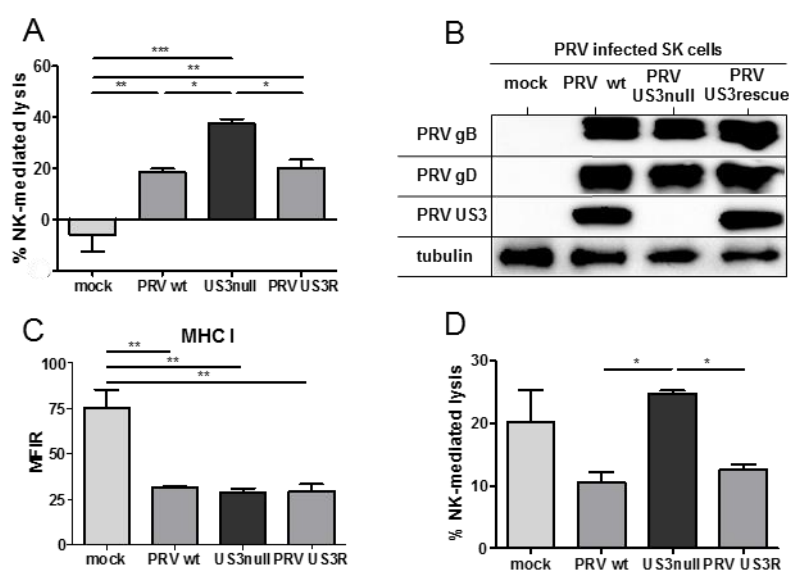
Figure 1. US3 suppresses susceptibility of PRV-infected cells to porcine and human NK cell-mediated lysis. (A) SK cells were mock-infected or infected with WT PRV, US3null PRV or US3rescue PRV for 10h and subsequently incubated with IL2-primed primary porcine NK cells at a target:effector ratio of 1:25 for 4h. Viability of target cells was assessed by propidium iodide and flow cytometry, and the percentage NK cell-mediated lysis was calculated. Data represent mean + SEM of three independent experiments (* p<0.05, ** p<0.01, *** p<0.001). (B-C) SK cells were mock-infected or infected with WT, US3null or US3rescue PRV for 12h and subsequently (B) analyzed by Western blotting for expression of PRV gB, PRV gD, PRV US3 and tubulin or (C) assessed for MHC I cell surface expression by flow cytometry. Data in (C) represent mean + SEM of three independent repeats (** p<0.01). (D) SK cells were infected with WT, US3null or US3rescue PRV for 10h and subsequently incubated with IL2-cultivated human NK cells at a target:effector ratio of 1:1 for 4h. Viability of target cells was assessed by propidium iodide and flow cytometry, and the percentage NK cell-mediated lysis was calculated. Data represent mean + SEM of three independent repeats (* p<0.05).

2) <u>PRV US3 enhances resistance to NK cell-mediated killing by increasing binding of the inhibitory NK cell receptor CD300a to infected cells.</u>

We next investigated whether the PRV US3 affects resistance of infected cells to NK cell-mediated lysis by modulating the specific binding of NK cell receptors on infected cells. The inhibitory CD300a NK cell receptor and its binding with the polar lipids PS and PE is highly conserved across animal species (28, 29). Given the ability of PRV US3 to protect infected cells from both human and porcine NK cells, we investigated whether US3 affects binding of CD300a to the infected cell surface. A CD300a-Fc soluble chimeric molecule was generated and analyzed in a flow cytometric binding assay. As shown in Figure 2A, SK cells infected with WT PRV or US3rescue virus displayed a substantially increased binding of recombinant CD300a compared to US3null PRV-infected cells or mock-infected cells. We then addressed whether this US3-dependent increase in binding of CD300a to PRV-infected cells is involved in the US3-mediated protection from NK cell-mediated lysis. To this end, cytotoxicity assays were performed in the presence or absence of the anti-CD300a blocking mAb KS153. Reactivity of KS153 towards CD300a was confirmed on transfected HEK293T cells (Suppl. Fig. 1A). Addition of KS153 allowed to calculate CD300a-dependent protection of cells against NK cell-mediated lysis (see Material and Methods). Figure 2B shows that US3 provides CD300a-mediated protection of infected cells towards NK cell-mediated lysis. In conclusion, PRV-infected cells show a US3-dependent increase in CD300a binding and CD300a is involved in the protective effect of PRV US3 against NK cell-mediated lysis.
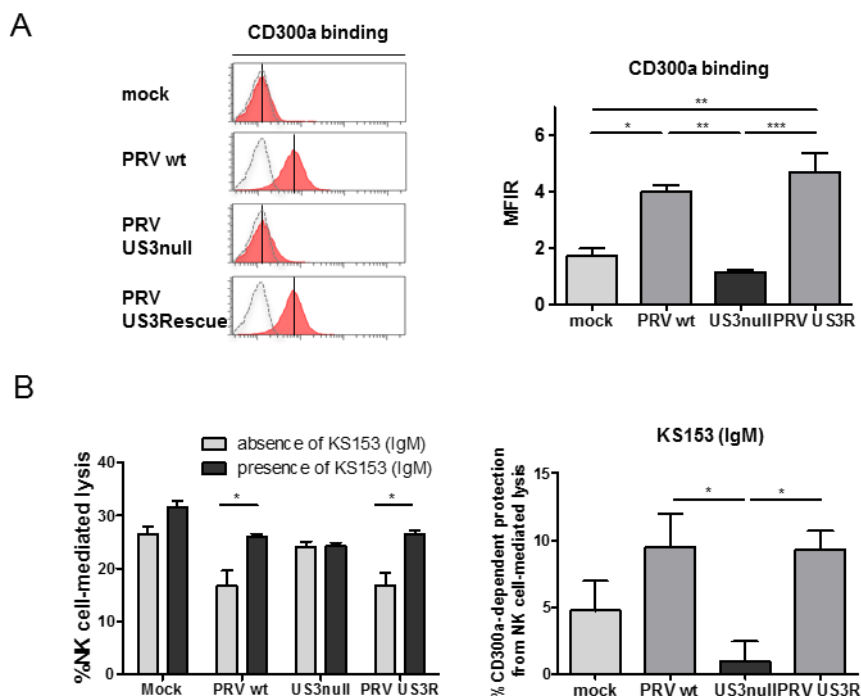
Figure 2. PRV triggers US3-dependent increased binding of the inhibitory NK cell receptor CD300a to the infected cell surface and increased CD300a-mediated protection of infected cells against NK cell-mediated lysis. (A) SK cells were infected with WT PRV, US3null PRV or US3rescue PRV for 12h and subsequently assessed by flow cytometry for binding of recombinant CD300a-Fc (20 μg/ml). Graphs represent mean + SEM of three independent repeats (* p<0.05, ** p<0.01, *** p<0.001). Vertical lines indicate median fluorescence intensity. Dotted line histogram represents isotype-matched antibody control signal. (B) SK cells were infected with WT, US3null or US3rescue PRV for 10h and subsequently incubated with IL2-cultivated human NK cells at an effector:target ratio of 1:1 in medium alone and with anti-CD300a antibodies (KS153). Viability of target cells was assessed by propidium iodide and flow cytometry, (left) the % NK cell-mediated lysis and (right) % CD300a-dependent protection against NK cell-mediated lysis was calculated. Data represent mean + SEM of three independent repeats (* p<0.05).

3) CD300a ligands phosphatidylserine (PS) and phosphatidylethanolamine (PE) are involved in the PRV US3-mediated binding of CD300a

Two highly conserved cellular ligands, PS and PE, have been identified for the inhibitory NK cell receptor CD300a (25, 26). To investigate whether these cellular ligands are involved in the observed PRV US3-dependent increase in CD300a binding to PRV-infected cells, binding of CD300a-Fc was assessed in the presence of increasing concentrations of milk fat globule-EGF factor 8 (MFG-E8, lactadherin) or duramycin, which have been previously reported to interfere with the ability of CD300a to bind PS and PE, respectively (25). As shown in Figure 3A, addition of MFG-E8 resulted in a significant dose-dependent reduction in CD300a-Fc binding to PRV-infected SK cells, indicating that PS is involved in the binding of CD300a to PRV-

infected SK cells. Suppression of CD300a binding was also observed using duramycin, although the effect appeared less dose-dependent.

The involvement of PS and PE in US3-mediated CD300a binding suggests that US3 may modulate the cell surface exposure of these CD300a ligands. No assays have been described to specifically detect cell surface PE. However, cell surface PS can be detected, for example using the PS-specific antibody 1H6 (27). To determine whether US3 modulates cell surface PS, mock-infected SK cells or SK cells infected with WT PRV, US3null PRV or US3rescue PRV were analyzed by flow cytometry using the anti-PS antibody 1H6. Figure 3B shows that SK cells infected with WT PRV or US3rescue PRV indeed expose PS at much higher levels compared to cells infected with US3null PRV. Surprisingly, mock-infected cells also showed substantial PS exposure. Increased PS exposure is one of the hallmark characteristics of apoptotic cells. Nevertheless, the Sytoxblue live/dead staining indicated that neither of the conditions (mock, WT PRV, US3null PRV, US3rescue PRV) resulted in substantial or different amounts of cells in a late apoptotic stage. To further assess whether the US3-mediated PS exposure observed on PRV-infected cells portrayed an apoptotic cell surface profile of these cells, an Annexin-V binding assay was used, which is widely used to detect PS on apoptotic cells. Figure 3C confirmed that neither mock-infected nor PRV-infected cells appears to be in an apoptotic state, as no apparent Annexin V binding could be detected.

To better assess the potential biological significance of our findings, we investigated whether expression of PRV US3 also led to increased CD300a binding and modulated PS cell surface exposure in porcine primary epithelial cells. Figure 4A&B shows that the effects of PRV US3 on CD300a binding and PS cell surface exposure in infected porcine primary epithelial cells are very similar to the effects observed in the SK cell line. Again, Western blot analysis confirmed that this US3-induced phenotype is not caused by differences in virus replication levels (Fig 4C). In conclusion, we report that PS and PE are involved in the PRV US3-dependent recognition of infected cells by CD300a and that US3 modulates PS exposure on the surface of infected cells.
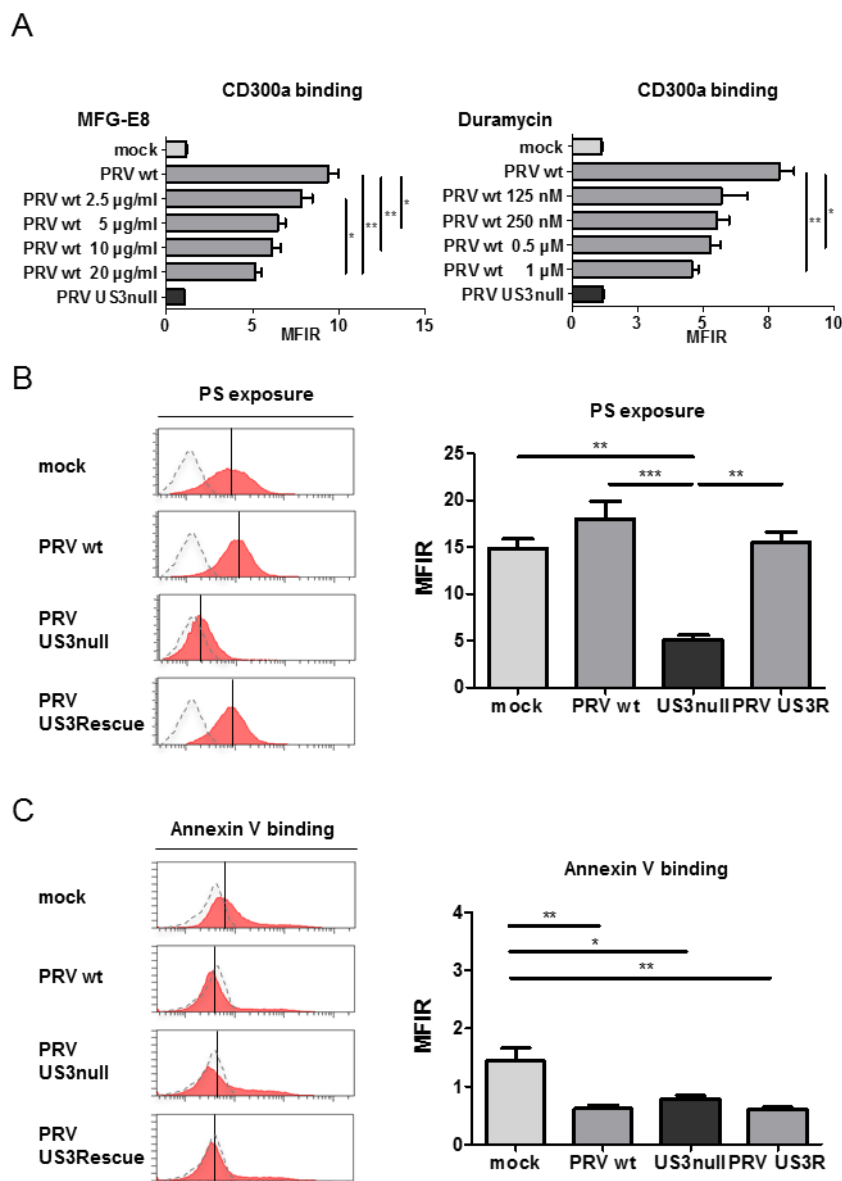
Figure 3. CD300a ligands PS and PE are involved in the US3-dependent increased binding of CD300a to the infected cell surface and US3 modulates PS cell surface exposure. (A) SK cells were mock-infected or infected with WT PRV or US3null PRV for 12h, subsequently incubated with MFG-E8 or duramycin at given concentrations and assessed by flow cytometry for binding of recombinant CD300a-Fc (20 μg/ml). Data represent mean + SEM of three independent repeats (* p<0.05, ** p<0.01). (B-C) SK cells were mock infected or infected with WT, US3null or US3rescue PRV for 12h and subsequently assessed by flow cytometry for cell surface exposure of PS using antibody 1H6 (B) or annexin V (C). Graphs represent mean + SEM of three independent repeats (** p<0.01, *** p<0.001). Vertical lines indicate median fluorescence intensity. Dotted line histogram represents isotype-matched antibody control signal.
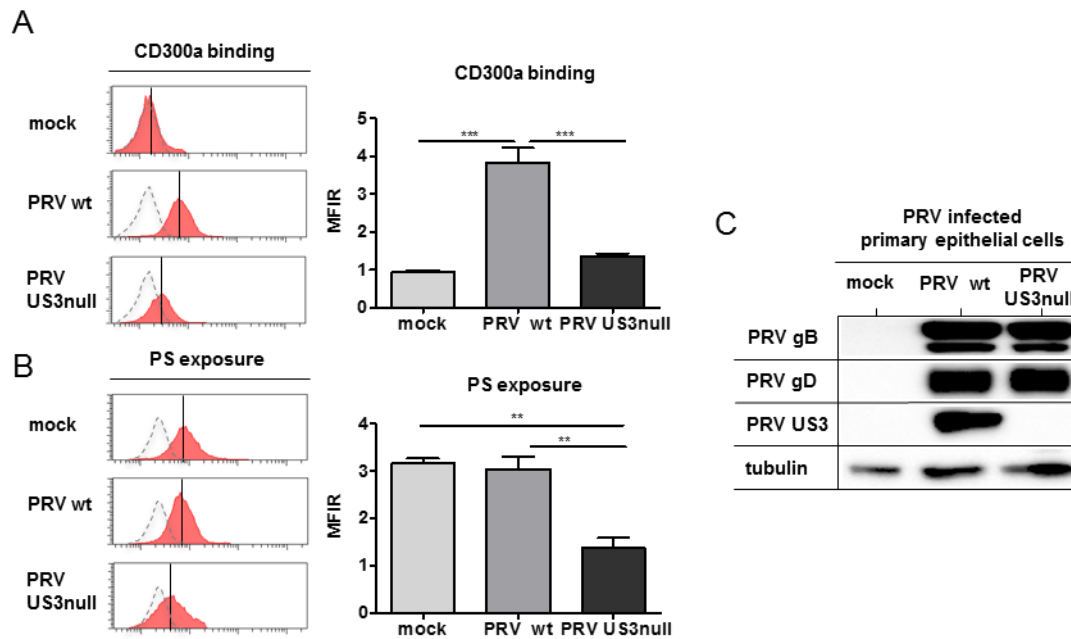
Figure 4. Effects of US3 on CD300a binding and PS exposure also occur in primary epithelial cells. Porcine primary epithelial cells were mock infected or infected with WT PRV or US3null PRV for 12h and assessed by flow cytometry (A-B) for binding of recombinant CD300a-Fc (20 μg/ml) (A) and cell surface exposure of PS using antibody 1H6 (B) or assessed by Western blotting for expression of PRV gB, PRV gD, PRV US3 and tubulin (C). Graphs in (A) and (B) represent mean + SEM of three independent repeats (** $p<0.01$, *** $p<0.001$). Vertical lines indicate median fluorescence intensity. Dotted line histogram represents isotype-matched antibody control signal.

4) <u>The kinase activity of PRV US3 and cellular p21-activated kinases are required for increased CD300a binding and modulation of cell surface PS</u>

PRV US3 is a viral serine/threonine protein kinase and can directly phosphorylate and activate cellular group I p21-activated kinases (PAKs), which are central regulators in Rac1/CDC42 Rho GTPase signaling (34). Activation of group I PAKs has been reported recently to trigger increased PS cell surface exposure during thrombin-mediated activation of platelets (35). To investigate a possible involvement of the US3-PAK kinase signaling axis in increased CD300a binding and modulation of PS exposure, we first assessed whether the kinase activity of PRV US3 was required for the observed effects. Therefore, a kinase-inactive US3 mutant PRV virus was used which harbors a point mutation (D223A) in the conserved aspartate in PRV US3 that constitutes the catalytic base required for phosphotransfer (34, 36). SK cells were mock-infected or infected with WT PRV, isogenic US3 kinase-inactive PRV or isogenic US3null

PRV and assessed for binding of recombinant CD300a and modulation of cell surface PS. Figure 5A&B shows that cells infected with PRV expressing kinase-inactive US3 show a similar phenotype as US3null PRV-infected cells with regard to CD300a binding and cell surface exposure of PS. Western blotting demonstrated a similar infection efficiency of cells infected with WT, US3null or US3 kinase-inactive PRV (Figure 5C). The effect of PRV on PS exposure in the assay shown in Figure 5 is somewhat less pronounced compared to our earlier data (Figures 3). This mild discrepancy can possibly be attributed to the fact that in the assays to determine kinase involvement of US3, PRV strain Becker and isogenic mutants were used, whereas in the former experiments, the highly virulent field strain NIA3 (and isogenic mutants) was used.
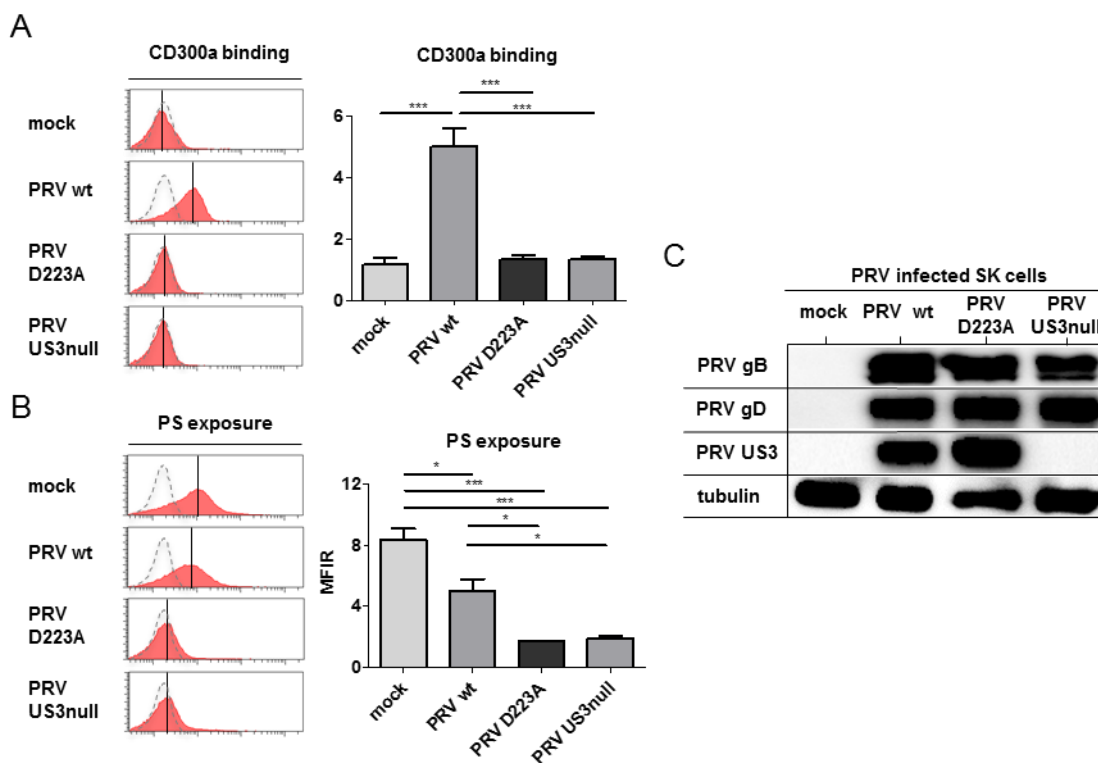
Figure 5. Kinase activity of PRV US3 is required for its effects on CD300a binding and PS exposure. SK cells were mock infected or infected with wt PRV, kinase-inactive D223A US3 PRV or US3null PRV for 12h, and subsequently assessed by flow cytometry (A-B) for binding of recombinant CD300a-Fc (20 μg/ml) (A) and cell surface exposure of PS using antibody 1H6 (B), or assessed by Western blotting (C) for expression of PRV gB, PRV gD, PRV US3 and tubulin. Graphs in (A) and (B) represent mean + SEM of three independent repeats (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Vertical lines indicate median fluorescence intensity. Dotted line histogram represents isotype-matched antibody control signal.

To investigate whether group I PAKs are involved in the PRV US3-mediated effects on CD300a binding and PS cell surface exposure, group I PAKs were specifically inhibited using the selective allosteric group I PAK inhibitor IPA-3 (37). As shown in Figure 6A, treatment with IPA-3 abrogated the increase in CD300a binding and reduced PS cell surface exposure in WT PRV-infected cells and mock-infected cells to levels observed in US3null PRV-infected cells. These inhibitory effects of IPA-3 are not caused by suppressive effects on PRV infection or the expression level of US3, as indicated by Western blot analysis shown in Figure 6B. In conclusion, these experiments show that the PRV US3-dependent increase in CD300a binding and modulation of cell surface PS exposure depend on the kinase activity of US3 and on the group I PAK cell signaling pathway.
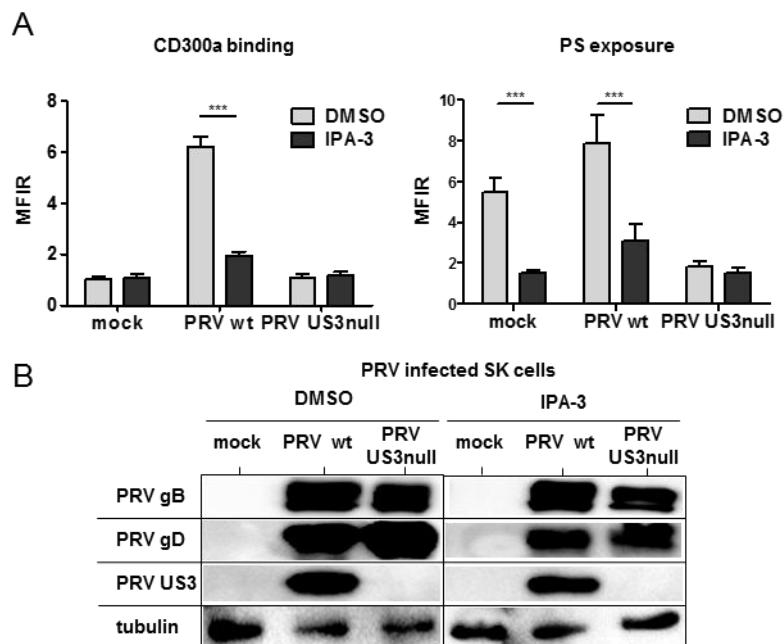
Figure 6. Group I PAKs are involved in the effects of US3 on CD300a binding and PS exposure. SK cells were mock infected or infected with wt or US3null PRV. At 2hpi, group I PAK inhibitor IPA-3 or DMSO diluent were added. At 12hpi, cells were assessed by flow cytometry for binding of recombinant CD300a-Fc (20 μg/ml) and cell surface PS exposure using antibody 1H6 (A), or by Western blotting for expression of PRV gB, PRV gD, PRV US3 and tubulin (B). Data in (A) represent mean + SEM of three independent repeats (*** p<0.001).

5) CD300a in primary porcine NK cells

The CD300a receptor is highly conserved across mammals and recently also characterized as an inhibitory receptor in birds (28, 29). The natural host of PRV is the pig and, currently, porcine

NK cell receptors are poorly characterized. CD300a function in porcine NK cells has not yet been addressed. Functionality of activating/inhibitory NK cell receptors can be assessed using a P815-based antibody redirected lysis assay. P815 cells express mouse Ig receptors. Coincubation of NK cells with P815 cells and bridging IgG antibodies directed against activating or inhibitory NK cell receptors triggers increased or decreased NK-mediated lysis of P815 cells, respectively. A P815-based antibody redirected lysis assay using human NK cells and anti-human CD300a antibodies leads to reduced P815 killing, demonstrating that human CD300a serves as an inhibitory NK cell receptor (27, 38). Here, we used mouse monoclonal antibodies directed against human CD300a in a similar P815-based antibody redirected lysis assay using primary porcine NK cells to evaluate cross-reactivity of the anti-human CD300a IT144 antibody with the porcine CD300a homologue and to determine whether, like in human NK cells, CD300a serves as an inhibitory receptor in porcine NK cells. Therefore, P815 cells and IL2-activated porcine primary NK cells were coincubated at different effector/target ratios with the mouse monoclonal antibody IT144 directed against huCD300a, an isotype matched mouse IgG1 control antibody, a mouse monoclonal IgG1 anti-porcine CD16 antibody (which generates an activating effect on NK cells) or medium alone, and the percentage of NK cell-mediated P815 lysis was calculated. Figure 7 shows that, as reported before (39), the porcine CD16 receptor serves as an activating NK cell receptor, since triggering of CD16 resulted in substantially increased porcine NK cell-mediated P815 killing. Importantly, the CD300a directed antibody triggered a significant inhibitory response towards porcine NK cell-mediated lysis of P815 cells as compared to isotype-matched control or medium alone, indicating that also in the porcine system, CD300a serves as an inhibitory NK cell receptor.
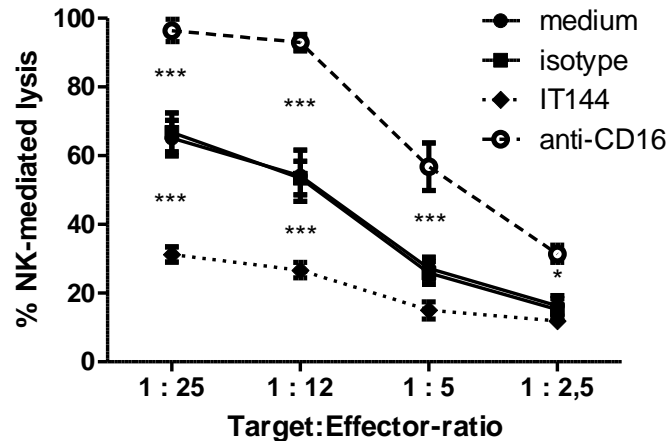
Figure 7. Porcine NK cells express a functional homolog of the inhibitory NK cell receptor CD300a. An antibody redirected killing assay based on the redirected killing of Fc-receptor bearing P815 cell line was performed with IL2-activated porcine primary NK cells, at indicated target:effector ratio, in the presence of medium, anti-huCD300a antibody IT144, anti-porcine CD16 antibody or isotype-matched control antibody. Viability of target cells was assessed by propidium iodide and flow cytometry, and the percentage NK cell-mediated lysis was calculated. Data represent mean + SEM of three independent repeats (* $p<0.05$, *** $p<0.001$).

DISCUSSION

In the current report, we describe a previously uncharacterized viral NK cell evasion strategy and the involvement of CD300a in the recognition of virus-infected cells. By using PRV mutants and specific inhibitors we demonstrate that this trait depends on the expression of the catalytically intact US3 and the activation of the group I PAKs. Interestingly, different tumor cell lines have recently been reported to show increased PS exposure and CD300a binding (27). In addition, blocking of PS using MFG-E8 enhanced NK cell-mediated killing of tumor cells, leading to the hypothesis that tumor cells may subvert NK cell-mediated lysis via increased CD300a binding (27). Our data for the first time directly demonstrate that manipulation of CD300a indeed represents a *bona fide* NK evasion strategy, indicating that both viruses and tumor cells may manipulate this NK cell inhibitory pathway for their own benefit.

CD300a is not only expressed on NK cells, but also on several other immune cell populations, where it typically also triggers inhibitory signaling pathways. Thus, CD300a not only has been implicated in inhibition of NK cell activity (23, 27), but also of mast cells (40), neutrophils (41), eosinophils (42), B and T cells (43, 44). As such, the consequences of viral triggering of the

CD300a inhibitory receptor may stretch beyond the effects on NK cells described here. On the other hand, CD300a shows significant overlap regarding ligand specificity with the closely related activating receptor CD300c, which is particularly expressed on monocytes and mast cells (45, 46). Hence, in future research, it will be interesting to study whether the US3-mediated modulation of CD300a binding may have consequences on other immune cells and whether or not US3 also affects CD300c binding.

The polar lipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) have been described as principal CD300a ligands (25). Both PS and PE typically show an asymmetric distribution in the plasma membrane lipid bilayer and are enriched at the inner, cytoplasmic leaflet (47). Although externalization of PS is a hallmark of early stages of apoptosis, there is increasing evidence that cells may show PS exposure independent of programmed cell death, exemplified by reports on apoptosis-independent PS exposure on tumor cells and during activation of mast cells, B cells, T cells and platelets (27, 48-52). Our results are in line with the possibility of PS exposure that is independent of apoptotic cell death, since we observed PS exposure using the PS-specific antibody 1H6, which was not accompanied by an apoptotic cellular phenotype, as indicated by Sytoxblue dead/alive marker and lack of staining with Annexin V, a widely used reagent to detect PS exposure during apoptotic cell death. Further in line with this, apoptosis is considered to trigger an eat-me signal for phagocytosis, and PS exposure was found not to be sufficient to induce this process *in vivo* (53). Alternatively, one could argue that 1H6 may not specifically bind PS. However, several reports indicate that 1H6 antibody specifically binds to PS and not to other lipids (54-56). Nevertheless, we cannot formally rule out that 1H6 may possibly bind other lipid(s) in the cell membrane that are modulated by PRV US3 during infection. Irrespective of whether this is the case, we found that exposure of 1H6-reactive lipids is not sufficient to trigger substantial recombinant CD300a binding. Indeed, mock cells showed 1H6 reactivity that was comparable (and sometimes even higher) than observed for PRV-infected cells, but, unlike PRV-infected cells, did not show significant CD300a binding. Hence, additional CD300a ligands appear to be involved in the US3-triggered CD300a binding. This is in line with our blocking assays, which showed that not

only the PS-blocking reagent MFG-E8, but also the PE-blocking agent duramycin suppressed the US3-mediated increase in CD300a binding. Functional recognition reporter assays indicated that CD300a may bind stronger to PE than to PS (25, 46, 57). This could account for the discrepancies in the observed correlation of CD300a binding and PS exposure. In conclusion, our data suggest that US3 expression affects cell surface exposure of different CD300a ligands, which are associated with substantially increased CD300a binding.

We had previously shown that PRV US3 directly phosphorylates and thereby activates group I p21-activated kinases, critical downstream effectors of the Cdc42/Rac1 signaling pathways (34). Here, we report that inhibition of group I PAK activity inhibits the ability of US3 to trigger increased CD300a binding or modulate PS exposure. Group I PAK activity has been reported before to be critically involved in PS exposure during platelet activation (35). Our current data therefore suggest that group I PAKs may be linked to PS exposure/CD300a binding in different cell types. In this context, it is of interest that several viruses, including HIV, have been reported to trigger group I PAK activity (58), and may therefore modulate similar effects as we describe here. In line with this, several viruses have been reported to trigger exposure of anionic phospholipids, like PS (59). This has been speculated to enable viruses to evade immune recognition and dampen inflammatory responses to infection (59). Our current report demonstrates that viral manipulation of exposure of anionic phospholipids like PS indeed may allow viruses to subvert important components of the antiviral immune response. Targeting anionic phospholipid exposure and signaling pathways, like group I PAKs, may therefore hold promise as therapeutic strategies for viral diseases (58, 59).

In addition, our findings may also be of relevance for cancer therapy since several cancer types have been associated with upregulated group I PAK activity and non-apoptotic PS exposure (60-62). Our observation that non-infected primary epithelial cells are substantially recognized by the PS-binding antibody 1H6 may indicate that perhaps caution should be taken when targeting PS for anti-cancer or anti-viral therapy (60). We found that, despite showing similar binding of 1H6, PRV-infected cells show substantially increased CD300a binding compared to mock cells. Combined with the observation that several cancer cell types also display

substantially increased CD300a binding (27, 59), this may indicate that targeting CD300a binding may be a more stringent strategy to target virus-infected or tumor cells compared to targeting PS exposure.

Finally, our data indicate that, like in human and other mammals, CD300a serves as an inhibitory receptor in swine. Several studies have reported that the CD300 receptor family is highly conserved across multiple species (28, 29). This is particularly true for CD300a, illustrated by its recent identification and characterization in chicken, showing inhibitory activity and affinity for PS and PE as described in mammals (29). Our data also indicate that the human inhibitory NK cell receptor CD300a recognizes porcine cells implying that, under certain circumstances, huCD300a can be involved in the recognition of porcine cells. The high conservation of the CD300a ligands, PS and PE, throughout species further emphasizes the possibility that these ligands may be of importance when considering the xenograft NK response of human versus pig (29).

In conclusion, we report a novel alphaherpesvirus NK evasion strategy, consisting of US3-dependent increased binding of the inhibitory NK cell receptor CD300a, which is orchestrated by group I PAK activity and anionic phospholipids like PS and PE. Our data provide novel insights in alphaherpesvirus and CD300a biology, and may have implications for antiviral and antitumor therapies.

MATERIALS AND METHODS

Viruses and Cells – PRV NIA3 wild-type virus, the isogenic US3null mutant and restored rescue virus were described before and were kindly provided by the ID-DLO, the Netherlands (63-65). The PRV Becker wild-type virus, isogenic US3null mutant and kinase-negative US3 mutant (D223A) were described before and kindly provided by Greg Smith (Northwestern University, Chicago) (34, 36). Porcine SK cells and porcine primary epithelial cells were obtained and cultivated as described before (19, 66). Mouse P815 cells were maintained in RPMI supplemented with 10% FCS, L-glutamine and antibiotics (penicillin and streptomycin)

(67). Human 293T cells were maintained in DMEM supplemented with 10% FCS, L-glutamine and antibiotics (penicillin and streptomycin) (19).

Antibodies and Reagents – Antibodies directed against PRV glycoproteins gB (mIgG2a, 1C11) and gD (mIgG1, 13D12) were provided by H. Nauwynck (Ghent University, Belgium) and described before (68). The mouse monoclonal antibody raised against PRV US3 was kindly provided by L. Olsen and L. Enquist (Princeton University, New Jersey). Antibodies directed against human CD300a (KS153, mIgM and IT144, mIgG1) were kindly provided by S. Parolini (University of Brescia, Italy) and the anti-human CD300a antibody E59/126 (IgG1) was previously generated and described (23, 69). Mouse monoclonal antibodies against porcine markers CD3ε (mIgG1, PPT3), CD4 (mIgG2b, 72-14-4), CD8α (mIgG2a, 11/295/33), and CD172a (IgG1, 74-22-15) were provided by E. Cox (Ghent University, Belgium), used and titered on freshly isolated porcine PBMC and were all described earlier (70-72). Primary antibodies raised against MHC I (PT85A, mIgG2a, VMRD, USA), phosphatidylserine (1H6, mIgG, Millipore, Netherlands), anti-porcine CD16 (G7, mIgG1, AbD Serotec, UK) and alpha-tubulin (DM1A, mIgG, Abcam, UK) were purchased. Recombinant CD300a-Fc chimera was provided by C. Cantoni (University of Genova, Italy) and produced as follows. The sequence encoding the extracellular portion of human CD300a receptor was amplified starting from the pcDNA3.1TOPO-CD300a plasmid using the following primers: 5' CAGGGGAACTCGAGAACGGACCATGTGGCTGCCTTG (CD300a XhoI up) and 5' GACTAGGATCCAAATGCTGTGAGTTCACCACCTC (CD300a BamHI dw). Amplification was performed with Platinum TAQ High Fidelity (Life Technologies, UK) for 20 cycles (30 s at 95°C, 30 s at 58°C, and 1 m at 72°C) followed by a 7-min elongation step at 72° C. The PCR product was digested with XhoI and BamHI restriction enzymes and subcloned in the SalI-BamHI–digested pRB1-2B4Fcmut vector (kindly provided by M. Falco, Istituto G. Gaslini, Genova, Italy) in frame with the sequence coding for the human IgG1 portion, which was mutagenized to obtain a mutated Fc that does not bind to Fc receptors. The pRB1-CD300aFcmut construct was stably transfected into the HEK293 cell line (human embryonic fibroblast) using Fugene 6 (Roche, Switzerland). Supernatants were collected from the cell

transfectant cultured in Dulbecco's modified Eagle's medium supplemented with 10% ultra-low IgG fetal bovine serum (Life Technologies, UK) and 0.5 µg/ml G418 (Calbiochem, USA), and CD300a-Fc molecule was purified by affinity chromatography using Protein A–Sepharose 4 Fast Flow (GE Healthcare, UK). Purified protein was checked by SDS-PAGE followed by silver staining and ELISA using CD300a-specific mAbs. For flow cytometric analysis, goat anti-human R-PE or AF647 and anti-mouse R-PE or Cy5 labeled antibodies (Life Technologies, UK) were used. Goat anti-mouse IgG1 R-PE, IgG2a AF647, and IgG2b FITC (Life Technologies, UK) and goat anti-mouse IgG MACSbeads (Miltenyi Biotec, Germany) were used for cell sorting. HRP-labeled polyclonal goat anti-mouse antibodies (Dako, Denmark) were used for Western blot detection.

Infections and IPA-3 treatment – SK cells were detached from the cell culture flask using trypsin, seeded in suspension flasks (Sarstedt, Germany) at 1.2 x 10^6 cells/ml, inoculated at a multiplicity of infection (MOI) of 10, and put on a rocking platform at 37°C basically as described before (73). Porcine primary epithelial cells were grown in 6-wells, inoculated the next day at a MOI of 10 and the virus was washed away 2 hours post inoculation (hpi), as described before (19). Cells were treated with group I PAK inhibitor IPA-3 (Tocris, UK) or DMSO control (Sigma Aldrich, Germany), as described previously (34, 37).

Flow cytometric analysis – Cells were harvested, incubated on ice for 40 minutes with mouse primary antibodies or recombinant CD300a-Fc (20 µg/ml), and subsequently washed and incubated for 40 minutes on ice with R-phycoerythrin (R-PE) or Cy5 labeled goat anti-mouse or anti-human secondary R-PE or AF647 labeled antibodies (Life Technologies, UK), respectively. Cells infected with PRV NIA3 strains were consistently stained using R-PE labeled secondary antibodies. Cells infected with PRV Becker strains, which encode an mRFP-expression cassette, were labeled with AF647 or Cy5 to avoid spectral overlap. Annexin V binding assays were performed according to the manufactures' protocol (BD Biosciences, Belgium). Analysis was performed after washing on 20,000 living cells using a FACSAria III and FACSDiva software (BD Biosciences, Belgium). Live/dead stain Sytoxblue (Life

Technologies, UK) was used to discriminate living cells. Analysis on primary cells was performed similarly, but on 10,000 living cells.

Western Blot – Cell lysis was performed on a shaker at 4°C for 1h with lysis buffer containing NP-40 (Roche, Switzerland) and protease inhibitors (Sigma Aldrich, Germany), nuclei were removed by centrifugation (13,000 x g, 10 min), and protein content was measured using the BCA protein assay kit (Thermo Scientific, USA) (74). Per sample, 20 μg protein was loaded on 10% acrylamide SDS/PAGE gels and transferred to a P-Hybond membrane (GE Healthcare, UK), which was afterward blocked using 5% milk powder diluted in PBS-T [PBS supplemented with 0.1% Tween 20 (Sigma Aldrich, Germany)]. Incubations with primary monoclonal or HRP-labeled secondary antibodies were performed for 1h in 5% milk powder PBS-T at room temperature. Bands were detected by chemiluminescence using the ECL Prime kit (GE Healthcare, UK) and visualized using a ChemiDoc MP Imager (Biorad, USA), according to the manufacturer's instructions.

NK cells – Human NK cells were isolated from peripheral blood mononuclear cells (PBMC) using the RosetteSep^{TM} NK cell enrichment kit (Stemcell Technologies, Canada), cultured in the presence of 100 units/ml huIL2 (Chiron, USA) as described before (75) and used within 3 weeks. Porcine primary NK cells were isolated from porcine PBMC by negative MACS depletion and a FACS purification step using antibodies against porcine CD172a, CD3, CD4 and CD8α, as described before (19). Afther isolation, porcine NK cells were incubated for 18h in the presence of 40 units/ml huIL2 (Life Technologies, UK). CD16 expression on sorted cells confirmed ≥ 98% NK purity.

Cytolytic and Antibody redirected killing assays – A flow cytometric prodium iodide / carboxyfluorescein succinimidyl ester-based assay was used to quantify the NK cell-mediated lytic activity against infected target cells, as described before (19). Viability of 5,000 target cells after incubation for 4h at 37°C with NK cells was evaluated by flow cytometry using propidium iodide (Life Technologies, UK). Unless stated otherwise, cytolytic assay with human NK cells were performed at a target:effector ratio of 1:1 and assays with porcine NK cells at a ratio of 1:25. The percentage of NK-mediated lysis was calculated using the formula: (%dead

target$_{NK}$ - %dead target$_{spont}$)/(%dead target$_{maximum}$ - %dead target$_{spont}$), as described before (19). To determine CD300a-dependent protection against NK cell-mediated killing, NK cell-mediated cytotoxicity was evaluated in the absence or presence of the CD300a-blocking IgM antibody KS153 (10 µg/ml). Followingly, the percentage of CD300a-dependent protection from NK cell-mediated lysis was calculated by subtracting the percentage of NK-mediated lysis in absence of KS153 with the percentage obtained in presence of KS153. For antibody redirected killing assays, cytolytic activity of NK cells was assessed against the murine mastocytoma FcγR$^+$ P815 cell line in the presence of medium alone, IT144 (mIgG1) anti-CD300a antibody, IgG1-isotype control antibody or anti-porcine CD16. The added specific mAbs (of IgG isotype) are bound by FcγRs on the target cells and mimic the expression of the ligands of the analyzed NK receptors, thus revealing their function.

Statistics – Statistical analysis was performed using Prism software (Graphpad, USA) based on the mean and the SEM of at least three independent replicates using a one-way ANOVA test.

SUPPLEMENTAL FIGURES

Suppl. Figure 1. Mouse monoclonal antibodies raised against human CD300a (E59/126, IT144 and KS153) recognize human CD300a. 293T cells were transfected with either a huCD300a expressing plasmid or control plasmid for 48h, and subsequently the binding of monoclonal antibodies (E59/126, IT144 an KS153) was assessed by flow cytometry. Empty profiles represents appropriate isotype-matched controls.

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Chapter 5: Pseudorabies glycoprotein B activates NK cell-mediated cytotoxicity

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ABSTRACT

Natural killer (NK) cells are members of the innate immunity and play a central role in the defense against viral infections and cancer development. NK cells are regulated by a diversity of activating and inhibitory germ-line encoded receptors on their cell surface that recognize specific ligands on the plasma membrane of potential target cells. Pseudorabies (PRV) virus, an alphaherpesvirus, has previously been reported to induce NK cell-mediated cytotoxicity towards infected cells, although the viral factors that trigger NK cell reactivity are unknown. Here, we report that glycoprotein gB of PRV triggers NK cell-mediated cytotoxicity. The ability of gB to induce NK cell reactivity does not depend on sialylation of gB. PILRβ is an activating NK cell receptor that is closely related to the inhibotory PILRα protein that has been described previously to interact with PRV and herpes simplex virus 1 (HSV-1) gB. We found that recombinant mouse PILRβ does not interact with PRV gB, but does bind HSV-1 gB. In conclusion, we found that PRV gB provides an activating signal to NK cells, but cannot draw firm conclusions as of yet regarding a possible involvement of the activating NK cell receptor PILRβ.

INTRODUCTION

Natural killer (NK) cells are members of the innate immunity and play a central role in the defense against viral infections and cancer development. NK cells are regulated by a diversity of activating and inhibitory germ-line encoded receptors on their cell surface that recognize specific ligands on the plasma membrane of potential target cells. By these, still increasing, group of NK receptors, NK cells can sense a wide array of alterations in cell surface profile of target cells and respond immediately without the need to clonally expand (1, 2). The pseudorabies (PRV) virus, an alphaherpesvirus, has previously been reported to induce NK cell-mediated cytotoxicity towards infected cells ((3) and Chapter 4), although the viral factors that trigger NK cell reactivity are unknown. Previous studies have suggested that viral envelope glycoproteins, that are expressed in the plasma membrane of infected cells, may modulate NK cell reactivity. Both for HSV-1 and PRV, viral glycoproteins gB and/or gC have been suggested to possibly influence the activity of NK-like cells (4-6).

Here, we report that PRV gB directly triggers NK cytotoxicity towards PRV-infected cells. Intriguingly, the glycoprotein B is highly conserved among alphaherpesviruses and herpesviruses in general. During viral entry in host cells, HSV-1 and PRV gB have previously been demonstrated to interact with a member of the paired immunoglobulin-like type 2 (PILR) family, PILRα. In infected cells, gB is expressed in different host cell membranes, including the plasma membrane (7), where it may interact with receptors on other cells, including immune cells. PILRα is an inhibitory receptor that is closely related to PILRβ, an activating receptor. In man, the inhibitory PILRα is not expressed on NK cells, while NK cells do express the activating PILRβ (8, 9). Mouse NK cells also express the activating PILRβ receptor, but whether PILRα is also expressed remains elusive (10).

In this chapter, we characterize the PRV gB-dependent activation of NK cytotoxicity, we report that mouse PILRβ can bind HSV-1 gB and we discuss whether the activating PILRβ receptor may be involved in PRV gB-mediated activation of NK cells.

RESULTS

1. Expression of PRV gB triggers NK cell-mediated killing

First, we investigated whether PRV gB is involved in the recognition of PRV-infected cells by NK cells. SK cells were mock-infected or infected with wild-type (WT) PRV or isogenic gBnull virus, coincubated with IL2-primed primary porcine NK cells and subsequently assessed for viability by flow cytometry. Figure 1A shows that PRV-infected SK cells are more susceptible to NK cell-mediated lysis as compared to mock-infected SK cells, and that this increased susceptibility largely depends on expression of PRV gB. This difference in susceptibility of infected cells to NK cell-mediated lysis was not due to possible differences in virus replication efficiency, as expression levels of other viral proteins (PRV gC and PRV gD) were similar for both viruses (Figure 1B).
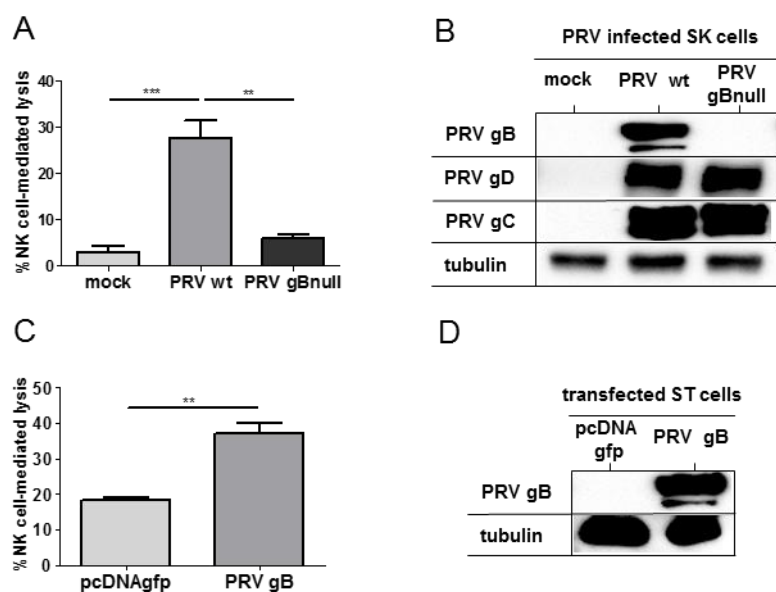
Figure 1. Expression of PRV gB is sufficient to increase NK cell-mediated killing. (A-B) SK cells were mock-infected or infected with WT PRV or isogenic gBnul PRV mutant. (A) SK cells were collected at 10 hpi and subsequently incubated with IL2-primed porcine primary NK cells at a target:effector ratio of 1:25 for 4h. Viability of target cells was assessed by propidium iodide and flow cytometry, and the percentage NK cell-mediated lysis was calculated. Data represent mean + SEM of three independent repeats (** p<0.01, *** p<0.001). (B) SK cells were collected at 12 hpi and subsequently analyzed by Western blotting for expression of gB, gC, gD and tubulin. (C-D) ST cells were transfected with eGFP-expressing plasmid or cotransfected with eGFP- and an endocytosis-negative PRV gB-expressing plasmid, and subsequently at 24 hpt (C) incubated with IL2-primed porcine primary NK cells at a target:effector ratio of 1:25 for 4h or (D) assessed by Western blotting for expression of gB and tubulin. Viability of target cells was assessed by propidium iodide and flow cytometry, and the percentage NK cell-mediated lysis was calculated. Data represent mean + SEM of three independent repeats (** p<0.01).

To further investigate whether expression of PRV gB is sufficient to increase susceptibility of cells towards NK cell-mediated lysis, transfection assays were performed. Therefore, ST cells

were co-transfected with an eGFP- and an endocytosis negative PRV gB-expressing plasmid or transfected with the an eGFP-expressing plasmid alone as a control. This endocytosis-negative PRV gB mutant was initially chosen to ensure high expression of PRV gB on the plasma membrane of transfected cells. Due to low transfection efficiency of porcine cell lines, transfected cells were enriched by FACS cell sorting based on eGFP-expression before coincubation with IL2-primed porcine primary NK cells. As shown in Figure 1C, ST cells expressing PRV gB were substantially more susceptible to NK cell-mediated lysis, compared to cells transfected with the eGFP plasmid alone. Western blot analysis confirmed expression of PRV gB in transfected ST cells (Figure 1D) and cell surface expression was previously described (11). In conclusion, these data indicate that PRV gB has the previously uncharacterized ability to trigger NK cell-mediated cytotoxicity.

2. <u>PRV gB activates porcine NK cells independent of sialic acids on infected cells and is not recognized by a recombinant murine homologue of the activating NK cell receptor PILRβ.</u>

Sialylation of HSV-1 gB and another PILR ligand, CD99, has been reported to be crucial for their interactaction with PILRα (10, 12, 13). To further investigate the mechanism by which PRV gB increases susceptibility towards NK cell-mediated lysis and the possible involvement of the activating NK receptor PILRβ, the contribution of sialylation on the infected cell surface towards NK cell-mediated lysis was assessed, and binding of recombinant murine PILRβ to cells infected with WT or gBnull PRV was examined.

SK cells were mock-infected or infected with WT PRV virus or isogenic gBnull virus for 10h and subsequently treated or not with neuraminidase, which removes cell surface associated sialic acids, and subsequently assessed for susceptibility to porcine NK cell-mediated lysis. Figure 2A shows that neuraminidase treatment did not alter NK cell susceptibility of PRV-infected cells. Although we do not provide direct evidence of sialic acid removal, identical neuraminidase treatment of the porcine NK cells instead of the target SK cells abrogated the cytotoxic response towards PRV-infected SK cells, while still retaining the (sialic acid independent) cytolytic activity against mouse RAW cell line (Suppl. Fig. 1A). Unfortunately, recombinant porcine PILR receptors are not available. However, murine recombinant PILRβ is available. As a first step to investigate a possible involvement of the activating PILRβ member of the paired PILR receptors in PRV gB-induced NK cell cytotoxicity, binding of recombinant

murine PILRβ towards mock-infected or cells infected with PRV WT or isogenic gBnull virus was assessed by flow cytometry. Although PRV-infected cells showed a trend of slightly increased murine PILRβ binding, no statistically significant differences were found between mock-infected and PRV-infected cells, indicating that PRV infection does not substantially alter ligand presentation towards the murine PILRβ receptor.
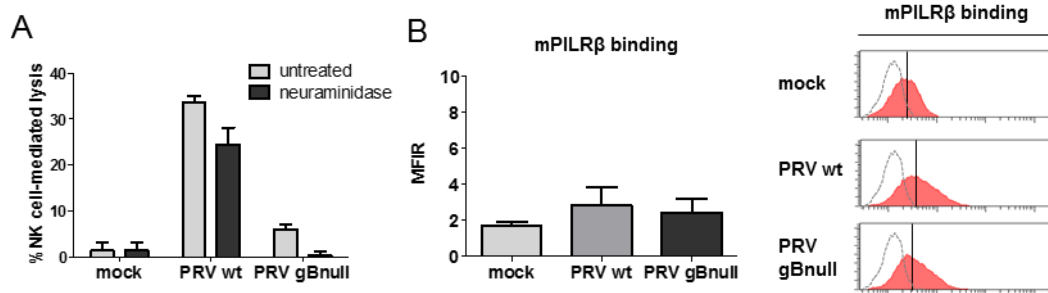
Figure 2. PRV gB activates porcine NK cells independently of cell surface sialylation on infected cells and isn't recognized by the mouse homologue of activating NK receptor PILRβ. (A-B) SK cells were mock-infected or infected with WT PRV or isogenic gBnul PRV mutant. (A) SK cells were collected at 10 hpi, mock-treated or treated with 30mU/ml neuraminidase to remove sialic acids, and subsequently incubated with IL2-primed porcine primary NK cells at a target:effector ratio of 1:25 for 4h. Viability of target cells was assessed by propidium iodide and flow cytometry, and the percentage NK cell-mediated lysis was calculated. (B) SK cells were collected at 12 hpi and subsequently assessed by flow cytometry for binding of recombinant mouse PILRβ-Fc. Graphs in (A) and (B) represent mean + SEM of three independent repeats. Vertical lines indicate median fluorescence intensity. Dotted line histogram represents isotype-matched antibody control signal.

3. <u>HSV-1 infected cells are recognized by the mouse homologue of the activating NK receptor PILRβ, which is partly dependent on the presence of sialic acids on the infected cell surface.</u>

To obtain a more complete picture of the putative interaction of alphaherpesvirus-infected cells with PILRβ, we assessed binding of recombinant murine PILRβ with HSV-1-infected cells. 293T cells were either mock-infected or infected with HSV-1, collected at 16 hpi and subsequently assessed for binding of mPILRβ-Fc and expression of HSV-1 gB by flow cytometry (Figure 3A-B). HSV-1 infected cells showed strongly increased binding of mPILRβ-Fc compared to mock-infected cells, suggesting that HSV-1 gB may be recognized by the murine homologue of this activating paired PILR receptor. The ability of HSV-1 gB to bind recombinant murine PILRβ was confirmed by transfection studies (Figure 3C). To determine whether sialic acids on HSV-1 infected cells are involved in murine PILRβ binding, as reported before for human PILRα (12), sialylation of HSV-1 infected cell surface was removed by neuraminidase treatment. To this end, 293T cells were mock-infected or infected with HSV-1 for 16h and subsequently treated with neuraminidase before being assessed for mPILRβ-Fc

binding by flow cytometry. Interestingly, removal of sialic acids only partially reduced binding of mPILRβ and increasing neuraminidase concentration did not provide an additional reduction in mPILRβ-Fc binding. This implicates that sialic acids are involved in but not absolutely required for binding of HSV-1 gB to murine PILRβ, in contrast to binding of HSV-1 to human PILRβ. In conclusion, HSV-1 gB is able to bind recombinant murine PILRβ and this partly depends on sialic acids on gB.
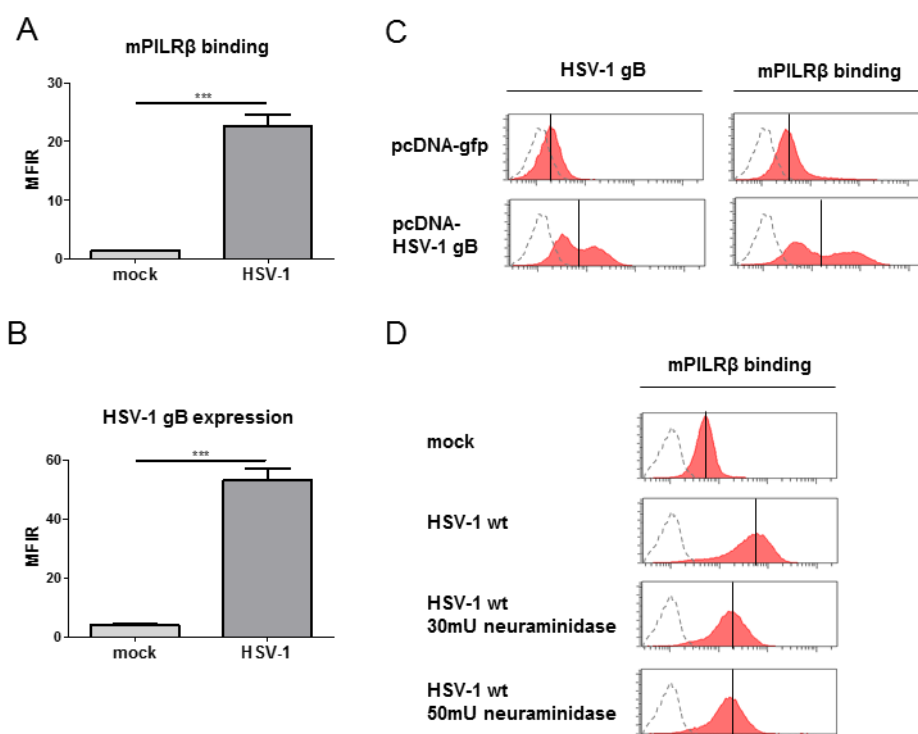
Figure 3. HSV-1 infected cells are recognized by the mouse homologue of activating NK receptor PILRβ, partly dependent on presence of sialic acids on infected cells. (A-B) 293T cells were mock-infected or infected with WT HSV-1 for 16h and assessed by flow cytometry for binding of recombinant mouse PILRβ-Fc and expression of HSV-1 gB. Data represent mean + SEM of three independent repeats (*** p<0.001). (C) 293T cells were transfected with either HSV-1 gB expressing plasmid or control plasmid pcDNA-gfp, and at 48h after transfection assessed by flow cytometry for binding of recombinant PILRβ-Fc. (D) 293T cells were mock-infected or infected with WT HSV-1 for 16h, treated with neuraminidase (30 or 50 mU/mL) for 30 min at 37°C and subsequently assessed by flow cytometry for binding of recombinant PILRβ-Fc. (C-D) Vertical lines indicate median fluorescence intensity. Dotted line histogram represents isotype-matched antibody control signal. Data represents one repeat of two independent repeats.

DISCUSSION

The current study demonstrates that PRV gB has the previously uncharacterized ability to trigger NK cell-mediated cytotoxicity. The ability of gB to induce NK cell reactivity does not depend on sialylation. In addition, we found that mouse PILRβ homologue does not interact with PRV gB, but does bind HSV-1 gB, which only partly depends on sialylation. The

mechanism by which PRV gB increases susceptibility of infected and transfected cells to a cytotoxic NK cell response remains elusive. One hypothesis is that PRV gB either increases ligand exposure towards activating NK receptors or decreases ligand exposure towards inhibitory NK receptors.

We found that sialylation of the infected cell surface was not involved in the recognition of infected cells by NK cells, since neuraminidase treatment of PRV-infected cells did not affect their susceptibility to NK cell-mediated lysis. Although we provide no direct evidence for sialic acid removal by the neuraminidase treatment, a similar neuraminidase treatment reduced the affinity of murine PILRβ to HSV-1-infected cells. In addition, a similar neuraminidase treatment of the porcine NK cells abrogated the NK cell mediated lysis of PRV-infected cells. This confirms the efficiency of the neuraminidase treatment and also suggests that sialic acids on the NK cell surface are involved in recognition of PRV-infected cells. Several studies indicate that multiple NK cell receptors, such as natural cytotoxicity receptors (NCRs) and the activating NK cell co-receptor 2B4, require sialylation for ligand recognition (14-16). It will therefore be interesting in future studies to investigate the possible involvement of such sialylated NK receptors in recognition of PRV-infected cells.

HSV-1 gB has been reported to interact with the human inhibitory receptor PILRα (hu PILRα), but not with the related activating huPILRβ receptor, a process that contributes to viral entry in host cells (17, 18). HSV-1 gB has also been reported to bind mouse PILRα (mPILRα) (17). Human and mouse PILRα have also been reported to mediate entry of PRV (19). To our knowledge, it had not been previously investigated whether HSV-1 gB and/or PRV gB are able to bind mPILRβ. Here, we show that PRV gB does not detectably bind mPILRβ while HSV-1 gB substantially binds mPILRβ. Neuraminidase treatment reduced this mPILRβ substantially. Again, we did not confirm absence of sialylation in neuraminidase-treated cells, but further increasing neuraminidase concentration did not result in further decreased mPILRβ binding, suggesting that HSV-1 gB-mediated mPILRβ binding is only partly sialic acid-independent. The interaction of HSV-1 gB with huPILRα has been extensively described. HSV-1 gB contains two O-glycolated amino-acids [threonine 53 (T53) and T480] that interact with a siglec (sialic acid-binding immunoglobulin-tupe lectin) domain on huPILRα. Removal of sialic acids or preventing O-glycolisation of HSV-1 gB abrogates the interaction of HSV-1 gB with huPILRα. This study also identified three important amino acids, Y2 (tyrosine), R95 (arginine) and W108 (tryptophan), in the siglec domain of huPILRα that were critical for HSV-1 gB binding. Mutation of either of these three amino acids separately was sufficient to inhibit huPILRα

binding to HSV-1 gB (12, 19, 20). Interestingly, huPILRβ differs from huPILRα in one of the three critical amino acid residues, containing a leucine at the W108 position, and this has been demonstrated to explain the inability of huPILRβ to engage HSV-1 gB (20). To better understand the possible binding affinities of gB of HSV-1 and PRV with PILR members of different species, we aligned the available human and murine PILR amino acid sequences and predicted porcine PILR amino acid sequences (predicted based on computational analysis of the complete mapped porcine genome) (Figure 4). This alignment provided that murine PILRβ contains two of the three critical amino acids residues, R95 and W108, necessary for HSV-1 gB binding. This observation is in line with our findings that recombinant mPILRβ binds HSV-1 gB.

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PILRalpha_human    MGRPLLLP-LLPL------LLPPAFLQPSGSTGSGPSYLYGVTQPKHLSASMGGSVEIPF 53
PILRbeta_human     MGRPLLLP-LLLL------LQPPAFLQPGGSTGSGPSYLYGVTQPKHLSASMGGSVEIPF 53
PILRalpha_pig      MGLPLLLP-LLLLLLLLLLLLPPASLQGDHWAQPKALFHFGMNQQKQLSAPEGGSIRIPF 59
PILRbeta_pig       MGLPLLLP-LLLP---LLLLLPPASLQGDHWAQLKARFDYGMNQPTQLSAPGGGSIRIPF 56
PILRalpha_mouse    MALLISLPGGTPAMAQILLLLSSACLHAGNSERSNRKNGFGVNQPESCSGVQGGSIDIPF 60
PILRbeta_mouse     MALLISLPGGTPAMAQVLLLLSSGCLHAGNSERYNRKNGFGVNQPERCSGVQGGSIDIPF 60
                   *.  : **          * ...*: .          :*:.*  *.  ***: ***

PILRalpha_human    SFYYPWELATAPDVRISWRRGHFHRQSFYSTRPPSIHKDYVNRLFLNWTEGQKSGFLRIS 113
PILRbeta_human     SFYYPWELAIVPNVRISWRRGHFHGQSFYSTRPPSIHKDYVNRLFLNWTEGQESGFLRIS 113
PILRalpha_pig      SFDYSQDSGRVSNVKISWRWKHFHGDFIYKTTPRLIHKNFKNRLLLNWEEDMKNGSLQIS 119
PILRbeta_pig       FFYYSQDSGSVSNVIISWRWKHFHGDFIYKTTPRLIHNNFKNRLLLNWEEGMKNGSLQIS 116
PILRalpha_mouse    SFYFPWKLAKDPQMSIAWRWKDFHGEFIYNSSLPFIHEHFKGRLILNWTQGQTSGVLRIL 120
PILRbeta_mouse     SFYFPWKLAKDPQMSIAWKWKDFHGEVIYNSSLPFIHEHFKGRLILNWTQGQTSGVLRIL 120
                    * :. .  .:: *:*: .** : :*.:   **:.: .**:*** :. .* *:*

PILRalpha_human    NLQKQDQSVYFCRVELDTRSSGRQQWQSIEGTKLSITQAVTTTQRPSSMTTTWRLSSTT 173
PILRbeta_human     NLRKEDQSVYFCRVELDTRRSGRQQLQSIKGTKLTITQAVTTTT--------TWRPSSTT 165
PILRalpha_pig      NLRREDEGLYFCQVEMITENYGEQKWQSIQGTKLTITHAAKTATPG----PTTSASTAAT 175
PILRbeta_pig       NLRREDEGLYFCQVEMITEKYGEQEWQSIQGTELTITHNQTTILGS----TTTAAATAG- 171
PILRalpha_mouse    NLKESDQTRYFGRVFLQT-TEGIQFWQSIPGTQLNVTNATCTPTTLP----STTAATSAH 175
PILRbeta_mouse     NLKESDQAQYFSRVNLQS-TEGMKLWQSIPGTQLNVTQALN--TTMR----SPFIVTSEF 173
                   **:..*:  ** :* : :   * :  *** **:*.:*:               .   ::

PILRalpha_human    TTTGLRVTQGKRRSDSWHISLETAVGVAVAVTVLGIMILGLICLLR--WRRRKG------ 225
PILRbeta_human     TIAGLRVTESKGHSESWHLSLDTAIRVALAVLKTVILGLLCLLLWWRRRKGSRAPSS 225
PILRalpha_pig      TAADLWDLEGKRSSGSWLLNKEAVVRLALPSAVLKIAILGLMVFLR--WKRSKGLQTKAR 233
PILRbeta_pig       ----LGTSEDQRHSP----STEAAVGIAAN--ELIIAIAGLILCL--------------- 206
PILRalpha_mouse    TQNDITEVKS-ANIG--GLDLQTTVGLATAAAVFLVGVLGLIVFLW--WKRRRQGQKTKA 230
PILRbeta_mouse     TTAGLEHTSDQRNPS--LMNLGAMVTMLLAKVLVIVLVYGWMIFLR--WKQR-------- 221
                       :  ..          . : : :     .  : * :  *

PILRalpha_human    ------------------------------------------------------------
PILRbeta_human     DF---------------------------------------------------------- 227
PILRalpha_pig      TPARGSFQNLEEKYENIGNKEQQTDPKECHKRSSSDLA---------------------- 271
PILRbeta_pig       ------------------------------------------------------------
PILRalpha_mouse    EIPAREPLETSEKHESVGHEGQCMDPKENPKDNNIVYASISLSSPTSPGTAPNLPVHGNP 290
PILRbeta_mouse     --PAH------------------------------------------------------- 224


PILRalpha_human    ------------
PILRbeta_human     ------------
PILRalpha_pig      ------------
PILRbeta_pig       ------------
PILRalpha_mouse    QEETVYSIVKAK 302
PILRbeta_mouse     ------------
```

Figure 4. Alignment of human, porcine and murine PILR receptors. The human, porcine and murine PILR r amino acid sequences were aligned with the ClustalW software. The three residues (Y2, R95 and W108) previously determined to be important for PILRα ligand recognition are highlighted in green, while variations are highlighted in blue. Porcine PILR sequences are computational predicted sequences based on the fully sequenced porcine genome.

The difference in affinity of HSV-1 gB for huPILRβ versus mPILRβ may be of relevance when studying and interpreting mouse models of HSV-1 infection, particularly regarding any effects HSV-1 and HSV-1 gB may have on the effect of specific immune cells, particularly NK cells. Interestingly, both porcine PILRβ and PILRα lack Y2 and R95, but the other critical amino acid is conserved. The Y2 and R95 of human PILR is replaced by, respectively, a phenylalanine and a glutamine in the porcine PILR sequences. Like tyrosine and arginine, phenylalanine and glutamine are polar amino acids. This makes it difficult to predict whether porcine PILRs contain a functional siglec domain, but does not exclude the possibility. Remarkably, we found that recombinant mPILRβ does not obviously bind PRV gB, although huPILRα and mPILRα have been reported to mediate PRV entry, and thus are likely to interact with PRV gB (19). This suggests that other, yet unidentified factors also play a role in determining ligand recognition by the PILR homologs.

In conclusion, we demonstrate that PRV gB triggers NK cell cytotoxicity. We also provide additional information concerning the reported ligand recognition of the PILR receptors and cannot exclude at present the possibility that porcine PILRβ may interact with PRV gB to enhance NK cell reactivity.

MATERIALS AND METHODS

Viruses and Cells – PRV Kaplan wild type virus, isogenic gBnull mutant virus and the RN009 cell line, an PRV gB-expressing RK13 cell line, were kindly provided by Thomas Mettenleiter (Friedrich Loeffler Institute, Germany) and have been described before (21). The PRV gBnull mutant virus was grown and titered on the complementing PRV gB-expressing cell line RN009. HSV-1 F strain wild type virus was kindly provided by B. Roizman (University of Chicago, USA). Porcine SK cells and ST cells were cultivated in MEM supplemented with 10% FCS, L-glutamine, sodium pyruvate and antibiotics (penicillin, streptomycin and gentamycine), as described before (3, 22). Human 293T cell line and mouse RAW cells were cultivated in DMEM supplemented with 10% FCS, L-glutamine and antibiotics (penicillin, streptomycin and gentamycin) (3, 23).

Antibodies and Reagents – Antibodies directed against PRV glycoproteins gB (mIgG2a, 1C11) and gD (mIgG1, 13D12) were provided by H. Nauwynck (Ghent University) and described before (24). Mouse monoclonal anti-PRV gC antibody (mIgG1, 8P19) was kindly provided by A. Brun (Laboratoire Institut Français de la Fièvre Aphteuse, Lyon, France) and

rabbit polyclonal anti-HSV gB antibody was kindly provided by R. Eisenberg and G. Cohen (University of Pennsylvania, USA). Mouse monoclonal antibodies against porcine markers CD3 (mIgG1, PPT3), CD4 (mIgG2b, 72-22-15), CD8α (mIgG2a, 11/295/33), and CD172a (IgG1, 74-22-15) were provided by E. Cox (Ghent University, Belgium), used and titered on freshly isolated porcine PBMC and were all described earlier (25-27). Mouse monoclonal antibodies against porcine CD16 (mIgG1, G7) and alpha-tubulin (mIgG1, DM1A) and recombinant mouse PILRβ-Fc chimera were purchased from AbD Serotec (UK), Abcam (UK) and Sino Biological Inc. (USA), respectively. Goat anti-mouse IgG1 R-PE, IgG2a AF647, and IgG2b FITC (Life Technologies, UK) and goat anti-mouse IgG MACS beads (Miltenyi Biotec, Germany) were used cell sorting. HRP-labeled polyclonal goat anti-mouse antibodies (Dako, Denmark) were used for Western blot detection. For transfection studies, control plasmid pcDNA3.1(-) was purchased from Life Technologies (UK), a pcDNA3.1-based plasmid expressing eGFP was constructed and a pcDNA3.1-based plasmid expressing endocytosis-negative PRV gB was constructed based on a previously described gB AQRL-expressing plasmid (28, 29). Insertion of the eGFP and gB AQRL genes into the pcDNA3.1(-) backbone was reported and verified in the Master thesis of B. Spiesschaert (Faculty of Veterinary Medicine, UGent, Belgium)(11).

Infections and Transfections – SK cells were detached from cell culture flasks using trypsin, seeded in suspension culture flasks (Sarstedt, Germany) at 1.2×10^6 cells/mL, inoculated at a multiplicity of infection (MOI) of 10, and put on a rocking platform at 37°C basically as described before (30). 293T cells were seeded at 2×10^5 cells/mL and inoculated the next day at an MOI of 10 (3). For transfection studies, ST cells were detached from cell culture flasks using trypsin and seeded in 6-well plates at 0.2×10^6 cells/ml. The following day, cells were transfected with JetPRIME (Polyplus Transfection, USA) according to the manufacturer's specifications for cotransfection of adherent cells. For cytolytic assays, cells were collected at 10 hours post inoculation (hpi) or 24 hours post transfection (24 hpi), and for expression analysis, cells were collected at 12 hpi or 24 hpt. When stated, cells were re-suspended in RPMI and treated with neuraminidase from *Vibrio cholerae* (Roche, Switzerland) at the indicated concentration for 30 minutes at 37°C, as previously described (12). Prior to the treatment, cells were washed and resuspended in RPMI medium. 293T cells were seeded and transfected using JetPEI (Polyplus Transfections, USA) according to the manufacturer's protocol and harvested at 48 hpt.

Western blot – Cell lysis was performed on a shaker at 4°C for 1h, nuclei were removed by centrifugation (13,000 x g, 10 min), and protein content was measured using the BCA protein assay kit (Thermo Scientific, USA) (31). Per sample, 20 μg protein was loaded on 10% acrylamide SDS/PAGE gels and transferred to a P-Hybond membrane (GE Healthcare, UK), which was afterwards blocked using 5% milk powder diluted in PBS-T [PBS supplemented with 0.1% Tween 20 (Sigma Aldrich, Germany)]. Incubations with primary monoclonal or HRP-labeled secondary antibodies were performed for 1h in 5% milk powder PBS-T at room temperature. Bands were detected using a ChemiDocMP Imager (Biorad, USA), according to the manufacturer's instructions.

Porcine primary NK cells – Blood was collected from the vena jugularis of swine (2,5 to 8 months old) and mixed with heparin (LEO Pharma, Denmark) at a final concentration of 50 units/ml blood. PBMCs were collected by density centrifugation on Lymfoprep (Axis-Shield, Scotland) density gradient medium, accordingly to the manufacturer's protocol. Red blood cells were lysed by a 10 minute osmotic shock at RT by incubating the collected buffy coat in lysis buffer [composed of 90% of NH_4Cl 0,83% (w/v) and 10% TRIS 2,06% (w/v) in distilled water at a final pH of 7,2]. After lysis of red blood cells, PBMCs were washed and, subsequently, a negative MACS selection was performed by depleting CD3- and CD172a-expressing cells. Finally, NK cells were isolated by FACS cell sorting, characterized by markers CD172a, CD3, CD4 and CD8α (3, 32). Upon isolation, NK cells were primed for 18h with 40 units/ml recombinant huIL2 (Life Technologies, UK). Expression of CD16 on sorted cells confirmed ≥98% NK purity.

Cytolytic Assays – A flow cytometric propidium iodide (PI)-based assay was used to quantify NK cell-mediated lytic activity against infected or transfected target cells, basically as described before (3). Briefly, infected or transfected target cells were coincubated with IL2 primed NK cells at an effector:target ratio of 25:1 for 4h at 37°C and subsequently analyzed by flow cytometry. To discriminate target cells from NK cells, infected target cells were treated with 5μM CFSE (Life Technologies, UK) prior to the assay and transfected target cells were cotransfected with an eGFP-expressing plasmid. Due to the low transfection efficiency of porcine cell lines, transfected cells, identified by eGFP expression, were enriched by FACS cell sorting to a final purity of 80% before the cytotoxicity assay. Viability of 5,000 target cells was evaluated by flow cytometric using PI (Life Technologies, UK). The percentage of NK cell-mediated lysis was calculated using the formula: (%dead target$_{NK}$ - %dead target$_{spont}$)/(%dead

target$_{maximum}$ - %dead target$_{spont}$). The percentage dead target$_{maximum}$ was determined by incubating target cells with 0.1% Triton X-100 (Sigma Aldrich, Germany).

Flow Cytometric Analysis – Cells were collected by gently pipetting, incubated on ice with primary mouse antibodies or recombinant mouse PILRβ-Fc (10 μg/mL), and subsequently washed and incubated on ice with R-PE labeled goat anti-mouse or goat anti-human secondary antibodies (Life Technologies, UK), respectively. Analysis was performed on 20,000 living cells [discriminated via Sytoxblue dye staining (Life Technologies, UK)] with a FACSAria III flow cytometer and FACSDiva software (BD Biosciences, Belgium).

Statistics – Statistical analysis was performed using Prism software (GraphPad) based on the mean and the SEM of at least three independent replicates, using a one-way ANOVA test.

SUPPLEMENTAL FIGURES
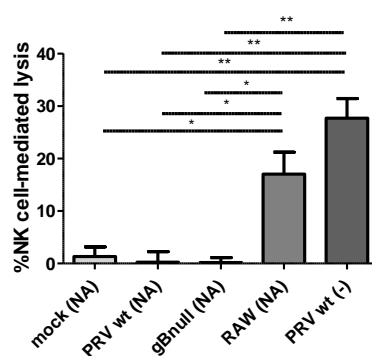
Supplemental Figure 1. Recognition of PRV-infected cells by porcine NK cells is highly dependent of NK cell surface sialylation. SK cells were mock-infected or infected with WT PRV or isogenic gBnull PRV mutant for 10 hpi. Infected SK cells and mouse RAW cells, as control, were collected and subsequently incubated with IL2-primed porcine primary NK cells, which were previously treated with 30mU/mL neuraminidase (NA), at a target:effector ratio of 1:25 for 4h. WT PRV-infected SK cells incubated with untreated IL2-primed porcine primary NK cells were included in the assay and indicated as PRV wt (-). Viability of target cells was assessed by propidium iodide and flow cytometry, and and the percentage NK cell-mediated lysis was calculated. Data represent mean + SEM of three independent repeats (** p<0.01).

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Chapter 6: General discussion

Herpesviruses have developed complex and often subtly fine-tuned interactions with the immune system of their host. As a result, these viruses typically cause relatively mild, although economically important and socially discomforting disease symptoms and are able to persist lifelong in a latent state in their host. The innate immunity, Natural Killer (NK) cells in particular, has previously been extensively reported to be important in alphaherpesvirus biology (1-9). Consequently, NK cell related deficiencies are associated with aggravated, potentially life-threatening, alphaherpesvirus disease, including encephalitis (1-3). Remarkably, the mechanisms by which alphaherpesviruses activate or suppress NK cells remain largely unstudied (5, 6).

In the current doctoral dissertation we identified three alphaherpesviral proteins, PRV/HSV-2 gD (Chapter 3), PRV US3 (Chapter 4) and PRV gB (Chapter 5), that modulate NK cell activity and investigated the NK cell receptors and ligands involved.

1) <u>The interference of alphaherpesvirus glycoprotein D with CD112, a ligand for the activating NK receptor DNAX accessory molecule-1 (DNAM-1)</u>

We reported that the gD glycoprotein of PRV and HSV-2 interferes with binding of the activating NK cell receptor DNAM-1 to the surface of infected cells, thereby identifying an NK evasion strategy of alphaherpesviruses. Expression of gD leads to reduced cell surface availability and degradation of its cellular ligand CD112 (nectin-2). This, in turn, reduces binding of the activating NK cell receptor DNAM-1 and, consequently, decreases NK-mediated lysis of gD expressing cells.

Interestingly, the UL141 protein of the betaherpesvirus HCMV is reported to similarly protect infected cells from NK cell-mediated lysis by reducing the cell surface availability of the DNAM-1 ligands CD112 and CD155 (10, 11). In HCMV-infected cells, UL141 sequesters both CD112 and CD155 in the endoplasmic reticulum. In the absence of other viral HCMV proteins present, UL141 can only efficiently reduce CD155 cell surface expression, but does not affect CD112. Another HCMV protein UL2 has been identified to co-operate with UL141 to reduce and degrade CD112. UL2 was found to mediate TRC-8 ubiquitin ligase-dependent ubiquitination of CD112, thereby licensing CD112 for proteasomal degradation, while UL141 aids in promoting CD112 retention and UL2 accessibility (12, 13). Neither HCMV UL141 nor UL2 displays any amino acid sequence similarity to alphaherpesvirus gD, pointing to a convergent evolution in the herpesvirusfamily. Both alphaherpesvirus and betaherpesvirus

families have developed distinct mechanisms to evade DNAM-1-dependent NK cell-mediated lysis, emphasizing the importance for different viruses to circumvent the activating NK receptor DNAM-1 (14).

We observed that during infection, HSV-2 gD decreases CD112 cell surface availability, and consequently DNAM-1 binding, less drastically compared to PRV gD. Interestingly, PRV gD contains a functional endocytosis motif (YRLL) in its cytoplasmic domain, which is not present in HSV-2 gD. This motif drives endocytosis of PRV gD from the cell surface early in infection and contributes to the efficient internalization of antibody-antigen complexes in PRV-infected monocytes (15-17). Potentially, this endocytosis motif could contribute to CD112 internalization and degradation. However, as reported in the Master thesis of T. Delahaye (UGent), we observed that transfection with endocytosis-negative PRV gD mutants led to a downregulation of CD112 cell surface levels to a similar extent as compared to transfection with PRV gD wt (18). The latter indicates that PRV gD reduces CD112 cell surface availability independently of its endocytosis motif.

PRV and HSV-2 gD also differ in the way they engage the cellular ligand CD111 (19, 20). Although it is not known whether this is also reflected in a difference in CD112 binding, this may account for the observed difference in CD112 reduction observed between PRV and HSV-2 gD. Of potential interest in this context, we found that transfection with HSV-2 gD, in contrast to PRV gD, did not reduce CD112 cell surface availability (Chapter 3 and Suppl. Figure 1). Since HSV-2 gD-mediated downregulation of CD112 is only observed during infection, this may suggest that HSV-2 gD relies on the expression of another viral protein to downregulate CD112. This would be in line with the co-operation between HCMV UL141 and UL2 to downregulate CD112 (12). Such co-operation with putative other viral proteins appears to be less critical for PRV gD, although we did observe that PRV gD-mediated CD112 degradation is less dramatic during transfection than during infection. For HCMV, the accessory viral protein UL2 that is required for CD112 downregulation contributes to CD112 ubiquitination (12). Possibly in line with this, infection with both wt and gDnull PRV and HSV-2 increased the apparent molecular weight of CD112, detected by Western blotting analysis, suggesting a post-translational modification of CD112 (Suppl. Figure 1). It remains to be studied whether or not this putative post-translational CD112 modification represents ubiquitination. We hypothesize that gD of PRV, but not of HSV-2, has the ability to internalize and to a lesser extent degrade CD112 on itself during transfection, while (unidentified) accessory viral protein(s) are beneficial (PRV) or required (HSV-2) for CD112 degradation during infection.

Our data also underscore the conservation of the DNAM-1 receptor activity over different species, as we showed that PRV gD-mediated interference with CD112 and consequent protection against NK-mediated cell lysis was also observed in a system with porcine target cells and porcine effector NK cells. Although the porcine genome was known to encode a DNAM-1 homologue, our RT-PCR data for the first time demonstrated the expression of DNAM-1 in porcine NK cells. Remarkably however, alignment of human, mouse, rat, bovine and porcine DNAM-1 homologues revealed that the porcine DNAM-1 homologue has only one immunoglobulin (Ig)-like domain in its extracellular region, while DNAM-1 homologues of other species contain two Ig-like domains (Figure 2). This is particularly interesting as a study on the crystal structure of human DNAM-1 found indications that these Ig-like domains directly engage CD112 for ligand binding (21). This study proposed two possibilities for CD112 engagement by DNAM-1, as shown in Figure 1.
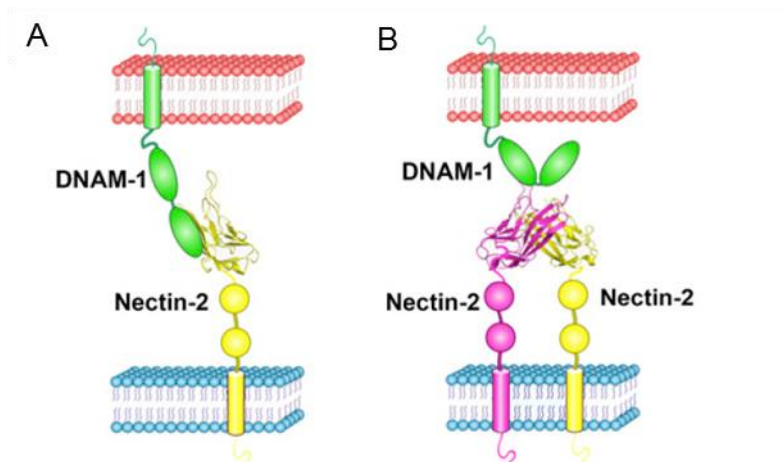
Figure 1. Two possible binding models of CD112 with human DNAM-1. Human DNAM-1 is hypothesized to engage CD112 via two possible ways: (A) binding to CD112 monomers by the outer Ig-like domain in DNAM-1 or (B) binding to CD112 cis-homodimers by the inner Ig-like domain (Figure adapted from: (21)).

The outer Ig-like domain of DNAM-1 could potentially bind CD112 monomers, while the inner Ig-like domain is suggested to preferably bind CD112 homodimers. The single Ig-like domain in pig shows homology to the outer Ig-like domain of other species. Interestingly, in mice, ligand recognition of a naturally occurring DNAM-1 splice variant, lacking the outermost Ig-like domain, was drastically reduced (22). In addition, ligand recognition of a human DNAM-1 mutant lacking the inner Ig-like domain was found to be similar to recombinant full length human DNAM-1 (23). Taking these data together, we hypothesize that the outer Ig-like domain of DNAM-1 homologues is of uttermost importance for CD112 binding. The role of the inner Ig-like domain largely remains to be addressed.

```
bovine_DNAM-1   ------------MDHLTLLLAILYVYTAPCEEIFWDTTVKLAKNMTLECVYPLADTVTQ 47
porcine_DNAM-1  ------------MDYLTFFLVILYVYKALCEEVFWDTTVKLAENMTLECVYPSMNTLTQ 47
human_DNAM-1    ------------MDYPTLLLALLHVYRALCEEVLWHTSVPFAENMSLECVYPSMGILTQ 47
mouse_DNAM-1    ------------MAYVTWLLAILHVHKALCEETLWDTTVRLSETMTLECVYPLTHNLTQ 47
rat_DNAM-1      MWKAVQPPEPFPEMAYLTWLLAILHVHKALCEETLWDTTVRLSETMTLECVYPLTDNLTQ 60
                           * : * :*.:*:*: * *** :*.*:* :::.*:***** :**

bovine_DNAM-1   MEWFKI-KMERESMAIFNPAFHGIIREAYTDRVYFSNHTMASNDMTLSFHNASEADVGFY 106
porcine_DNAM-1  MEWFKINMMERESIAIFNPTYGVIVRKPYADRVYFSNLTNASNDMSLSFHNASEADIGFY 107
human_DNAM-1    VEWFKI-GTQQDSIAIFSPTHGMVIRKPYAERVYFLNSTMASNNMTLFFRNASEDDVGYY 106
mouse_DNAM-1    VEWTKNTGTKTVSIAVYNPNHNMHIESNYLHRVHFLNSTVGFRNMSLSFYNASEADIGIY 107
rat_DNAM-1      AEWTKDTGTKKVNIAVYHPNHNMHIDPNYLHRVHFQNATVGFRNMSLSFYNASEADIGIY 120
                 ** *     :  .:*:: * . :   * .**:* * *  . .:*:* * **** *:* *

bovine_DNAM-1   SCLLHTFPHGTWEKVIQVVPPDNFEDAGPPNSQMVSEPGKNITLTCEPRENHLLEEVSWE 166
porcine_DNAM-1  SCSLHTFPHGPWKKMIQVVPSD-----------------------------LPAATW- 135
human_DNAM-1    SCSLYTYPQGTWQKVIQVVQSDSFEAAVPSNSHIVSEPGKNVTLTCQPQMTWPVQAVRWE 166
mouse_DNAM-1    SCLFHAFPNGPWEKKIKVVWSDSFEIAAPSDSYLSAEPGQDVTLTCQLPRTWPVQQVIWE 167
rat_DNAM-1      TCLFHAFPRGSWEKKIKVVQSDSFETTAPLDSYLSAEPGQNVTLSCQLERTWLVQLVIWE 180
                :* ::::*.*.*:* *:** .*             : . *

bovine_DNAM-1   KIQPQQIDLLISCNLSQGRNYTSKYPRQILSNCNKGMKKAFVTMPNVIGSDSGLYRCGFK 226
porcine_DNAM-1  -----------------------FP-------------------------KCSF- 141
human_DNAM-1    KIQPRQIDLLTYCNLVHGRNFTSKFPRQIVSNCSHG-RWSVIVIPDVTVSDSGLYRCYLQ 225
mouse_DNAM-1    KVQPHQVDILASCNLSQETRYTSKYLRQTRSNCSQGSMKSILIIPNAMAADSGLYRCRSE 227
rat_DNAM-1      KVQPHQVDILASCNLSQGTRYTSKYLRQTWSDCSQESMKSALIIPYAMAADSGLYRCRSE 240
                       :                                    :*

bovine_DNAM-1   ASTGEKETFVMRLTVTNGETKNSYIPFVAGGAVLLFLLVILITTITVITYKRKKRQQKRG 286
porcine_DNAM-1  ---------------------IFQFLGSGQALCHLLP---PPIASCVYRG---QQSTH 172
human_DNAM-1    ASAGENETFVMRLTVAEGKTDNQYTLFVAGGTVLLLLFVISITTIIVIFLNRRRRRERRD 285
mouse_DNAM-1    AITGKNKSFVIRLIITDGGTNKHFILPIVGG--LVSLLLVILIIIIFILYNRKRRRQVRI 285
rat_DNAM-1      ASTGTNKTCVIRLIITDGGTNNHFILLIVGG--LASLLLVILITIIIILFNRKRRRQVRT 298
                             : .*  *  *:      *      .   ::

bovine_DNAM-1   LFKDVWITQNRATNNYRNPFPPNPPAGG----AGEDIYVNHPVFCLGPRARV 334
porcine_DNAM-1  DFSPALHLQN-----------------------------------------  182
human_DNAM-1    LFTESWDTQK-APNNYRSPISTGQPTNQSMDDTREDIYVNYPTFSRRPKTRV 336
mouse_DNAM-1    PLKEPRDKQSKVATNCRSPTSPIQSTDD----EKEDIYVNYPTFSRRPKPRL 333
rat_DNAM-1      PLKELRDKQSKVATNCRSPTSPIQTTDD----EKEDIYVNYPTFSRRPKPRF 346
                :.      *.
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Figure 2. Alignments of bovine, porcine, human, mouse and rat DNAM-1 protein sequences reveal that porcine DNAM-1 homologues lacks the second Ig-like domain. Alignments were made using the ClustalW software with available sequences (Genbank identifiers: bovine DNAM-1, XP_010817044.1; porcine DNAM-1, XP_003480484.1; human DNAM-1, AAC50560.1; mouse DNAM-1, BAD90660.1; rat DNAM-1, NP_001100840.1). The signal sequence (--), the first and second Ig-like domains (--) and the transmembrane domain (--) of human DNAM-1 are marked in respective colors, as predicted by Expasy and Phobius prediction software. The absence of the second Ig-like domain of the porcine DNAM-1 homologue was confirmed by sequencing the obtained DNAM-1 sequence from freshly isolated porcine NK cells (Chapter 3).

Another peculiarity is the relationship of HSV-1 with CD112. HSV-1 does not typically interact with CD112, but several single mutations in HSV-1 gD can alter ligand recognition in favor of CD112 (24). We demonstrated that the interaction of PRV and HSV-2 gD with CD112 provides a protective effect to NK cell-mediated killing. Since HSV-1 gD does not typically interact with CD112, there may be specific reasons for this virus not to deploy this NK evasion strategy. Interestingly, particular clinical HSV-1 isolates derived from patients suffering from HSV-1 encephalitis were found to display the uncommon ability to interact with CD112 (25). Also, impaired NK cell functionality has been associated with HSV-1 encephalitis (1-3). Hence, for HSV-1, the virus/NK cell balance may be particularly delicate. Disturbance of this balance, e.g.

via dysfunctional NK cells or increased NK-evasive properties of the virus, may lead to aggravated disease that has no evolutionary benefit, either for host or virus. Of course, this reasoning is still speculative at this point. Therefore, it would be important to study whether CD112 interacting HSV-1 clinical isolates do show increased NK-evasive properties and whether these mutants are indeed associated with increased encephalitic prevalence and/or aggravated disease.

2) PRV US3 protein kinase protects from NK cell-mediated lysis via increased binding of the inhibitory NK receptor CD300a

In Chapter 4, we reported a previously uncharacterized viral NK cell evasion strategy consisting of a virus-triggered increased binding of the inhibitory NK cell receptor CD300a to infected cells. By using PRV mutants and specific inhibitors we demonstrated that this effect depends on the kinase activity of US3, on the activity of cellular group I p21-activated kinases (PAK) and on exposure of CD300a ligands like phosphatidylserine (PS) and phosphatidyl-ethanolamine (PE).

Surprisingly, HSV-1 US3 has been reported to trigger increased NK cell activity. Indeed, expression of US3 during HSV-1 infection of mouse embryonic fibroblasts was associated with a downregulation of MHC I (26). This downregulation resulted in suppressed killing by cytotoxic T lymphocytes but, since MHC I is a ligand for the murine inhibitory Ly49 NK cell receptor, also triggered an increased NK cell-mediated IFNγ response (26). Expression of HSV-1 US3 during infection of a human lymphoblastoid cell line, in which HSV-1 US3 does not mediate MHC I downregulation, did not affect lysis of these cells by NK cell-like lymphokine-activated (LAK) cells (27). These studies indicate that HSV-1 US3 may not have NK cell-evasive properties, but rather may elicit, in particular cell types, an NK cell response through MHC I downregulation on the infected cell surface. In line with the lack of NK cell-evasive properties of HSV-1 US3, we found that HSV-1 US3 was only minimally involved in increased binding of CD300a to HSV-1-infected 293T cells (Suppl. Figure 2). Nevertheless, also in HSV-1-infected cells, we did see a strongly increased CD300a binding (Suppl. Figure 2), indicating that this effect is conserved over different alphaherpesviruses, although the underlying mechanisms may be different.

Several of the effects that have been earlier associated with US3 may have an impact on NK cell activity.

First, similar to HSV-1 US3, PRV US3 downregulates MHC I. However, this effect appeared to be highly cell type-specific, and was only observed in ST (swine testicle) cell cultures, but not in PK-15 (porcine kidney) cell cultures or primary porcine alveolar macrophages (28). In line with this, although we did observe PRV-induced MHC-I downregulation in SK (swine kidney) cell cultures and primary porcine kidney epithelial cells in our studies, this did not depend on US3. Hence, it would be interesting to determine in ST cells whether PRV US3 expression is associated with MHC I-dependent decreased NK cell cytotoxicity.

Secondly, PRV US3 also dramatically alters the actin cytoskeleton of several adherent cell types by breaking down actin stress fibers and formation of large cell protrusions (29-31). This is particularly interesting as the HCMV pUL135 protein has been reported to induce a similar break-down of filamentous actin in adherent human foreskin fibroblasts, which has been associated with impaired recognition of HCMV-infected cells by NK cells, possibly through impaired synapse formation between the NK cell and its target cell (32). Possibly, PRV US3-mediated disruption of the actin cytoskeleton may similarly disturb NK cell synapse formation and hence cytotoxicity. This was not addressed in our studies, since we focused on suspension cells, and therefore did not display actin stress fibers or PRV-induced actin rearrangements. Hence, other experimental set-ups and/or other cell types may substantially affect the NK-modulating activity of PRV US3 and should be investigated further.

Our research group showed previously that PRV US3 phosphorylates and thereby activates group I p21-activated kinases (PAKs), critical downstream effectors of the Cdc42/Rac1 signaling pathways (30). Here, we demonstrated that inhibition of group I PAK activity prevents the ability of US3 to trigger increased CD300a binding or modulate PS exposure, as determined by anti-PS antibody 1H6. Group I PAK activity has been reported before to be critically involved in PS exposure during platelet activation (33). This interaction of PRV US3 with group I PAK kinases to modulate PS exposure and increase CD300a binding towards the infected cells can be of particular interest for several viruses and virus biology in general. For example, cells infected with Pichinde virus, a model for the Lassa fever virus, also display increased PS exposure and several other viruses, among which HIV and influenza virus, have been reported to activate PAK signaling in infected cells (34-38). Furthermore, several enveloped viruses expose PS on their surface, which disguises the virus particles as apoptotic bodies, tricking cells to engulf these virions via macropinocytosis. This cell entry mechanism is termed apoptotic mimicry and involves several PS binding receptors (39-42). Therefore, targeting group I PAK signaling and/or PS exposure during virus infection may possibly have

therapeutic potential, as decreased PS exposure could decrease spread of enveloped viruses and increase the susceptibility of infected cells to NK cell cytotoxicity (34, 43).

Finally, our data indicate that, like in human and other mammals, a functional CD300a homologue may serve as an inhibitory receptor in swine. Several studies have reported that the CD300 receptor family is highly conserved across multiple species (44, 45). This is particularly true for CD300a, illustrated by its recent identification and characterization in chicken, showing inhibitory activity and affinity for PS and PE, in line with what has been described in mammals (44).

3) The activating interaction of PRV gB with NK cells

We demonstrated that PRV gB triggers NK cell-mediated cytotoxicity. The ability of gB to induce NK cell reactivity did not depend on sialylation. In addition, we found that the murine homologue of the activating receptor PILRβ does not interact with PRV gB, but does bind HSV-1 gB. The latter was found to only partly depend on sialylation. The mechanism by which PRV gB increases susceptibility of infected and transfected cells to a cytotoxic NK cell response remains elusive. It is conceivable that PRV gB either increases ligand exposure towards activating NK receptors or decreases ligand exposure towards inhibitory NK cell receptors. The interaction of NK cells with viruses is shaped by co-evolution, where virus and NK cell have evolved mechanisms to avoid or enhance recognition, respectively (46). Our identification of PRV gB as a NK cell-activating factor is particularly interesting as this protein is essential for virus entry and spread, and represents the viral glycoprotein that shows the highest conservation across the different herpesviruses (47). Hence, the PRV virus cannot drastically adapt PRV gB to suppress its NK cell-activating effect. Identifying how PRV gB increases susceptibility could contribute greatly towards NK cell and virus biology.

Previously, specific sialylation sites on HSV-1 gB, which is highly homologous to PRV gB, have been reported to enable HSV-1 gB to interact with human PILRα (48, 49). PILRα is an inhibitory immune receptor, but is not expressed on human NK cells (50). HuPILRα not only depends on sialylation to recognize HSV-1 gB, but also to other cellular ligands, like CD99 and PILR-associating neural protein (PANP) (51). PILRα is closely related to the activating receptor PILRβ, which is expressed on human and mouse NK cells (52). Nevertheless, HSV-1 gB has been reported not to interact with human PILRβ (53).

Since we observed that, both in infection and transfection assays PRV gB expression increased susceptibility of cells towards porcine NK cell-mediated lysis, we hypothesized that PRV gB potentially could be a ligand for the porcine activating NK cell receptor PILRβ. Since the interactions of human PILRα with its targets are sialic acid dependent, we tested the effect of sialic acid removal from the surface of PRV-infected cells and found that sialylation of the infected cell surface does not appear to be involved in the recognition of PRV-infected cells by NK cells. Hence, based on the available interaction studies of huPILRα with HSV-1 gB (48, 49), this suggests that the porcine PILR homologues are not involved in the recognition of PRV-infected cells. However, we also demonstrated that HSV-1 gB substantially binds the mouse homologue of PILRβ and that neuraminidase treatment reduced but did not abolish mPILRβ binding. The latter indicates that the requirement of ligand sialylation for PILR recognition may be less conserved in other species than human. This may be particularly true for porcine PILR homologs, since analysis of predicted porcine PILR amino acid sequences showed that both porcine PILRα and PILRβ display a mutation of an arginine that is typically critical for the sialic acid binding properties of a receptor (54-56), so the interaction of porcine PILR homologs with its ligands may perhaps be entirely sialic acid-independent. It will be crucial to generate recombinant porcine PILR homologs and porcine PILR-specific antibodies to be able to set up conclusive experiments concerning a possible role for porcine PILRβ in the NK cell-activating effect of PRV gB.

Interestingly, unlike neuraminidase treatment of infected target cells, neuraminidase treatment of the porcine NK cells abrogated the NK cell-mediated lysis of PRV-infected cells. This indicates that sialic acids on the NK cell surface are involved in recognition of the PRV-infected cells. Several studies indicate that multiple NK receptors, such as natural cytotoxicity receptors (NCRs) and the activating NK co-receptor 2B4, require sialylation for ligand recognition (57-59). NCRs are involved in the recognition of cells infected by various viruses, among which cells infected with the alphaherpesvirus HSV-1 (5, 60). Transfection studies showed that the HSV-1 ICP0 protein was sufficient to trigger an upregulation of ligands for all three NCRs: NKp30, NKp44 and NKp46 (5). Interestingly, HSV-1 ICP0 homologues are present in other alphaherpesviruses, like PRV and HSV-2. These ICP0 homologues share a common RING-finger domain, involved in protein-protein interactions, but lack further homology (61). We studied the ability of recombinant human NCRs to bind to PRV-, HSV-1- and HSV-2-infected 293T cells, which was described in the Master thesis of R. Haeck (UGent) (62). Remarkably, only infection of HSV-1, as previously reported (5), resulted in increased binding of

recombinant human NCR receptors to infected cells. Neither HSV-2 nor PRV triggered increased human NCR binding. These data indicate that, in this particular experimental model, the NCRs are not involved in the recognition of PRV- and HSV-2-infected cells. However, this does not exclude the possibility that PRV-infected cells may be recognized by porcine NCR homologues, present in the porcine genome (63).

Unfortunately, current reagents and tools directed against porcine NK receptors are limited and only antibodies directed against porcine NKp46 have been reported (64). This single porcine NK receptor tool has already generated interesting and unexpected information. Indeed, porcine blood NK cells are heterogeneous in their expression of NKp46, which was previously suggested to be a pan-marker protein for NK cells across mammalian species (64). Both NKp46$^+$ and NKp46$^-$ porcine NK cell populations were reported, and both appeared to display similar cytolytic capability (64). Using the porcine NKp46 antibody VIV-KM1, we have performed cytolytic assays with FACS sorted NKp46$^+$ and NKp46$^-$ porcine NK cell subpopulations, which showed that the NK cell-activating effect of PRV gB could be observed with either NK cell subpopulation (Suppl. Figure 3). This suggests that NKp46 is not involved in the activating effect of PRV gB on NK cell activity. However, we cannot formally exclude the involvement of porcine NKp46 in the recognition of PRV-infected cells by NK cells, since no positive control, i.e. cells reported to be efficiently lysed by porcine NKp46, was available for our assay. Expanding the porcine NK cell receptor toolkit would be invaluable to further address the interaction of porcine NK cells with PRV and other porcine viruses, and would allow to analyze the homology and differences between porcine and human NK cells.

4) Conclusions

In conclusion, this doctoral dissertation describes different new insights in the complex interaction between alphaherpesviruses, particularly PRV, and NK cells, which are summarized in Figure 3.
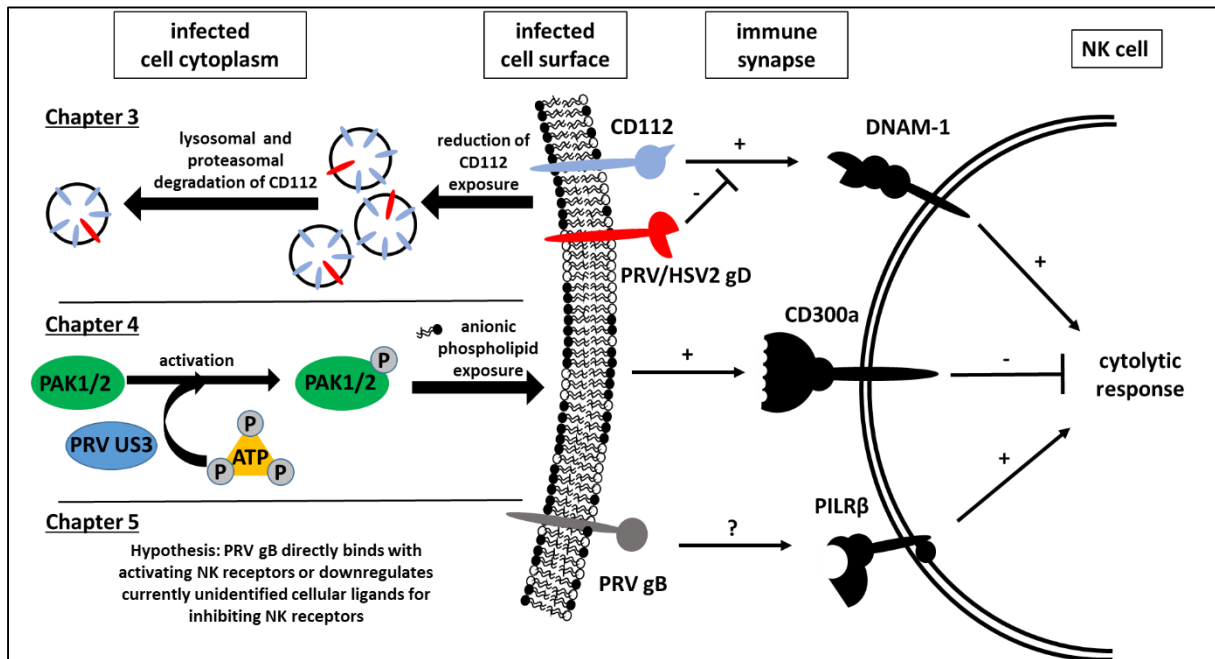

Figure 3. A schematic overview and interpretation of the main findings presented in this doctoral dissertation. Chapter 3: PRV/HSV-2 gD interferes with DNAM-1-dependent NK cell-lysis by degradation of the DNAM-1 ligand CD112. Chapter 4: Kinase intact PRV US3 activates group I PAK kinases to increase exposure of anionic phospholipids and binding of the inhibitory NK receptor CD300a. Chapter 5: Expression of PRV gB enhances susceptibility of cells for NK cell-mediated lysis by a yet unidentified mechanism.

5) Implications and applications

The findings of the current doctoral dissertation may be of value in different fields of research.

Xenotransplantation

Cross-species transplantation, or xenotransplantation, may have substantial potential to solve the critical need for organ, tissues and cells for clinical transplantation. The increasing availability of genetically engineered pigs may speed up progress in pig-to-nonhuman primate experimental models and potent pharmacological immunosuppressive regimens can largely prevent T-cell-mediated rejection and T-cell-dependent antibody responses (65). Observations in pig-to-primate and rodent models indicate that NK cells also play a prominent role in the host immunity response towards porcine xenografts (65). Several mechanisms through which

human NK cells recognize porcine endothelial cells have been elucidated and appear to be more diverse than those involved in NK cell alloreactivity (66).

Human NK receptors NKG2D, NKp44 and NKp46 have been demonstrated to be involved in the NK cell-mediated killing of porcine cells (67-69). Upon activation by TNFα, porcine cells have also been reported to increase expression of human NKp30 ligands (68). Our data, reported in chapter 4, indicate that the human inhibitory NK cell receptor CD300a recognizes porcine cells implying that, under certain circumstances, huCD300a can be involved in the recognition of porcine cells. The high conservation of the CD300a ligands, PS and PE, throughout species further emphasizes the possibility that these ligands may be of importance when considering the xenograft NK response (44).

Furthermore, a study characterizing porcine CD112 homologues suggested, based on 3D-modelling and human CD112 homology, that porcine CD112 may be a ligand for the human NK receptor DNAM-1 (70). In line with this, using blocking antibodies directed against human DNAM-1, we observed that human DNAM-1 is substantially involved in the killing of porcine cells by human NK cells (Figure 4). Infection of porcine cells by PRV, which leads to gD-dependent CD112 downregulation, drastically reduced DNAM-1-dependent killing of the infected porcine cells by human NK cells (Figure 4). These data suggests that porcine CD112 is a *bona fide* ligand for the human DNAM-1 NK receptor.
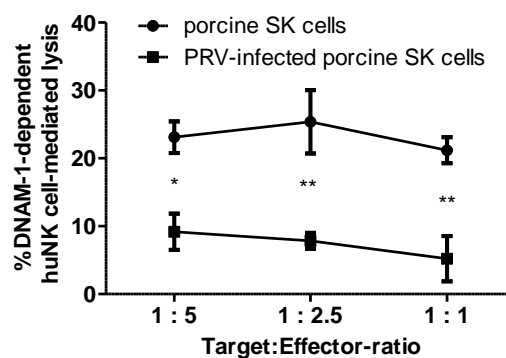

Figure 4. DNAM-1 is involved in the recognition of porcine SK cells by human NK cells and DNAM-1-dependent lysis of PRV-infected porcine cells is strongly reduced. Porcine SK cells were mock-infected or infected for 10h with PRV wt virus, and subsequently incubated with IL2-cultured human NK cells, in the absence or presence of DNAM-1 blocking antibody F5, at the given target:effector ratio for 4h. Viability was assessed by flow cytometry using propidium iodide and the %DNAM-1-dependent huNK cell-mediated lysis was calculated. Data represent mean + SEM of three independent repeats (*p<0,05, **p<0.01). Experiments were performed as described in chapter 3 and 4.

In conclusion, our data add human CD300a and DNAM-1 to the set of human NK receptors that may interact with porcine cells, further contributing to the idea that the xenograft response of human NK cells to porcine cells is mediated by multiple human NK receptors.

Oncolytic virotherapy

In Europe, yearly, 13,000 people are diagnosed with glioblastoma multiforme (GBM), a devastating type of brain tumor (71). GBM is the most lethal CNS tumor, with a 14.6 months median survival and a 5-year survival rate of 2% (72). Novel strategies, including therapeutic viruses, are needed and may hold promise to interfere with this deadly disease. One approach is to engineer viruses to specifically replicate in tumor cells and destroy these cells in the course of progeny virus release. This new progeny virus can further infect other tumor cells, thereby penetrating solid tumors and potentially removing the complete tumor (73). In the field of glioblastoma research, oncolytic HSV-1 (oHSV-1) vectors have shown substantial therapeutic potential during clinical phase I/II trial (72).

However, in mice, NK cells have been shown to interfere with the therapeutic effect of the promising oncolytic oHSV-1 virus rQNestin34.5 via rapid lysis of rQNestin34.5-infected cells, thereby preventing efficient spread of the oncolytic virus in the GBM tumors and thereby reducing the therapeutic efficacy (4). In chapter 3, we demonstrated that expression of PRV/HSV-2 gD in U87-MG, a glioblastoma cell line, reduced cell surface expression of the DNAM-1 ligand CD112 and consequently DNAM-1 binding. This could be of particular interest as DNAM-1 is highly involved in the recognition of oHSV-1-infected GMB tumors collected from patients and in oHSV-1-infected tumor cell lines (4, 74). An oncolytic oHSV-1 virus expressing PRV/HSV-2 gD could therefore potentiate the oHSV-1 virus to evade DNAM-1-dependent NK cell-mediated lysis and possibly increase therapeutic efficacy. In support of such an approach, a vesicular stomatitis virus-based oncolytic vector expressing the DNAM-1-interfering HCMV protein UL141 protein displayed increased oncolytic potential through decreased activity of NK cells (75). Along the same lines, our finding that HSV-1-infected cells display increased binding of the inhibitory NK receptor CD300a (Suppl. Figure 2) could be interesting to further study, as one could investigate whether this trait is conserved in current oHSV-1-based oncolytic vectors and in infected glioblastoma cells. This knowledge may allow to further fine-tune the interaction of the oncolytic virus vectors with the host immunity.

HSV-2 vaccine development

Many alphaherpesviruses are still widely spread and infection is incurable. Although vaccination has been successful for some alphaherpesviruses, vaccines are still lacking or inadequate for several of these pathogens, most notably HSV-2. Over 500 million people are infected worldwide with the sexually transmitted HSV-2, with an estimated 23 million new infections annually (76). This is of major importance as HSV-2-infected individuals have a 3-fold increased chance to acquire HIV-1 and this risk increases up to 8-fold if the exposure occurs soon after acquiring HSV-2 infection (77-79). Mathematical models suggest that even moderately effective prophylactic HSV-2 vaccines would lead to a marked decrease in HIV-1 incidence if given at high coverage (80, 81).

Our findings possibly may have implications for the development of HSV-2-based vaccines. The gD protein is a highly immunogenic protein, and several HSV-2 vaccines have focused on the induction of gD-specific antibodies. These antibodies may prevent virus infection by binding to the virus and blocking its ability to infect cells and may also trigger ADCC (antibody-dependent cellular cytotoxicity), which is mediated in large part by NK cells. Unfortunately, thus far the outcomes of clinical trials of HSV-2 vaccines have been disappointing (82). Our findings show that expression of HSV-2 gD reduces susceptibility of the infected cell to NK cell-mediated lysis, as reported in chapter 3, which may interfere with NK cell activity, including NK cell-dependent ADCC. In line with this, recently, an HSV-2 vaccine virus lacking the gD gene and supplemented with HSV-1 gD (obtained by replication of the virus in HSV-1 gD expressing cell lines) elicited a strong ADCC response and protection against disease in a mouse model (83).

PRV vaccine development

PRV causes Aujeszky's disease, a serious illness with high morbidity and mortality leading to severe losses in the pig industry (47). Various vaccination strategies based on modified life or inactivated vaccines have been successfully employed to control Aujeszky's disease. Among the vaccines, the Bartha-K61 attenuated PRV strain is widely used and has played a key role in the eradication of PRV in large areas of Western Europe and the US. This vaccine is an attenuated strain of PRV produced by extensive in vitro passaging and contains a deletion of several viral proteins that attenuates virulence (84-86). However, vigilance is still needed and improved PRV vaccines may still be needed at some point. Of particular importance in this context is the recent emergence of highly virulent PRV strains in China. The Bartha-K61

vaccine, which provided 100% protection against a lethal challenge with a classic PRV strain, provided only 50% protection against a challenge with the Chinese PRV isolate HeN1 (87).

Current vaccine strategies focus mainly on the immunological memory mediated by the adaptive immune response, the T and B lymphocytes. There are indications that it may be worth testing the potential beneficiary effect of targeting NK cells during alphaherpesvirus vaccination. Indeed , studies in mice indicated that depletion of NK cells resulted in a reduced CD8+ memory T cell population upon HSV-1 infection, indicating that NK cells may have an important supportive role in the development of a memory T cell response against HSV-1 (88). In addition, although still relatively controversial, increasing evidence in mice indicates that NK cells may also display memory-like features against viruses (89). NK cell memory-like properties against viruses have been particularly well documented for the mouse betaherpesvirus murine cytomegalovirus (MCMV) (90), but also for other viruses including influenza virus, HIV, vesicular stomatitis virus (VSV), HCMV, Hantavirus and Chikungunya virus (91-95). Interestingly, a recent study in mice indicates that memory-like NK cells may also be triggered upon infection with alphaherpesvirus HSV-2 (96).

Our findings may have implications to investigate the potential of targeting NK cells in future PRV/alphaherpesvirus vaccine design. As reported in chapter 5, we found that expression of PRV gB is sufficient to increase susceptibility to porcine NK cells. The identification of NK receptors involved in the NK cell-activating effect of PRV gB could facilitate the study whether memory NK cells are generated in response to PRV infection and, if so, whether these memory NK cells could contribute towards vaccine design. In any case, the role of PRV gB in PRV vaccines is important to study, as PRV gB expression greatly contributes to the generation of PRV-specific cytotoxic T cells (97).

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SUPPLEMENTARY FIGURES
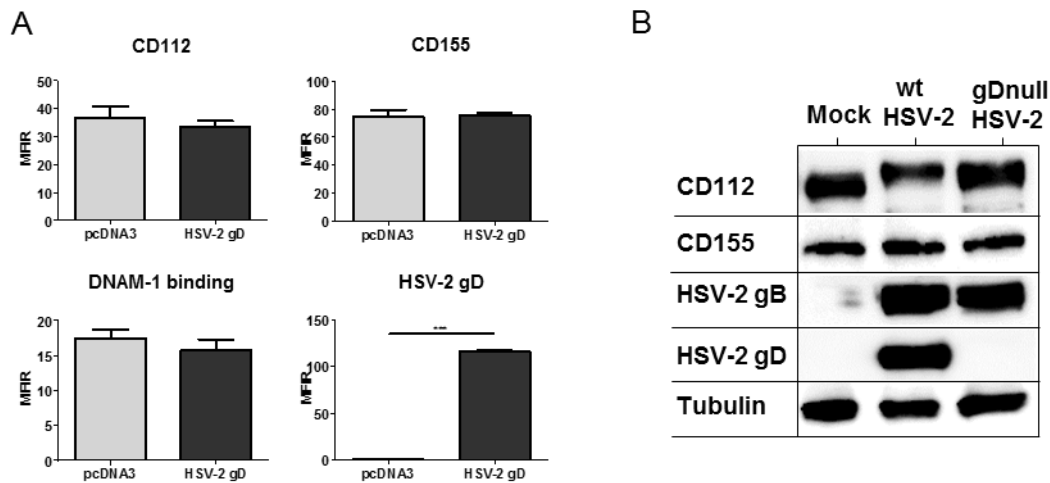
Suppl. Figure 1. HSV-2 gD alone is not sufficient for decreasing cell surface availability of CD112, and consequently DNAM-1 binding, but is involved in CD112 degradation during infection. (A) 293T cells were transfected for 48 h with HSV-2 gD expressing plasmid or empty control vector and subsequently assessed by flow cytometry for cell surface expression of CD112, CD155, HSV-2 gD and binding of DNAM-1-Fc (0.5 µg/sample). Data represent mean + SEM of three independent repeats (*** $p<0.001$). (B) 293T cells were infected for 24h with HSV-2 wt or isogenic HSV-2 gDnull mutant virus and subsequently assessed by Western blotting for expression of CD112, CD155, HSV-2 gB, HSV-2 gD and tubulin (loading control). Experiments were performed as described in chapter 3. The HSV-2 gD expressing plasmid was previously described (98).
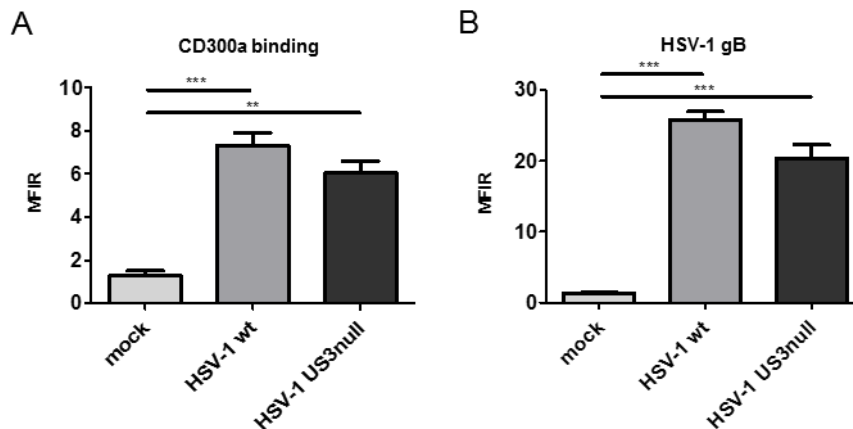
Suppl. Figure 2. HSV-1 infection increases binding of CD300a to the infected cell surface, independent of HSV-1 US3. 293T cells were mock-infected or infected with F strain HSV-1 wt or isogenic US3null mutant virus for 16h and subsequently assessed by flow cytometry for (A) binding of CD300a-Fc (0.5 µg/sample) and (B) expression of HSV-1 gB. Data represent mean + SEM of three independent repeats (**$p<0.01$, ***$p<0.001$). Experiments were performed as described in chapter 4. The US3null HSV-1 strain F mutant virus (R7041) has been previously described (99).
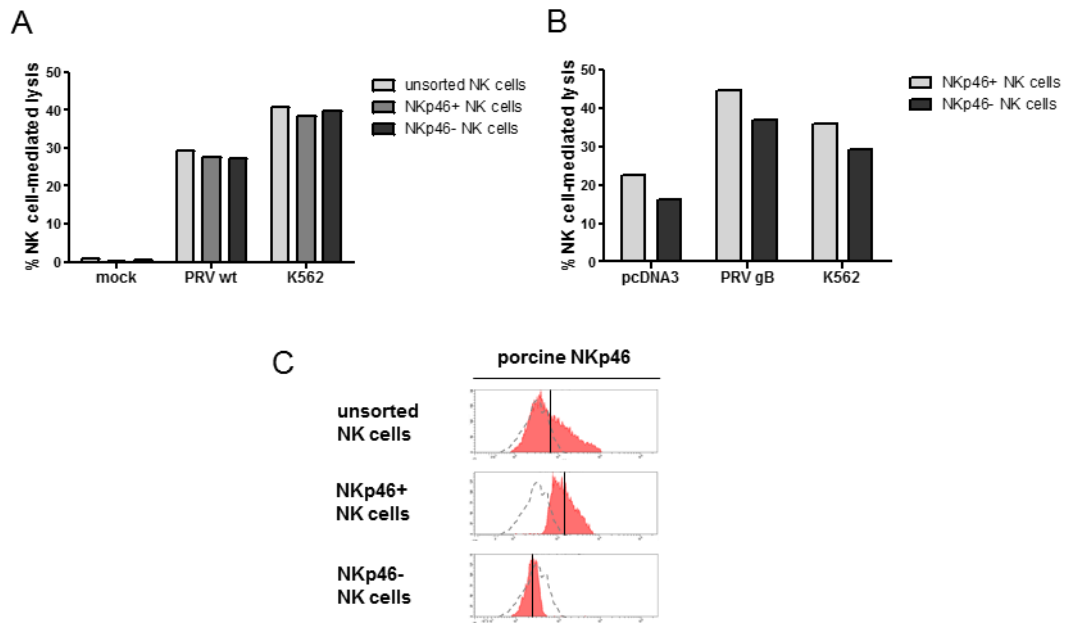
Suppl. Figure 3. NKp46 does not appear to be involved in recognition of PRV-infected SK cells or ST cells transfected with PRV gB-expressing plasmid by porcine primary IL2-primed NK cells. (A) SK cells were mock-infected or infected for 10h with PRV wt virus or (B) ST cells were transfected for 24h with PRV gB – expressing plasmid or empty control plasmids and subsequently incubated with primary IL2-primed porcine NK cell NKp46$^{+/-}$ subpopulations at a target:effector ratio of 1:25 for 4h. Primary porcine NK cells were isolated, IL-2 primed for 18h, stained with anti-porcine NKp46 antibody (VIV-KM1) and NKp46^{+} and NKp46^{-} NK cell subpopulations were isolated by FACS cell sorting immediately before use in the cytolytic assay. Viability was assessed by flow cytometry using propidium iodide. (C) Sorting analysis of of the NKp46^{+} and NKp46^{-} subpopulations. Vertical lines indicate median fluorescence intensity. Dotted line histogram represents isotype matched antibody control signal. The porcine NKp46 antibody (VIV-KM1) has been previously described (64). Experiments were performed as described in chapter 5.

Chapter 7: Summary / Samenvatting

SUMMARY

Herpesviruses have developed complex and often subtly fine-tuned interactions with the immune system of their host. As a result, these viruses typically cause relatively mild, although economically important, and socially discomforting disease symptoms and are able to persist lifelong in a latent state in their host. Natural killer (NK) cells are key players in the innate response to viral infections. In alphaherpesvirus biology, NK cells are of particular importance. Impaired NK cell activity has been associated with life-threatening encephalitis for the human alphaherpesviruses herpes simplex virus 1 (HSV-1) and varicella zoster virus (VZV). NK cells have also been identified as a limiting factor in HSV vector-based oncotherapy, since NK cells prematurely clear the viral vector. Therefore, unravelling the largely unstudied interactions of alphaherpesviruses with NK cells could provide essential insights in alphaherpesvirus biology and help optimize viral-based therapeutics towards the host immune response.

In the first chapter a brief introduction is given on alphaherpesviruses in general and more specifically on the alphaherpesvirus glycoprotein B (gB), gD and US3 protein kinase, as these are of particular relevance for the current dissertation. Furthermore, an overview of the current knowledge on NK cell function and regulation by NK receptors is given, with special attention for studies investigating porcine NK cells. In addition, this chapter also reviews the current knowledge on alphaherpesvirus interactions with NK cells.

In chapter 2, the aims of this doctoral dissertation are described.

In chapter 3, we demonstrated that expression of PRV/HSV-2 gD lowers the susceptibility of cells towards NK cell-mediated lysis through reduced cell surface availability of the DNAM-1 ligand CD112, representing a novel mechanism of alphaherpesvirus immune evasion. We found that porcine SK cells infected with the wild type (wt) porcine alphaherpesvirus pseudorabies virus (PRV) are less susceptible to lysis by porcine NK cells than cells infected with a PRV variant that lacks the glycoprotein gD, indicating that gD has an inhibitory effect on NK cell activity. Transfection studies confirmed the protective effect of PRV gD to NK cell-mediated lysis and showed that expression of PRV gD also reduced the susceptibility of 293T cells to human NK cytotoxicity.

PRV gD has been reported to interact with immunoglobulin-like superfamily members CD111 (nectin-1), CD112 (nectin-2) and CD155 (poliovirus receptor). Interestingly, the latter two are known ligands for the activating NK receptor DNAM-1. Therefore, the involvement of DNAM-

1, CD112 and CD155 in gD-mediated protection of cells against NK cell-mediated lysis was investigated. To this end, cell surface expression of CD112 and CD155 and binding of recombinant DNAM-1-Fc were assessed by flow cytometry in PRV-infected/gD-transfected 293T cells. Expression of gD resulted in an almost complete reduction of cell surface CD112 and a less pronounced downregulation of CD155. Importantly, these effects were associated with suppressed binding of recombinant DNAM-1 to the cell surface. In addition, the use of DNAM-1-specific antibodies to block the binding of DNAM-1 with its ligands demonstrated that expression of PRV gD decreased DNAM-1-dependent NK cell cytotoxicity. Western blotting further showed that the observed modulation of CD112 is not due to epitope masking by PRV gD, but to internalization or sequestering, and subsequent intracellular degradation. The natural host of the PRV virus is the swine and we confirmed in porcine SK cells and in primary porcine epithelial cells that PRV gD degrades CD112. Furthermore, we confirmed by RT-PCR that DNAM-1 is expressed in freshly isolated porcine NK cells.

In addition, we also found that a similar NK cell-evasive mechanism is also present in the human alphaherpesvirus HSV-2. Indeed, infection of 293T cells with HSV-2 resulted in a gD-dependent degradation of CD112, decreased binding of DNAM-1-Fc to the infected cell surface, and protection against NK cell cytotoxicity.

In chapter 4, we demonstrated another previously uncharacterized NK cell evasion mechanism orchestrated by the PRV protein kinase US3 via increased ligand presentation to the inhibitory NK cell receptor CD300a. We report for the first time the involvement of the CD300a NK receptor in the recognition of virus-infected cells. We reported that SK cells infected with a PRV variant that lacks the viral protein kinase US3 (US3null PRV) are more susceptible to lysis by porcine and human NK cells than cells infected with wt PRV, indicative for an NK cell-evasive role of US3. Interestingly, we found that recombinant CD300a specifically binds to wt PRV-infected cells and that this binding does not occur in cells infected with US3null PRV. In line with this, the use of CD300a-specific antibodies to block the binding of CD300a with its ligands, demonstrated that expression of US3 in infected cells increased CD300a-mediated protection of these cells against NK cell-mediated lysis.

Phosphatidylserine (PS) and phosphatidylethanolamine (PE) are known CD300a ligands. Blocking PS or PE using MFG-E8 and duramycin, respectively, indicated that both CD300a ligands are involved in the US3-triggered CD300a binding to PRV-infected cells. In line with this, anti-PS antibodies showed higher PS exposure on cells infected with wt PRV as compared

to cells infected with US3null PRV. Similar observations with regard to US3-mediated PS exposure and CD300a binding were made in primary porcine epithelial cells. Previously, it was reported that PRV US3 triggers activation of cellular group I p21-activated kinases (PAK). Treatment of wt PRV-infected cells with the PAK inhibitor IPA3 reduced PS exposure and CD300a binding to the level of cells infected with US3null PRV. In addition, an antibody redirected NK cell killing assay using human CD300a antibodies and porcine NK cells indicated the presence of a functional CD300a homologue in swine.

In chapter 5, we demonstrated that the gB glycoprotein of PRV triggers NK cell-mediated cytotoxicity. We did some initial experiments to address the potential involvement of sialylation and the activating NK receptor PILRβ in PRV gB-mediated activation of NK cells. We found that SK cells infected with wt PRV were more susceptible to lysis by porcine NK cells as compared to cells infected with a PRV variant lacking the gB glycoprotein. In addition, transfection studies confirmed that expression of gB is sufficient to trigger NK cell-mediated lysis of SK cells. These results indicate that PRV gB expression increases ligand expression towards activating NK receptors or decreases ligand expression towards inhibitory NK receptors. One hypothesis is that PRV gB is a direct ligand for the activating porcine NK receptor PILRβ. HSV-1 gB has been identified earlier to be a ligand for the closely related human inhibitory receptor PILRα and this interaction was reported to depend on sialylation of HSV-1 gB. However, removal of sialic acids on PRV-infected cells by neuraminidase treatment did not abolish PRV gB-mediated activation of NK cells. Interestingly, desialylation of the NK cell surface drastically impaired the recognition of PRV-infected cells by NK cells. In addition, although we could not demonstrate binding of the mouse PILRβ homologue to PRV gB, we did find evidence for mouse PILRβ binding to HSV-1 gB. In conclusion, we found that PRV gB triggers NK cell cytotoxicity by a yet unidentified mechanism and that sialic acids on the NK cell surface are required for recognition of PRV infected cells.

In chapter 6, the observations of the current doctoral dissertation are situated in the context of the existing literature and potential implications and applications of the findings are discussed.

SAMENVATTING

Herpesvirussen hebben complexe en vaak subtiele interacties ontwikkeld met het immuun stelsel van hun gastheer. Hierdoor veroorzaken deze virussen over het algemeen relatief milde, maar economisch en sociaal belangrijke ziektesymptomen en kunnen ze levenslang in een latente staat in de gastheer aanwezig blijven.

Natural Killer (NK) cellen behoren tot de hoofdrolspelers in de aangeboren immuunrespons tegen virale infecties en ook bij alfaherpesvirus infecties zijn NK cellen van cruciaal belang. Een verstoorde NK cel-activiteit kan er voor zorgen dat deze virussen veel ernstigere ziektebeelden veroorzaken, zoals levensbedreigende encefalitis geassocieerd met de humane alfaherpesvirussen, herpes simplex virus 1 (HSV-1) en varicella zoster virus (VZV). Daarenboven kunnen NK cellen een beperking vormen voor de ontwikkeling van herpesvirus-gebaseerde therapeutische vectoren. HSV-gebaseerde therapeutische vectoren hebben potentieel voor de bestrijding van zeer agressieve vormen van kanker, zoals glioblastoma multiforme. Experimenten in muizenmodellen hebben echter aangetoond dat NK cellen hierbij een limiterende factor kunnen vormen, doordat ze de virale vector vroegtijdig elimineren. Het ontrafelen van de nog grotendeels ongekende interacties van alfaherpesvirussen met NK cellen kan dan ook essentiële inzichten verschaffen in de biologie van alfaherpesvirussen en kan mogelijks bijdragen tot een geoptimaliseerde interactie van alfaherpesvirus vectoren en vaccins met het immuunsysteem van de gastheer.

In het eerste hoofdstuk wordt een korte inleiding gegeven over alfaherpesvirussen en, wegens hun belang en relevantie in dit doctoraal proefschrift, meer specifiek over drie alfaherpesvirus eiwitten, namelijk glycoproteïn B (gB), gD en het US3 proteïne kinase. Voorts wordt er ook een overzicht gegeven omtrent de huidige kennis over de functies en regulatie van NK cellen, met speciale aandacht voor studies over porciene NK cellen. Ten slotte wordt in dit hoofdstuk ook de huidige kennis over de interactie van alfaherpesvirussen met NK cellen beschreven.

In hoofdstuk 2 worden de doelstellingen van dit doctoraal proefschrift toegelicht.

In hoofdstuk 3 hebben we aangetoond dat de expressie van het gD eiwit van PRV/HSV-2 de vatbaarheid van cellen tegenover NK cel-gemedieerde lyse vermindert, doordat gD de beschikbaarheid van het DNAM-1 ligand CD112 aan het celoppervlak reduceert. We stelden vast dat porciene SK cellen die geïnfecteerd werden met het wild type (WT) porciene alfaherpesvirus pseudorabies virus (PRV) minder vatbaar zijn voor NK cel-gemedieerde lyse in vergelijking met cellen die geïnfecteerd werden met een PRV variant waarin gD ontbreekt.

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Transfectie studies in 293T cellen bevestigden dat expressie van PRV gD zorgt voor een beschermend effect tegenover NK cel-gemedieerde lyse en dit zowel voor porciene als humane NK cellen.

Eerdere studies toonden aan dat PRV gD een interactie kan aangaan met CD111 (nectin-1), CD112 (nectin-2) en CD155 (poliovirus receptor). Interessant hierbij is dat de CD112 en CD155 gekende liganden zijn voor de activerende NK receptor DNAM-1. Daarom werd de rol van DNAM-1, CD112 en CD155 onderzocht in het kader van de gD-gemedieerde bescherming van cellen tegenover NK cel-gemedieerde lyse. Hiertoe werd in eerste instantie het effect nagegaan van gD expressie (tijdens infectie en transfectie) op de hoeveelheid CD112 en CD155 aan het celoppervlak van 293T cellen met behulp van flow cytometrie. Expressie van gD resulteerde in een bijna volledige reductie van CD112 aan het celoppervlak en een minder uitgesproken reductie van CD155, wat op zijn beurt leidde tot een verminderde binding van recombinant DNAM-1 aan het celoppervlak. Daarenboven werd, met behulp van DNAM-1-blokkerende antistoffen, aangetoond dat expressie van PRV gD de vatbaarheid van cellen voor DNAM-1-afhankelijke NK cel-cytotoxiciteit vermindert. Western blotting toonde verder aan dat de geobserveerde modulatie van CD112 door gD niet het gevolg is van het maskering van epitopen, maar het gevolg is van (vermoedelijk) internalisatie en daarop volgende intracellulaire afbraak van CD112. De natuurlijke gastheer van het PRV virus is het varken en we bevestigden, gebruik makend van porciene SK cellen en primaire porciene epitheliale cellen, dat PRV gD ook in porciene cellen leidt tot degradatie van CD112. Bovendien werd met behulp van RT-PCR bevestigd dat DNAM-1 tot expressie komt in porciene NK cellen.

Tot slot werd aangetoond dat een analoog NK cel-evasief mechanisme voorkomt bij het humane alfaherpesvirus HSV-2. Ook bij dit virus resulteerde infectie van 293T cellen in een gD-afhankelijke afbraak van CD112, verminderde binding van recombinant DNAM-1 aan het celoppervlak en bescherming van de cellen tegen NK cel-cytotoxiciteit.

In hoofdstuk 4 werd een tweede voorheen ongekend NK cel evasie-mechanisme van alfaherpesvirussen geïdentificeerd, georkestreerd door het US3 proteïne kinase van PRV via verhoogde presentatie van liganden voor de inhiberende NK receptor CD300a. Dit is de eerste studie die de betrokkenheid van CD300a bij de herkenning van virus-geïnfecteerde cellen aantoont. We stelden vast dat SK cellen die geïnfecteerd werden met WT PRV minder vatbaar waren voor lyse door porciene of humane NK cellen in vergelijking met cellen die geïnfecteerd werden met een PRV variant waarin US3 ontbreekt (US3null PRV). Hierbij werd aangetoond dat SK cellen geïnfecteerd met WT PRV een verhoogde binding vertoonden van de inhiberende

NK receptor CD300a, wat niet het geval was bij cellen geïnfecteerd met US3null PRV. Gebruik makend van CD300a-blokkerende antistoffen werd bevestigd dat expressie van US3 in geïnfecteerde cellen leidt tot een verhoogde CD300a-afhankelijke bescherming tegen NK cel-gemedieerde lyse. Fosfatidylserine (PS) en fosfatidylethanolamine (PE) zijn gekende liganden van CD300a. Experimenten waarbij PS of PE geblokkeerd werden met respectievelijk MFG-E8 en duramycine, toonden aan dat beide liganden betrokken zijn bij de US3-afhankelijke CD300a binding aan PRV-geïnfecteerde cellen. Hierop verdergaand kon, met behulp van anti-PS antistoffen, een hogere PS blootstelling aangetoond worden in cellen geïnfecteerd met WT PRV in vergelijking met cellen geïnfecteerd met US3null PRV. Deze US3-afhankelijke PS blootstelling en CD300a binding werden eveneens vastgesteld in primaire porciene epitheliale cellen.

Eerdere studies aan onze onderzoeksgroep toonden aan dat PRV US3 leidt tot de activatie van groep I p21-geactiveerde kinasen (PAK). Behandeling van WT PRV-geïnfecteerde cellen met de PAK inhibitor IPA3 onderdrukte de PS blootstelling en CD300a binding tot op het niveau van US3null PRV-geïnfecteerde cellen. Dit toont aan dat de US3-gemedieerde PAK activatie belangrijk is voor de PS blootstelling en CD300a binding. Daarnaast werd, gebruik makend van een 'antibody redirected NK cell killing assay', met behulp van humane CD300a antistoffen en porciene NK cellen, aangetoond dat een functioneel CD300a homoloog aanwezig is in porciene NK cellen.

In hoofdstuk 5 werd aangetoond dat het gB glycoproteïne van het PRV virus de NK cel-gemedieerde cytotoxiciteit activeert. Verscheidene initiële experimenten werden uitgevoerd om de potentiële betrokkenheid van sialylatie en de activerende NK receptor PILRβ in deze PRV gB-gemedieerde activatie van NK cellen te onderzoeken.

SK cellen die geïnfecteerd werden met WT PRV bleken meer vatbaar te zijn voor lyse door porciene NK cellen, in vergelijking met cellen geïnfecteerd met een PRV variant waarin gB ontbreekt. Transfectie studies bevestigden dat expressie van PRV gB voldoende is om de gevoeligheid van cellen voor lyse door porciene NK cellen te verhogen. Op basis van deze resultaten kan de hypothese opgesteld worden dat PRV gB de expressie van liganden voor activerende NK receptoren doet toenemen of de expressie van liganden voor inhiberende NK receptoren doet afnemen. Eén mogelijke hypothese is dat PRV gB een ligand vormt voor de activerende NK receptor PILRβ. Eerde studies toonden aan dat HSV-1 gB een ligand vormt voor de nauw verwante humane inhiberende receptor PILRα en deze interactie bleek afhankelijk te zijn van siaalzuren op HSV-1 gB. Na het verwijderen van siaalzuren op PRV-

geïnfecteerde cellen door middel van neuraminidase-behandeling werd echter aangetoond dat siaalzuren niet betrokken zijn bij het NK-activerend effect van PRV gB. Interessant hierbij is dat het verwijderen van siaalzuur van het NK cel oppervlak wel een onderdrukkend effect had op de NK cel-gemedieerde lyse van PRV-geïnfecteerde cellen. Dit suggereert dat siaalzuren op het oppervlak van NK (receptoren) een belangrijke rol spelen bij de herkenning en/of lyse van PRV-geïnfecteerde cellen. Daarnaast werd aangetoond dat PRV gB niet lijkt te binden met het muis PILRβ, maar dat muis PILRβ wel bindt met HSV-1gB. In conclusie werd aangetoond dat PRV gB leidt tot activatie van porciene NK cellen via een nog ongekend mechanisme.

In <u>hoofdstuk 6</u> worden de bevindingen van dit doctoraal proefschrift gesitueerd in de context van de beschikbare literatuur en worden de mogelijke implicaties en applicaties van deze bevindingen besproken.

Curriculum Vitae

PERSONALIA

Korneel Grauwet werd geboren op 3 september 1985 te Brugge. Hij behaalde in 2007 het diploma van Master in de Industriële wetenschappen (Chemie) aan het KHBO te Oostende en behaalde in 2010 het diploma van Master in de bio-ingenieurswetenschappen (cel- & gentechnologie) aan de Universiteit Gent met onderscheiding. Tijdens zijn studies aan het KHBO en de UGent was Korneel een actief lid van de studentenclub Sd'A Oostende/Gent en zetelde 2 jaren in het presidium. Geboeid door virologie en het wetenschappelijk onderzoek, startte hij in 2010 een doctoraatsstudie aan de vakgroep Virologie, Parasitologie en Immunologie aan de Faculteit Diergeneeskunde van de Universiteit Gent. Deze studie werd gefinancierd door het Bijzonder Onderzoeksfonds van de Universiteit Gent. Dit onderzoek handelt over de herkenning van alfaherpesvirus geinfecteerde cellen door Natural Killer (NK) cellen.

Korneel Grauwet is auteur en mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Tevens nam hij actief deel aan nationale en internationale bijeenkomsten, symposia en congressen over alfaherpesvirussen en de aangeboren immuniteit.

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CONFERENTIES, ABSTRACTEN EN POSTER-SESSIES

*Indien het abstract geselecteerd werd voor presentatie, werd de **presenterende auteur** aangeduid.*

European Cancer Conference 2015 (Vienna, Austria):

The alphaherpesvirus gD glycoprotein suppresses DNAM-1-dependent Natural Killer cell-mediated lysis of infected cells through modulation of CD112

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International Herpesvirus Workshop 2015 (Boise, Idaho, USA):

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International conference on oncolytic virus therapeutics 2015 (Boston, Massachusetts, USA):

Alphaherpesvirus US3 protein kinase protects infected cells from NK cell-mediated lysis via increased binding of the inhibitory NK cell receptor CD300a

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Belgian Society for Virology 2014 (Brussels, Belgium):

The pseudorabies US3 protein interferes with presentation of the CD300a ligand phosphatidylserine and protects infected cells from NK mediated killing

***Grauwet K**, Cantoni C, Jacob T, De Pelsmaeker S, Claessen C, Parodi M, De Maria A, Moretta L, Vitale M, Favoreel H*

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The pseudorabies gD protein suppresses NK-mediated cell lysis through interference with DNAM-1 binding

Grauwet K, Cantoni C, Parodi M, De Maria A, Moretta L, Vitale M, Favoreel H

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fact I replaced all the names in the box by my name and everybody eventually knew what had happened (it was supposed to be a secret! santa though). Well that backfired a bit. But upon dividing the name cards, I did know that all of you had gotten my name. The facial expressions were better than any present ☺ I will remember the carting as well. Not only because I had won or Rudy his wife was faster than Rudy himself, but rather because you are kind and fun people.

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(dit noemen ze een paragraaf afsluiten met een cliffhanger)

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Hoogachtend,
Korneel

THE UNSUCCESSFUL SELF-TREATMENT OF
A CASE OF "WRITER'S BLOCK"[1]

DENNIS UPPER

VETERANS ADMINISTRATION HOSPITAL, BROCKTON, MASSACHUSETTS

REFERENCES

COMMENTS BY REVIEWER A

I have studied this manuscript very carefully with lemon juice and X-rays and have not detected a single flaw in either design or writing style. I suggest it be published without revision. Clearly it is the most concise manuscript I have ever seen—yet it contains sufficient detail to allow other investigators to replicate Dr. Upper's failure. In comparison with the other manuscripts I get from you containing all that complicated detail, this one was a pleasure to examine. Surely we can find a place for this paper in the Journal—perhaps on the edge of a blank page.