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Natural Killer cells in viral infections

clinical implications and therapeutic intervention

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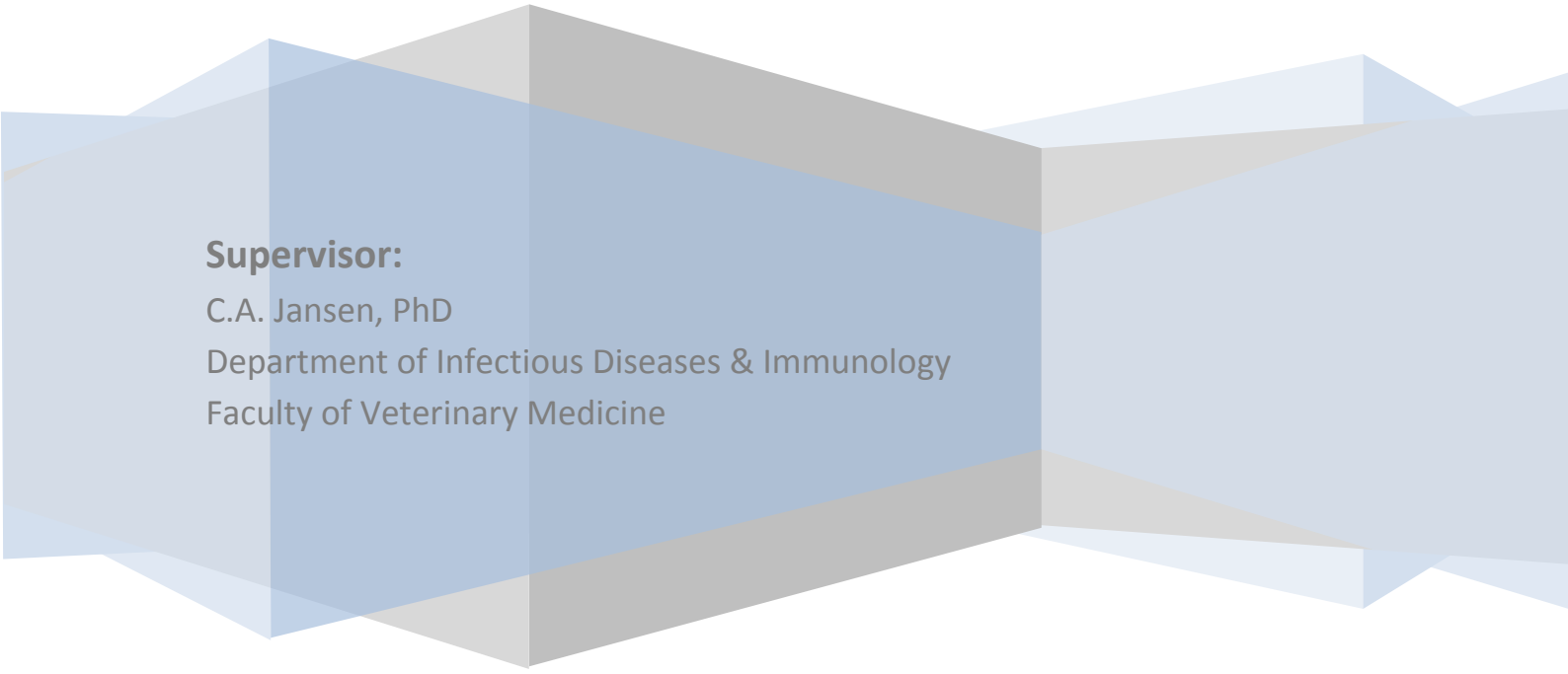

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Abstract:
Natural Killer (NK) cells, a subset of lymphocytes characterized by the expression of CD56 and the lack of CD3, are important effector cells of the innate immune system and provide a first-line defense against viruses. NK cells express both activating and inhibiting receptors, and the combined input of the receptors determines activation of the cell. For a long time, killing of cells that express a reduced level of MHC complexes such as tumor cells and virally infected cells was considered to be the major function of NK cells. Recent studies have suggested additional functions for NK cells. It was shown that NK cells have an important regulatory role in the initiation, progression and termination of an immune response. Furthermore, various discoveries have led to the hypothesis that the NK cell represents an evolutionary bridge between the innate and adaptive immune system.

During evolution, many viruses have developed numerous strategies to escape NK cells, which highlights the importance of NK cells in the protection against viruses. The majority of the viruses utilize MHC-I mimicry/up-regulation/redistribution, down-regulation of NK activating ligands or interference with interferon signaling as NK cell inhibiting strategies. Viruses form a continuous threat to humans, due to their ability to mutate frequently. Studying the escape mechanisms of viruses may provide a basis to identify targets that can be addressed therapeutically so that specifically NK cell immunity is enhanced. With the newly discovered innate and adaptive abilities of NK cells, targeting NK cells may stimulate both parts of the immune system, resulting in more efficient anti-viral immune responses. This report will review the recently discovered regulatory role of NK cells, and it will focus on the interaction of NK cells with viruses to survey potential targets for therapeutic intervention.

Key words: Natural Killer, HIV, HCV, HBV, EBV, viral infection, inflammation, immune escape, therapy

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General characteristics of Natural Killer cells

Natural Killer (NK) cells are a subset of lymphocytes that have the ability to lyse infected or transformed cells without prior activation (Brunetta et al., 2010; Sun and Lanier, 2009). NK cells are present in blood and the spleen, where NK cells comprise 5-15% of all lymphocytes (Brunetta et al., 2010; Cheent and Khakoo, 2009; Cooper et al., 2001). In the liver, 40-60% of the complete lymphocyte pool consists of NK cells (Mondelli et al., 2010). In contrast to other lymphocytes – attributed to the adaptive immune system – NK cells are considered to belong to the innate immune system, since they recognize common molecular patterns instead of specific pathogen-derived antigens (Cheent and Khakoo, 2009; Cooper et al., 2009; Lanier, 2008; Mondelli et al., 2010). Upon recognition of target cells NK cells may produce cytokines, like interferon-γ (IFN-γ), and/or perform cytotoxicity (Brunetta et al., 2010; Cooper et al., 2001; Horowitz et al., 2010; Lodoen and Lanier, 2006; Sun and Lanier, 2009).

Human NK cells are characterized by the lack of surface expression of CD3 and expression of a specific marker; CD56. CD56 is an isoform of the human neural adhesion molecule, but its function remains unclear. It has been postulated that CD56 is involved in target cell adhesion (Cooper et al., 2001). Additionally, NK cells express a varying level of CD16 (Fcγ receptor III), the low affinity receptor for immunoglobulin G (IgG), which is involved in induction of antibody-dependent cellular cytotoxicity (ADCC) (Brunetta et al., 2010; Cooper et al. 2001; Lanier, 2008). Based on the expression of these two markers, two NK cell subsets can be identified.

Firstly, $CD56^{bright}CD16^{dim/-}$ NK cells comprise approximately 10% of the total NK cell population (Brunetta et al., 2010; Cooper et al., 2001; Iannello et al., 2008). These cells are nearly absent in peripheral blood and primarily found in tissues and lymph nodes. Due to the low or absent expression of CD16, these cells are no efficient inducers of ADCC. In contrast, $CD56^{bright}CD16^{dim/-}$ NK cells produce abundant levels of cytokines upon stimulation, for example IFN-γ, tumor necrosis factor α/β (TNF-α/β), Interleukin 10 (IL-10), IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Cooper et al., 2001; Marcenaro et al., 2006; Moretta et al., 2008; Walzer et al., 2005). This suggest a regulative function for $CD56^{bright}CD16^{dim/-}$ NK cells within the immune response. Since $CD56^{bright}CD16^{dim/-}$ NK cells express CC-chemokine receptor 7 (CCR7) they are able to migrate into lymph nodes via high endothelial venules, where T and B cell responses are generated. $CD56^{bright}CD16^{dim/-}$ NK cells also express CXC-chemokine receptor 3 (CXCR3) and react strongly to interferon-inducible T-cell α chemoattractant (I-TAC) and interferon-γ inducible protein 10 (IP-10). These two proteins induce homing of $CD56^{bright}CD16^{dim/-}$ NK cells into inflamed tissues, in which they can influence the cytokine milieu (Cooper et al., 2001). Perrit et al., have proposed a further classification of $CD56^{bright}CD16^{dim/-}$ NK cells into NK1 and NK2 cells based on their distinct cytokine profiles. NK1 cells can be derived from naïve NK cells cultured with Th1 cytokines and produce IFN-γ,

TNF-β and IL-10. When naïve NK cells are cultured in a Th2 milieu, they give rise to NK2 cells which produce IL-5 and IL-13 (Peritt et al., 1998).

Secondly, the majority (90%) of NK cells express low levels of CD56 and high levels of CD16 (Brunetta et al., 2010; Cooper et al., 2001; Iannello et al., 2008). These cells, mainly present in peripheral blood, are specialists in cytotoxicity and ADCC, due to their high expression of CD16. $CD56^{dim}CD16^{+}$ NK cells have the ability to produce cytokines, although in lesser extent than $CD56^{bright}CD16^{dim/-}$ NK cells (Cooper et al., 2001). During inflammation $CD56^{dim}CD16^{+}$ NK cells migrate into the inflammatory site, where they recognize and lyse infected cells, bacteria and parasites (Lodoen and Lanier, 2006). $CD56^{dim}CD16^{+}$ NK cells also migrate into tumors and elicit cytolysis of transformed cells (Cooper et al., 2001). Just like other lymphocytes, $CD56^{dim}CD16^{+}$ NK cells show chemotactic response to fractalkine due to expression of CXCR1 (Sechler et al., 2004). $CD56^{dim}$ NK cells express CX_3C-chemokine receptor 1 (CX_3CR1) and are therefore attracted by IL-8, formerly solely demonstrated in neutrophils (Campbell et al., 2001; Moretta et al., 2008).This observation indicates that $CD56^{dim}$ NK cells may contribute to the acute phase of an immune response.

A third subset of NK cells was recently discovered in mucosa-associated lymphoid tissues. These NK cells produce IL-22, which is involved in maintaining the barrier function of epithelia via induction of anti-apoptotic and bactericidal protein expression (Cooper et al., 2009).

In mice, NK cells have similar functions as their human counterparts, like cytotoxicity and cytokine production. A clear distinction of subtypes based on CD56/CD16 expression has not been described, due to the absence of a CD56 homolog (Cooper et al., 2001). Murine NK cells express other specific markers, namely DX5, NK1.1, NKp46 and Ly49. Expression of these markers distinguishes NK cells from other murine lymphocytes and can be used to characterize murine NK cells (Cheent and Khakoo, 2009; Lanier, 2008; Vosshenrich et al., 2005).

Activation of NK cells

As mentioned previously, NK cells are able to recognize infected - and transformed cells. The mechanisms by which NK cells recognize these cells have been subject of studies since the discovery of NK cells. It was found that NK cells recognize these cells via a plethora of inhibiting and activating NK cell receptors and the combined input of these receptors determines activation.

It is known that certain tumor cells down-regulate expression of major histocompatibility complex I (MHC-I) molecules, and that these cells are therefore protected from lysis by cytotoxic T cells. These tumor cells could still be killed by NK cells, indicating that NK-mediated lysis was independent of MHC-I expression. Moreover, *in vivo* NK depletion reduced survival of tumor-burdened mice compared to tumor burdened mice with normal NK cell levels. These observations formed the

Table 1 **Activating and inhibiting receptors of NK cells**

A.) Activating Receptors

Family	Receptor	Ligand
KIR	2DS1 & 2	HLA-C
	3DS1	HLA-Bw4?
	2DS3	?
	2DS4	HLA-Cw4
	2DS5	?
	2DL4	HLA-G
NCR	NKp30	BAT-3?
	NKp44	Haemagglutinin
	NKp46	Haemagglutinin
C-type lectin	CD94:NKG2C & E	HLA-E
	NKG2D	MIC-A/B, ULBP
Other	CD16	IgG
	CD244	CD48

B.) Inhibitory Receptors

Family	Receptor	Ligand
KIR	2DL1-3	HLA-C
	2DL5	?
	3DL1	HLA-Bw4
	3DL2	HLA-A3/A11
LIR	LIR1	HLA class I
C-type lectin	CD94:NKG2A	HLA-E
Other	CD244	CD48

KIR: Killer cell Immunoglobulin-like Receptor, NCR: Natural Cytotoxicity Receptor, LIR: Leukocyte immunoglobulin-like receptor, HLA: Human Leukocyte Antigen.

fundament for the 'missing self hypothesis', which proposed that MHC-I molecules are involved in inhibition of NK-mediated cytotoxicity (Ljunggren and Kärre, 1985; Sun and Lanier, 2009).

Inhibitory receptors

Several inhibitory receptors that engage with MHC-I or MHC-I-like molecules have been identified, for example killer cell immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LIRs), killer cell C type lectin-like receptors (KLRs), such as CD94:NKG2A in humans and Ly49 in mice (Lanier, 2008; Cheent and Khakoo, 2009). An important signal that inhibits NK cell activity is delivered by HLA-E molecules via CD94:NKG2A. HLA-E is an instable minor HLA antigen and dependent on expression of major HLA antigens, therefore HLA-E cannot be expressed when HLA-A, B or C alleles are down-regulated. Hence, HLA-E represents an additionally control for expression of major HLA alleles (Howell et al., 2010; Lanier, 2008; Cheent and Khakoo, 2009). Table 1b gives an overview of inhibitory receptors and their ligands.

Although the inhibitory receptors belong to different families and differ in extracellular domains, these receptors share a common signal motif, the immunoreceptor tyrosine-based inhibitory motif

(ITIM), and accordingly a common signal transduction pathway. When an inhibitory receptor associates with its ligand, the ITIM motif is phosphorylated resulting in recruitment of the lipid phosphatase SHIP and/or the tyrosine phosphatases SHP-1 or SHP-2. Consequently, these phosphatases dephosphorylate substrates – especially Vav-related proteins – downstream of activating NK receptors resulting in inhibition of NK cells (Cheent and Khakoo, 2009; Lanier 2008) (figure 1).

Activating receptors

Alongside inhibitory receptors, NK cells express a divers set of activating receptors (table 1a). Most of these receptors belong to the same families as the inhibitory receptors, namely the KIRs and KLRs, with the exception of the natural cytotoxicity receptor (NCR) family, which contains exclusively activating receptors. In parallel to their function as inhibitory receptors, KIRs and KLRs also recognize HLA antigens, although with much lower affinity than their inhibitory counterparts. For instance, the activating receptors CD94:NKG2C and CD94:NKG2E both bind HLA-E, just like the inhibitory receptor CD94:NKG2A. The exact function of this phenomenon remains unclear, however one could postulate that a certain level of HLA antigens must be expressed on a cell. Expression of HLA below a certain threshold results in lack of inhibitory signals to NK cells and thus permits cytolysis of the target cell. In contrast, expression of HLA antigens that exceeds the normal range – due to viral infection or transformation – also induces cytolysis, via the activating low affinity receptors (Cheent and Khakoo, 2009; Lanier, 2008).

The natural cytotoxicity receptor family includes the receptors NKp30, NKp44 and NKp46. Members of this family have the unique ability to recognize specific viral antigens (Cheent and Khakoo, 2009; Lanier, 2008; Mao et al., 2010).

Cross-linking of individual activating receptors by itself does not induce NK cell activation. This requires prior exposure to IL-2 or cross-linkage of multiple activating receptors at the same time. Hence, the name co-activating receptors would be more appropriate. An exception is the activating receptor CD16, since individual cross-linking of this receptor results in NK cell cytotoxicity and cytokine production (Bryceson et al., 2006).

Activating receptors associate with other co-receptors than inhibiting receptors, which are involved in signal transduction pathways that induce NK cell activation. The best understood activating receptor complexes include the DAP10-associated NKG2D complexes, the immunoreceptor tyrosine-based activation motif (ITAM)-bearing co-receptor complexes and the CD244 receptor system (Cheent and Khakoo, 2009; Lanier et al., 2008).

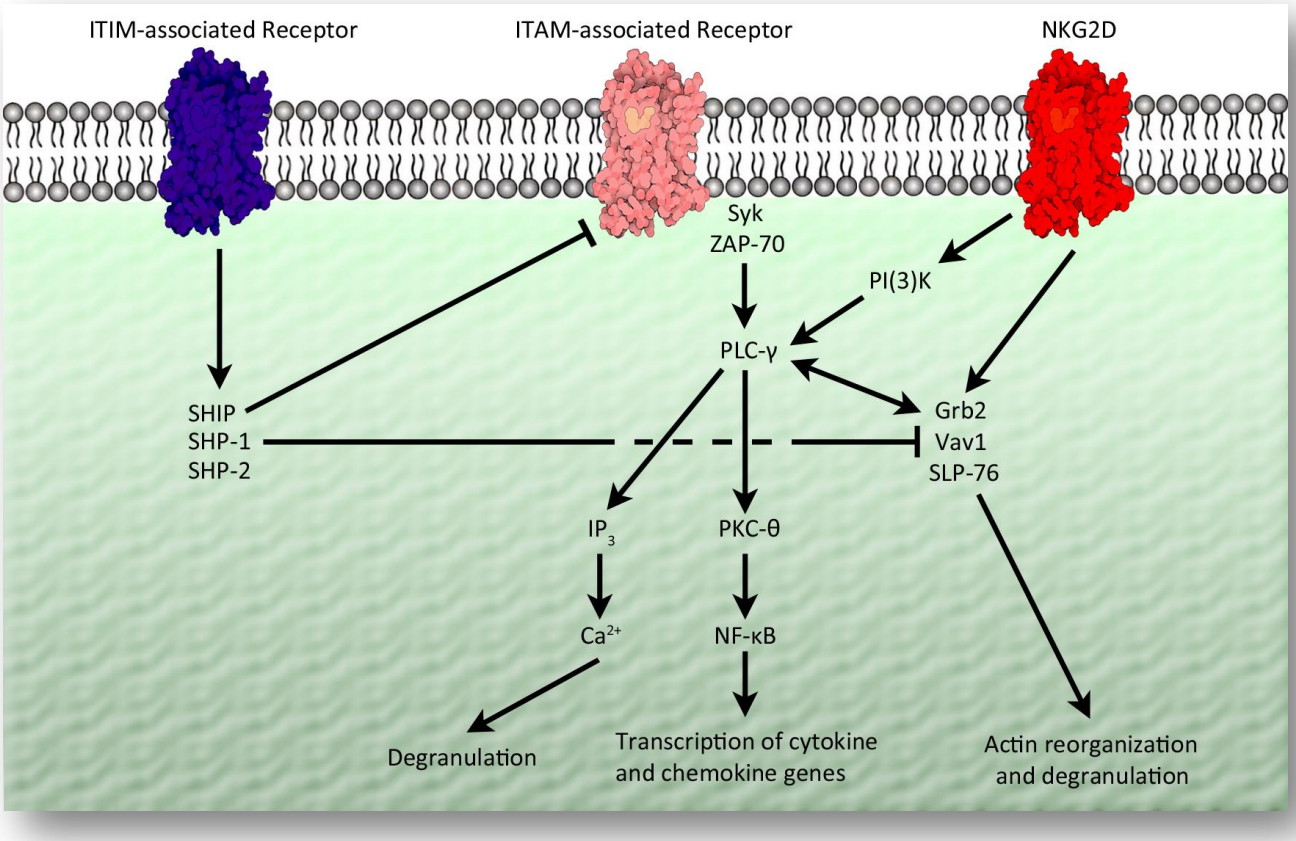

Figure 1 Schematic representation of the interaction of the activating (ITAM & NKG2D) and inhibiting (ITIM) receptor mechanisms of NK cells Inhibitory receptors share a common signaling motif: the immunoreceptor tyrosine-based inhibitory motif (ITIM). Ligand binding of inhibitory receptors results in recruitment of the phosphatases SHIP, SHP-1 and SHP-2 which dephosphorylate kinases downstream of activating receptors, mainly Vav-related proteins, and force dissociation of adaptor proteins from activating receptors. Many activating receptors associate with adaptor proteins that contain the immunoreceptor tyrosine-based activating motif (ITAM), the NKG2D receptor does not associate with adaptor proteins. Ligand binding of ITAM-associated receptors and NKG2D results in degranulation, transcription of cytokine and chemokine genes and actin reorganization via several pathways regulated downstream of PLC-γ.

DAP10-associated NKG2D complexes

NK2GD is a type II transmembrane glycoprotein belonging to the C-type lectin-like receptor superfamily. Unlike its family members, NKG2D does not form complexes with CD94 and DAP12. But NK2GD forms homodimers via disulfide-bonds and associates with the signaling subunit DAP10 (Lanier, 2008). NKG2D recognizes factors up-regulated by cells that are in cellular stress, such as MHC class I-like polypeptide-related sequences MICA and MICB, and UL16 binding protein 1-5 (ULBP1-5) (Cerboni et al., 2006; Cheent and Khakoo, 2009; Lanier, 2008).

In mice, NKG2D is expressed in two different isoforms, a long (NKG2D-L) and a short (NKG2D-S) isoform. Both isoforms bind identical ligands, namely RAE-1 family members, H60 and MULT1 (Jonjić et al., 2008; Lanier, 2008; Lodoen and Lanier, 2005). However, NKG2D-L exclusively associates with DAP10, while NKG2D-S can associate with both DAP12 and DAP10. Resting murine NK cells express low levels of NKG2D-S whereas activated murine NK cells express high levels of NKG2D-S. These observations suggest different signal transduction potential for the two isoforms that may be related

to activation state of the NK cell (Gilfillan et al., 2002). A similar mechanism has not been described in humans, but it was recently shown that prior exposure to IL-15 is necessary for cytolytic activity through NKG2D, since activity of DAP10 is regulated downstream of the IL-15R. (Cheent and Khakoo, 2009; Lanier, 2008).

In humans, DAP10 either binds the p85 subunit of phosphatidylinositol-3-OH kinase (PI(3)K) or Grb2, due to overlapping binding sites. It has been demonstrated that the ability to associate with both proteins is essential to induce cytotoxic activity via NKG2D stimulation (Upshaw et al., 2006). The mechanism behind this is that multiple DAP10 adaptor proteins recruit both Grb2-Vav1-Sos complexes and p85 to the immune synapse between NK cells and NKG2D ligand expressing cells. Subsequently, p85 phosphorylates among others Vav1 and phospholipase C (PLC) γ, resulting in enhanced immune synapse formation and cytotoxicity due to actin reorganization and Ca^{2+} influx (Lanier, 2008)(figure 1).

ITAM-bearing NK receptor complexes

All mature NK cells contain ITAM-bearing adaptor molecules, such as DAP12, FcεRI-γ and CD3-ζ that form complexes with a range of different receptors, like KIR2DS2, CD94:NKG2C, NKp30, NKp44, NKp46 and CD16 (Cheent and Khakoo, 2009; Lanier, 2008). CD16, NKp30 and NKp46 form an exception with respect to ITAM-bearing molecule preference, since these receptors are able to form complexes with either FcεRI-γ, CD3-ζ or both as FcεRI-γ/CD3-ζ heterodimers. The CD3-ζ adaptor protein contains three ITAMs and is therefore able to induce the down-stream signaling pathway more efficiently. This may explain why CD16 is able to activate NK cells without cross-linking of additional receptors. NKp30 and NKp46 are not able to activate NK cells upon cross-linking without co-activators, despite their ability to associate with CD3-ζ (Lanier, 2008). This may indicate that CD3-ζ associates with CD16 and NCRs with different affinities.

All ITAM-bearing receptor complexes use similar signal transduction pathways. Upon ligand cross-linking, the ITAM motif is phosphorylated by a member of the Src kinase family. Subsequently, the phosphorylated ITAM-bearing subunit recruits Syk and ZAP-70, two tyrosine kinases, which induce NK cell cytotoxicity and/or cytokine production via several pathways resulting in Ca^{2+} release, actin reorganization and/or NF-κB activation (figure 1). Eventually, the effect of ITAM-dependent signaling is regulated independently from ITAM-bearing receptors, since impairment of ITAM signaling via blockage of the Src kinase family members, abolishes cytokine production of CD16 stimulated NK cells, but has no effect on cytolytic activity (Lanier, 2008). In humans, PLC- γ1, PLC- γ2 and Vav1 play an important role in downstream ITAM signaling (Billadeau et al., 1998; Ting et al., 1992), whereas in mice PLC- γ2, Vav2 and Vav3 are the key players (Cella et al., 2004; Tassi et al., 2005). As mentioned before, phosphatases downstream of inhibitory receptors prevent NK activation by interfering with

Vav proteins (Cheent and Khakoo, 2009; Lanier, 2008). Moreover, it has been discovered that the lipid phosphatase SHIP is able to dephosphorylate CD3-ζ, thereby negatively regulating activating signals from CD16 and NCRs (Galandrini et al., 2002) (figure 1).

CD244 receptor system

Another abundantly expressed NK cell receptor is CD244 (or 2B4). CD244 recognizes CD48, a protein which is highly expressed by hematopoietic cells. CD244 is a unique NK receptor since it is able to function as both as an inhibitory and an activating receptor. In humans, CD244 activity is regulated by the downstream adaptor proteins SAP and EAT2. Mice NK cells express an additional third protein named ERT (Lanier, 2008).

Since a loss-of-function mutation of SAP – found in patients with X-linked lymphoproliferative disease (XLP) – impairs lysis of CD48-bearing cells, it was proposed that SAP plays a role in activating NK cells via CD244 cross-linking (Tangye et al., 2000). Indeed, SAP was found to recruit Fyn, a kinase from the Src family, which EAT2 and ERT do not. Therefore recruitment of SAP-Fyn complexes to CD244 results in phosphorylation of substrates downstream of CD244. Additionally, SAP is able to disrupt polarization of SHIP, SHP-1 and SHP-2 towards the immune synapse and thereby abolishes the inhibitory effect of these proteins (Tangye et al., 1999).

The inhibitory function of EAT2 and ERT is supported by the observation that either EAT2 or ERT-deficient murine NK cells or CD244 knock-out mice show increased cytolytic activity and IFN-γ production (Lee et al., 2004; Roncagalli et al., 2005), suggesting that CD244 acts predominantly as an inhibitory receptor. NK cells from CD244-KO mice lyse syngeneic NK and T cells, which implies a role for CD244 in regulation of fratricide (Taniguchi et al., 2007). Although the exact role of CD244 is currently unknown, its contribution to NK cell activation is regulated by its interplay with other inhibitory and activating receptors (Lanier, 2008).

Effector functions

NK cell activation is a complex and multi-factorial process, combining multiple activating stimuli, loss of inhibitory signals and the enhancing role of cytokines such as IL-2 and IL-15. When activated, NK cells are able to produce multiple cytokines – especially the $CD56^{bright}$ subset – of which IFN-γ is the most prevalent (Cooper et al., 2001; Lodoen and Lanier 2005; Walzer et al., 2005). IFN-γ is known to inhibit viral replication and proliferation of transformed cells directly and stimulates a Th1 response, resulting in activation and recruitment of cytotoxic T cells (Saha et al., 2010). Other cytokines that NK cells produce are TNF-β and GM-CSF, which both play a role in recruitment of granulocytes and monocytes (Cooper et al., 2001).

Activated NK cells (mainly $CD56^{dim}$ NK cells) have a strong cytotoxic capacity, but under the right conditions $CD56^{bright}$ NK cells, which are less cytotoxic, may acquire a $CD56^{dim}$ phenotype as well. The

exact signals that induce this differentiation is unknown, although studies have implied a role for IL-21 and KIR:MHC-1 contact with mature dendritic cells (mDC) in lymphoid structures (Miller et al., 2001; Parrish-Novak et al., 2000). $CD56^{dim}$ NK cells contain specialized granules in their cytoplasm, which upon activation polarize towards the target cell and release their content into the immune synapse (Moretta et al., 2007; Trambas and Griffiths, 2003). The polarization and release of granules is dependent on L-type calcium channels (Zocchi et al., 1998) and in the end results in death of the target cell. These granules contain the effector molecules perforin and granzymes. Perforin forms pores in the plasma membrane and granzymes induce rapid apoptosis (Podack et al., 1985; Shi et al., 1992). Perforin is stored in its active form in the cytosolic granules and has strong homology with C9, the pore-forming component of the complement system (Shinkai et al., 1988). Granzymes can either enter the cell via the pore created by perforin, or via endocytosis through association with the mannose-6-phosphate receptor, which requires perforin for the release granzymes from the endosome (Motyka et al,. 2000; Trambas and Griffiths, 2003). Once in the cytosol, granzyme B cleaves caspase 3, which induces the caspase-dependent cell death pathway. Granzyme A initiates the caspase-independent cell death pathway by nick formation in single-stranded DNA and prevention of cellular repair mechanisms (Shi et al., 1992). Thus, both cytokine production and cytolysis are involved in NK cell-mediated control of viral infections.

Innate or adaptive?

Since the discovery of the NK cells much question have been raised on their classification; do they belong to the innate or adaptive immune system, since NK cells demonstrate characteristics of both (Cooper et al., 2009; Lodoen and Lanier, 2006; Marcenaro et al., 2006; Moretta et al., 2008; Sun and Lanier, 2009).

On one hand, NK-cell receptor genes are not genetically rearranged to adapt to new pathogens, like the genomic VDJ regiments for the T cell receptor (Sun and Lanier, 2009). NK cells depend on molecular pattern recognition to identify pathogens, a specific feature of innate immune cells (Cheent and Khakoo, 2009; Cooper et al., 2009; Lanier, 2008; Mondelli et al., 2010). Similar to dendritic cells (DCs), macrophages and granulocytes, NK cells express Toll-like receptors (TLR). NK cells express TLR-3 and TLR-9 which recognize viral and bacterial products (Marcenaro et al., 2006). NK cells are readily present in tissues and can get activated without prior priming. Therefore NK cells are early responders - or even inducers - during inflammation, a feature that clearly distinguishes innate leukocytes from adaptive leukocytes (Moretta et al., 2008). In this sense, NK cells should be considered more innate than adaptive.

On the other hand, NK and T cells share some phenotypic and functional characteristics, indicating that they might be very closely related (Cooper et al., 2009). A large population of human NK cells

express CD8 upon activation, a molecule abundantly expressed by cytotoxic T cells (Brunetta et al., 2010). Furthermore, both NK cells and cytotoxic T cells maintain and depend on a Th1 milieu and function side-by-side in the eradication of transformed and infected cells. They also use the same mechanisms for killing. The only difference is that cytotoxic T cells recognize antigens MHC-dependently, while NK cells recognize antigens MHC-independently (Trambas and Griffiths, 2003). Similar to T-cells, NK cells have recently been shown to have the ability to "memorize". NK cells were found to respond more rapidly and efficiently in a second encounter with a previously encountered pathogen, months after the first exposure (Horowitz et al., 2010; Cooper et al., 2009; Sun and Lanier, 2009). Furthermore, NK cells undergo similar selection steps as T cells in bone marrow and the thymus to reduce auto-reactivity during NK-cell development. In the periphery naïve NK cells migrate to lymphoid organs to differentiate, previously thought to be limited to adaptive immune cells (Cooper et al., 2009; Bajénoff et al., 2006). Altogether these findings suggest that NK cells have evolved from or alongside T cells, which implies that NK cells may belong to the adaptive immune system.

In conclusion, NK cells have many similarities with both innate and adaptive cells, and therefore it is suggested that NK cells may represent the missing evolutionary link between the innate and adaptive immune system (Moretta et al., 2008; Sun and Lanier, 2009). It is important to further elucidated the exact role of NK cells in both the innate and adaptive immune system, since this may result in new targets for therapeutic intervention, or could provide a basis to fine-tune existing therapies in a NK cell-oriented scope, so that both immune systems are targeted. This may be especially useful in vaccinations, since it has been shown that activation of both the innate and adaptive immune systems increases the success of therapeutic vaccination (Berthoud et al., 2010; Butler and Harty, 2010; Horowitz et al., 2010).

NK cells as regulators of the immune response

(Figure 2) Although NK cells are present in tissues and do not need prior priming, their function is enhanced by activating cytokines provided by other innate cells, such as macrophages, neutrophils, DCs and mast cells. Mast cells play an important role in the activation or inhibition of NK cells as mast cells can either produce IFN-α or IL-4 and IL-5 dependent on the type of stimulus, resulting in either activation or inhibition of NK cells (Marcenaro et al., 2006). Macrophages, neutrophils, DCs and endothelial cells produce high levels of CXCL8 and CX_3CL1 during an inflammatory event, which induce chemotactic responses in NK cells (Robertson, 2002). As NK cells migrate into the inflamed tissue, they are likely to encounter immature dendritic cells (iDC) (Moretta et al., 2008). It was recently discovered that NK cells and DCs interact with each other and shape the subsequent

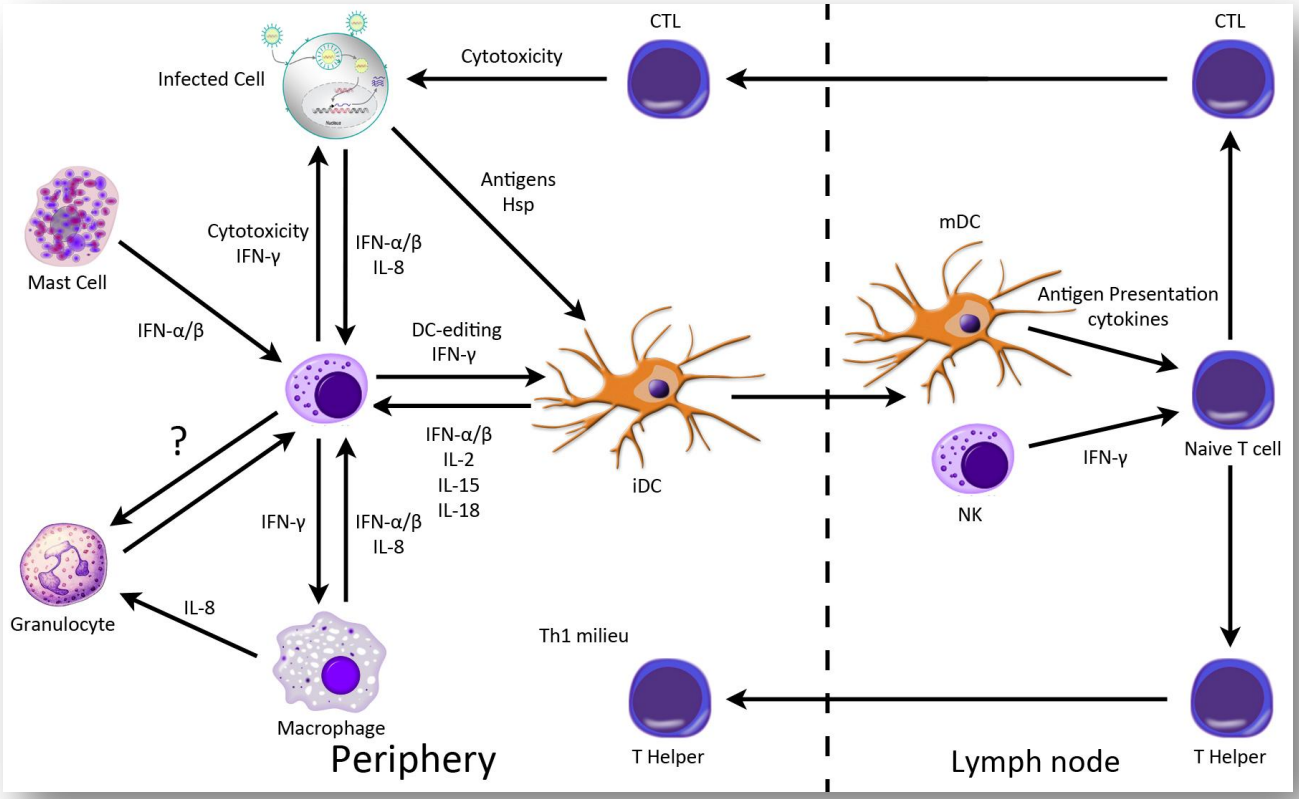

Figure 2 Schematic representation of the central regulatory role of the NK cell in the Th1 immune response When a cell becomes virally infected it releases IFN-α/β and IL-8 together with macrophages, which attracts NK cells and granulocytes. Mast cells are important cells since they determine the initial cytokine milieu. NK cells induce lysis of the infected cells and the contents of these cells activate iDCs and provide iDC with antigens. NK cells support iDCs in their maturation with DC editing, a process in which incorrectly matured DCs are lysed. The matured DCs will produce cytokines that have stimulating effects on NK cells and vice versa. mDCs and NK cells will migrate the lymph node, where they together induce naïve T cells to differentiate into antigen-specific T helper 1 cells and cytotoxic T cells (CTL). The T cells will migrate towards the inflamed tissue, where CTLs perform cytotoxicity on infected cells and Th1 cells produce cytokines that uphold the Th1 milieu that supports NK cells, CTLs and macrophages in the eradication of virally infected cells and cellular debris removal.

immune response (Bajénoff et al., 2006; Gerosa et al., 2005). This bidirectional interaction starts off with a process called DC editing, in which NK cells scan the surface of iDCs for maturation markers and expression of HLA molecules. iDCs express lower levels of HLA-E, making them more susceptible for NK-mediated lysis (Bajénoff et al., 2006; Gerosa et al., 2005; Marcenaro et al., 2006; Moretta et al., 2008; Walzer et al., 2005). When iDCs take up antigens from the environment and get the proper stimuli, they will mature and upregulate maturation markers CD80 and CD83 and HLA-I molecules. These ligands have inhibitory effects on NK cells, preventing lysis of mDC (Gerosa et al., 2005). Therefore, DC editing functions as a quality control for maturing DCs, since only the properly differentiated mDCs will survive.

mDCs produce high levels of IFN-α/β, IL-2, IL-12, IL-15 and IL-18. These cytokines have a stimulating effect on NK cells, which results in increased IFN-γ production by NK cells. IFN-γ has an effect on mDCs, resulting in a positive feedback loop that drives the overall immune response. As a

consequence, more antigens will be available for DC uptake due to increased NK-mediated lysis (Moretta et al., 2007). These antigens can be presented to naïve T cells in the lymph nodes or memory T cells in the periphery, shaping the adaptive immune response (Gerosa et al., 2005). Bajénoff et al. have demonstrated in a murine model that NK cells together with mDCs enter the lymph nodes via the high endothelial venules, facilitated by IL-15. In the lymph node, NK cells, mDCs and T cells accumulate in the cortical ridge of the paracortex, where T cell activation occurs. It was shown that NK cells produce high amounts of IFN-γ, skewing the T cell response towards the Th1 profile (Bajénoff et al., 2006).

When mast cells and eosinophils produce sufficient levels of IL-4, IL-5 or IL-13, NK cells are inhibited which results in reduced DC editing (Marcenaro et al., 2005; Marcenaro et al., 2006; Walzer et al., 2005), mDCs with an immature phenotype, and eventually a Th2 response upon contact with naïve T cells (Marcenaro et al., 2006; Walzer et al., 2005). TGF-β, one of the major cytokines that regulatory T cells and DCs produce in tolerogenic immune responses, is also able to inhibit NK cell functions (Castriconi et al., 2003).

One of the consequences of the Th1 milieu, which NK cells and T helper cells maintain together, is the differentiation of B cells into plasma cells that produce IgG directed against the pathogen. In a secondary response, IgG immunoglobulins are able to activate NK cells via CD16, and as a consequence pathogens are eradicated more efficiently and rapidly. Via this mechanism IgG may contribute to the memory effect of NK cells. This is in contrast with the observation that the memory effect of NK cells could be transferred to unexposed recipients when isolated pathogen-exposed NK cells were transplanted, suggesting an antibody-independent NK cell memory (Cooper et al., 2009).

NK cells are also involved in the termination of an inflammatory episode. The expanded T-cell population should decrease upon eradication of a pathogen, as it could otherwise result in excessive damage and even auto-reactivity. Initially it was thought that T cells undergo apoptosis when activating stimuli disappear from the inflammatory environment (Chong et al., 2010). Patients that suffer from a rare disease called Familial Hemophagocytic Lymphohistiocytosis, characterized by impaired cytotoxicity of NK and $CD8^+$ T cells due to a genetic defect in perforin or perforin-related proteins (Step et al., 1999), are not able to control an immune response. These patients suffer from a widespread accumulation of activated T cells and macrophages that release high amounts of cytokines. NK cells were reported to be involved in apoptosis induction of T cells - in a process called fratricide - to terminate an immune response. Fratricide is similar to cytotoxicity, but is directed to host leukocytes (de Saint-Basile, 2001). The exact triggers are unknown, but it has been shown that the CTL membrane may accidently fuse with target cell membrane, resulting in target cell-derived stress signals and MHC molecules on the surface of CTLs themselves, which makes them more susceptible for lysis (Carlin et al., 2001; Hudrisier et al., 2001). Interactions between CD244 on NK

cells and CD48 on DCs, macrophages and T cells are involved in fratricide as well (Trambas and Griffiths, 2003).

The functions of NK cells extend over all phases of inflammation and NK cells are important in the initiation, regulation and termination of typical Th1 responses against for example viral infection. As a result, viruses have evolved mechanisms to overcome NK cell surveillance during infection.

Viral modulations of NK cell immunity

Viruses are non-living pathogens that consist of an encapsulated viral envelope that encloses genetic material, which can either be DNA or RNA, single or double stranded. For replication, viruses are dependent on a host or multiple hosts (Mondelli et al., 2010). When viruses infect a host, they associate with a specific cell type within the host due to certain molecules they express on the surface of the viral envelope. These surface proteins enable viruses to attach to the target cell and induce endocytosis of the virus particle. Once in the cell, viruses will take over the replication machinery of the host cell, forcing it to duplicate the virus itself. Eventually, this results in an exponential increase in viral particles that are released from the infected cell and subsequently infect other cells. The release of viral particles is often accompanied with necrosis of the host cell (Fanales-Belasio et al., 2010). Continuous necrosis results in severe tissue damage which could eventually be fatal to the host.

During evolution, cellular organisms have developed an immune system with an increasing arsenal of very efficient tools to detect and eradicate viruses. This involves the detection of danger molecules or virally derived antigens in MHC complexes expressed by infected cells. Under pressure of the constant evolutionary battle between the immune system and viruses, viruses have developed strategies of their own to escape immune surveillance (Lodoen and Lanier, 2005; Scalzo, 2002).

Some viruses are so successful in escaping the immune system that they are able to asymptomatically persist within a host for a lifetime (Lodoen and Lanier, 2005; Scalzo, 2002). These latent viruses reside within the host cell, and do not replicate. When the immune system is weakened, these viruses rapidly replicate, sometimes accompanied with symptoms. During these outbreaks the virus is infectious and able to infect a new host (Reddehase et al., 2002).

Other viruses are extremely lethal due to their ability to suppress the host immune system. For instance the Ebola virus is extremely virulent due to its ability to suppress the host Th1 response (Reynard et al., 2009; Sullivan et al., 2003; Warfield et al., 2004).

Since NK cells play an important role in controlling and eradicating viral infections, viruses have developed numerous ingenious strategies to inhibit NK-cell function. Viruses that have developed the most efficient strategies to escape NK cell surveillance seem to be most successful in persistence within a host (Scalzo, 2002).

Hence, it is useful to survey the defensive strategies in order to identify new targets that could be exploited to therapeutically abolish the advantages that these strategies offer. In this report it was shown that the NK cell holds a central position in both the innate and adaptive immune system. Therefore targets for therapeutic intervention that augment NK cell functioning could prove useful as a new treatment for dangerous viruses. This may be applied to currently incurable viruses such as the human immunodeficiency virus (HIV) and the Ebola virus, but also to viruses that are curable or latently present like the influenza virus, the hepatitis virus B and C (HVB and HVC) and herpes viruses such as the cytomegalovirus. There is a potential threat that these currently relatively harmless viruses mutate and cause a potentially lethal worldwide pandemic and perhaps are NK cells key in counteracting such a threat. Hereafter these viruses will be described in more detail regarding their ability to suppress NK cells.

Influenza virus

Influenza virus is a RNA virus belonging to the *Orthomyxoviridae* family. Infection with the virus causes influenza or "the flu", a respiratory disease characterized by fever, headache, coughing, sneezing and fatigue. The Influenza virus, especially type A, forms a pandemic threat to the human population, as was shown by the recent swine flu outbreak (Mao et al., 2009). The influenza virus expresses some specific proteins that determine its virulence, which are hemagglutinin (HA) and neuraminidase (NA). These proteins are involved in cell adhesion and release from the host cell (Guo et al., 2010).

Since influenza-infected respiratory epithelial cells release chemokines that attract NK cells, it is likely that NK cells will encounter the influenza virus. Shortly after infection, NK cells show increased killing of influenza-infected cells, facilitated by NKp44 and NKp46 which recognize viral HA on the surface of infected cells (Guo et al., 2010; Mao et al., 2009). However the influenza virus is able to counteract NK cell-mediated killing via several mechanisms (Mao et al., 2009; Mao et al., 2010; Guo et al., 2010).

Firstly, influenza increases the rate of replication during the acute phase of infection, overwhelming the starting-up NK cell response. During the first few days of infection influenza virus particles outnumber NK cells in the lung and robust infiltration of NK cells only occurs after 5 days post infection. Thus, the rapid viral synthesis and delayed response of NK cells provides sufficient time for the influenza virus to establish a stable virus titer (Guo et al., 2010).

Secondly, influenza is able to modify the surface proteins HA and NA frequently and quickly. During replication certain sites are sensitive for mutation, resulting in new or removed glycosylation sites in these proteins. Modified glycosylation results in reduced recognition by antibodies and NCRs

on NK cells, thereby reducing CD16 and NCR-mediated cytotoxicity of influenza-infected cells (Guo et al., 2010).

Thirdly, viral HA has a dose-dependent negative effect on NCR-mediated killing. Mao et al., have demonstrated that administration of HA to NK cell culture reduces the capacity of NK cells to lyse cells opsonized by NKp46 antibodies. Pseudo-typed viral particles expressing HA and NA are able to inhibit NK activation in a dose-dependent manner as well (Guo et al., 2010). The mechanism responsible for the inhibition is that HA can be internalized via sialic acids, resulting in down-regulation of intracellular CD3-ζ and since NKp46 is dependent on CD3-ζ for signaling, HA reduces its activation (Mao et al., 2010).

Fourthly, the influenza virus stabilizes MHC-I expression in infected cells, and therefore inhibits NK-mediated cytotoxicity. Most viruses induce down-regulation of MHC-I molecules in infected cells to reduce susceptibility to CTL-mediated cytolysis. In influenza-infected cells, MHC-I molecules are redistributed into lipid rafts at the cell surface which increases their mobility, thus enhancing their interaction with inhibitory receptors on NK cells (Achdout et al., 2008).

Fifthly, the influenza virus interferes with the class I interferon production of infected cells. Haye et al., demonstrated that influenza virus-infected respiratory epithelial cells and DCs show reduced IFN-γ production. This reduction was absent in cells that were infected with modified influenza virus which produced non-functional NS1 protein, thus the inhibition of IFN-γ production is NS1-mediated (Haye et al., 2009). NS1 inhibits IFN-γ production via multiple interactions; 1.) NS1 can bind to the viral RNA-recognizing protein retinoic acid-induced gene-I (RIG-I), which results in inhibition of NF-κB and thus cytokine production, and 2.) NS1 prevents release of IFN-γ mRNA from the nucleus. NS1 also protects against the anti-viral effects of IFN released by activated NK cells via inhibition of PKR, an IFN-induced kinase that is able to shut down the translation machinery of an infected cell (Wolf and Ludwig, 2009).

Finally, the influenza virus is able to directly infect and induce apoptosis in NK cells. Mao et al., have shown that influenza enters NK cells via clathrin and caveolin-dependent endocytosis by binding to sialic acids on the cell surface. Once inside, the virus replicates inefficiently, but is able to induce apoptosis which contributes to reduced cytotoxic activity of the NK cell population (Mao et al., 2009).

Altogether these observations show that the influenza virus has many options to inhibit NK cell activation by which the influenza virus prolongs its presence within the host. The inhibitory strategies have *in vivo* relevance as well, since in more severe cases - mostly young and elderly patients - influenza causes potentially lethal pneumonia and it was shown that these patients have low NK cells numbers in peripheral blood, no pulmonary NK cells, and more apoptotic NK cells (Mao et al., 2009).

This indicates that NK cells have a prominent role in controlling influenza virus infection and therefore represent a promising target for therapeutic intervention in influenza virus infection.

Cytomegalovirus

The cytomegalovirus (CMV) belongs to the subfamily *alphaherpesvirinae* of the family *herpesviridae* and is one of the most prevalent viruses in humans; between 50-80% of all adults worldwide are infected. Although CMV infection rarely causes symptoms during adult live, CMV is of risk in immunocompromised persons, due to for instance HIV infection, leukemia or immune suppression after organ transplantation. In these patients, CMV infection is much more aggressive and may induce hepatitis, retinitis, colitis, pneumonitis and even encephalitis, which could result in death. CMV can cause congenital birth defects when the mother contracts a primary infection or suffers a reactivation of the virus during pregnancy (Loewendorf and Benedict, 2010). CMV is a latent virus and therefore very efficient in evading the host immune system. Studies assessing the capability of CMV to evade NK surveillance have put forward several mechanisms by which CMV suppresses NK cells (Jonjić et al., 2008; Lodoen and Lanier, 2005).

MHC-I Mimicry

CMV induces expression of structures resembling MHC-I molecules on the surface of infected cells. This strategy interferes with the “missing self” principle of NK cells and has been identified for both human (HCMV) as murine (MCMV) cytomegalovirus. In humans, UL18 is the decoy ligand expressed by HCMV-infected cells to mimic MHC-I, and it acts via the inhibitory NK receptor LIR-1 (Chapman et al., 1999; Reyburn et al., 1997).

In mice, MCMV expresses two MHC-I homologues, namely m144 and m157. The NK receptor that binds m144 remains unknown. However, MCMV strains that lack m144 show increased susceptibility to NK-mediated lysis, indicating its importance in NK evasion. The receptor for m157 is Ly49I, but in MCMV resistant mouse strains which express Ly49H, m157 contraversively activates NK cells. (Jonjić et al., 2008; Lodoen and Lanier, 2005).

Redistribution of MHC-I molecules

CMV down-regulates MHC-I molecules to escape $CD8^+$ T cell surveillance and as a results HLA-E molecules get destabilized and down-regulated through which the infected cell becomes more susceptible to NK-mediated killing (Lanier, 2008; Jonjić et al., 2008). To counteract HLA-E down-regulation HCMV expresses UL40, a protein very similar to the nonameric leader sequence of HLA-C, which is able to bind and stabilize HLA-E molecules (Ulbrecht et al., 2000). Furthermore, HCMV can selectively influence HLA expression via US2 and US11, resulting in down-modulation and proteasomal degradation of HLA-A and up-regulation of HLA-E. By this means CMV is able to escape both T cell and NK cell surveillance (Jonjić et al., 2008; Lodoen and Lanier, 2005).

MCMV uses a different strategy. The MCMV protein gp34 forms complexes with MHC-I molecules and induces translocation of these complexes from the ER to the cell surface (Kavanagh et al., 2001). The MHC-I-gp34 complex functions as a decoy MHC-I molecule and therefore inhibit NK cells. Additionally, gp34 contains an antigenic peptide that, in conjunction with MHC, does not activate $CD8^+$ T cells (Lodoen and Lanier, 2005).

NKG2D ligand down-regulation

Both HCMV and MCMV inhibit NK recognition by disrupting the up-regulation of NKG2D ligands to the cell surface. The responsible protein in HCMV is UL16, an intracellular protein localized in the membrane of the ER-cis-Golgi compartment. UL16 associates with NKG2D ligands MICB, ULBP1 and ULBP2 – but not with MICA – and retains these molecules within the ER-cis-Golgi compartment (Lodoen and Lanier, 2005; Welte et al., 2003). Chalupny et al. have demonstrated that another viral protein - UL142 - is responsible for down-modulation of MICA (Chalpuny et al., 2006; Jonjić et al., 2008). HMCV also impairs translation of MICB mRNA via a strand of micro interfering RNA (miRNA) miR-UL112 (Stern-Ginossar et al., 2007)

In MCMV, the protein gp40 has a similar function as UL16 and retains all RAE-1-related proteins within an intermediate ER to Golgi compartment. Identical to UL16, gp40 does not affect all NKG2D ligands since it does not alter expression of H60 and MULT1 (Lodoen et al., 2003; Lodoen and Lanier, 2005). MCMV utilizes two other viral proteins; m145 and m155 which down-modulate MULT1 and H60 via proteasomal degradation (Jonjić et al., 2008). An important finding is the viral protein m138, a Fcγ receptor expressed on MCMV-infected cells, which directs degradation of recycled MULT1 and H60. Furthermore, m138 interferes with the costimulatory molecule B7.1 on antigen presenting cells, as a result T cell activation and $Ly49^+$ NK cell expansion in the early phase of MCMV infection is hampered (Cook et al., 2009; Jonjić et al., 2008; Mintern et al., 2006).

NCR ligand interference

Although no distinct HCMV-ligand for NCRs is known, HCMV actively interferes with the NCR NKp30. The dominant viral protein pp65 (UL83) has shown to inhibit NKp30-dependent activation of NK cells by forcing dissociation from its adaptor protein CD3-ζ (Arnon et al., 2005). Whether MCMV uses a similar mechanism is currently not known. The observation that most mouse strains, based on NCR polymorphisms, fail to provoke a NK cell response subsequent to MCMV infection could indicate that an immune evasive mechanism directed to NCRs exists (Jonjić et al., 2008).

Hepatitis Virus B and Hepatitis Virus C

Hepatitis is an inflammation of the liver that can progress with limited or no symptoms, but it is often accompanied with malaise, loss of appetite and yellow pigmentation of the skin (jaundice). Most hepatitis cases worldwide are induced by hepatitis viruses, of which currently five have been

identified (A-E). Hepatitis Virus B (HVB) and Hepatitis Virus C (HVC) are responsible for a large number of infections worldwide and these viruses can induce severe complications like cirrhosis, liver failure and hepatocarcinomas in chronic cases. The underlying pathogenesis by which these viruses cause liver disease is still poorly understood, although it is known that immune evasion is crucial for the pathogenesis of both HVB and HVC (Mondelli et al., 2010; Pelletier et al., 2010). It was shown that acute HVB infection does not induce significant innate immune responses (Fisicaro et al., 2009; Webster et al., 2000; Wieland et al., 2004). In contrast, acute HCV infection induces IFN-α/β production (Foy et al., 2003; Loo et al., 2006), and is associated with increased NK cell degranulation of which the magnitude correlates with the efficiency of subsequent HCV-specific T cell responses. This highlights the importance of NK cell activation and interferon signaling in the control of acute HCV infection (Pelletier et al., 2010). To counteract the anti-viral effect of interferon and activation of the immune system, HVC interferes with the interferon signaling of the host (Foy et al., 2003; Loo et al., 2006). It was shown by Saito et al., that HCV maintains interactions of RIG-1 with its repressor domain so that it cannot associate with the adaptor protein LGP2 to induce IFN genes (Saito et al., 2007). In addition to interferon inhibition, two other mechanisms by which HBV and HCV evade NK cells have been identified.

Tseng et al. have shown that the HVC viral envelope molecule E2 can associate with CD81 which results in cross-linking of CD81. As a consequence, NK cells become dysfunctional and do not respond to CD16 cross-linking with or without presence of activating cytokines (IL-2, IL-12 and/or IL-15). The E2-exposed NK cells in this experiment did not produce or release IFN-γ, nor did they perform cytolysis on human tumor cells (Tseng et al., 2002).

Recently, Tang et al. have demonstrated that HVB down-regulates NKG2D ligand MICA in HVB-induced hepatocarcinoma cells and that these cells are resistant to NK cell-mediated cytotoxicity. By using siRNA strands, directed against HBV mRNA strands, MICA expression and susceptibility to NK cell-induced lysis could be restored (Tang et al., 2009). Even though these discoveries are based on a HVB-induced tumor cell line, it is plausible that MICA down-regulation also occurs during the acute infection phase of HVB.

Human immunodeficiency Virus

The Human Immunodeficiency Virus (HIV) belongs to the genus *Lentiviridae* of the family *Retroviridae* and acute infection with the virus results in mild to severe flu-like symptoms that last one to two weeks. After this period, HIV resides in the host $CD4^+$ T cells without causing any symptoms, which can last for several months to decades. HIV infection will slowly progress into Acquired Immune Deficiency Syndrome (AIDS), characterized by a strong reduction in $CD4^+$ T cell numbers, which compromises the immune system eventually resulting in opportunistic infections and tumors. AIDS

patients are unable to elicit a sufficient immune response and will eventually succumb as a result of otherwise un-lethal infections and abnormalities. HIV infection is currently incurable and only therapy that delays the progression to AIDS exists (Iannello et al., 2008; Kallings, 2008). Like all retroviruses, HIV is able to mutate fast, and therefore it is expected that HIV will ultimately become resistant to the current therapy (Iannello et al., 2008).

Due to the reduction in $CD4^+$ T helper cells, the immune system of a HIV-infected individual becomes solely dependent on cells that do not require prior activation, like NK cells. It has been shown by Martin et al. that HIV-infected individuals - in which AIDS progression is postponed - share a common KIR allele (KIR3DS1) and HLA-B allele (HLA-Bw4-I) combination, indicating that the NK cell is important in controlling HIV (Iannello et al., 2008; Martin et al., 2002). HIV-infected individuals also have high quantities of IgG antibodies against the viral protein gp120/41 and these antibodies induce NK-mediated ADCC of HIV-infected cells. Despite the ability of NK cells to delay disease progression in more resistant individuals, HIV infection will eventually advance to AIDS. This may be due to evasion strategies that HIV has evolved to escape NK cell immunity (Iannello et al., 2008).

HIV is able to frequently change its protein modifications, which neutralizes IgG antibody binding and thus ADCC of HIV-infected cells by NK cells. Additionally, HIV-directed IgG antibodies form complexes with HIV particles that attach to DCs. This induces NK-mediated lysis of these DCs, and as a consequence impairs activation of lymphocytes. NK cells are also affected by the HIV-antibody complexes, since exposure induces down-regulation of NKp30 and KIRs, which impairs NK cell functionality (Tha-In et al., 2007).

HIV proteins interfere with multiple NK cell surveillance mechanisms

It has been demonstrated that HIV can modulate MHC-I expression to counteract both $CD8^+$ T cells as well as NK cells. The viral protein Nef is responsible for this redistribution of HLA-I molecules, since it recognizes a motif of HLA-A and HLA-B molecules resulting in specific degradation of these structures. HLA-C and HLA-E expression is not affected but rather enhanced by Nef, resulting in inhibition of NK cells (Cerboni et al., 2007; Iannello et al., 2008; Jonjić et al., 2008; Lodoen and Lanier, 2005; Scalzo, 2002).

Alongside MHC-redistribution, Nef also induces down-modulation of NKG2D ligands in infected cells, causing a decreased susceptibility to NK cell-mediated cytolysis. Cerboni et al., have demonstrated that purified Nef protein reduces expression of MICA, ULBP1 and ULBP2, but not of ULBP3 in a T-cell line in a dose-dependent manner. Furthermore the Nef-treated cells were resistant to NK cell-mediated lysis (Cerboni et al., 2007).

Another HIV-derived protein that reduces NK cell function is Tat, which is either secreted or incorporated in the host cell membrane. Zocchi et al., have demonstrated that NK cells are

dependent on L-type calcium channels for the release of cytotoxic granules and that Tat blocks these channels *in vitro.* The *in vivo* relevance of Tat has yet to be confirmed, although nanomolar concentrations of Tat are detectable in serum of HIV-infected individuals and NK cells exposed to serum of these individuals show reduced cytotoxic capacity (Zocchi et al., 1998).

The main viral protein gp120 is able to inhibit NK activity by binding the α4β7 integrin on NK cells. This interaction results in phosphorylation of p38 MAPK, which leads to impaired cytolytic activity and lowered secretion of cytokines (Brunetta et al., 2010).

Pathologic redistribution of NK subpopulations

Alongside the previously mentioned direct effects, HIV also has an indirect impairing effect on NK cells, which results in pathological redistribution of NK subpopulations. The severity of the redistribution correlates with the level of HIV viremia (Brunetta et al., 2010). Previously it was thought that peripheral NK cells got depleted by HIV infection, since $CD56^{dim}CD16^{+}$ NK cells are almost undetectable in blood of HIV-infected individuals. However, Mavilio et al. discovered that NK cells in HIV-infected individuals are not depleted but that the NK cells do not express any CD56 on the cell surface. These $CD56^{-}$ NK cells are dysfunctional and express less NCRs, have impaired interactions with DCs, and functional defects in cytokine production and cytotoxicity (Mavilio et al., 2003; Mavilio et al., 2005; Mavilio et al., 2006). Furthermore, these NK cells down-regulate NKG2A and up-regulate NKG2C, and are therefore more susceptible for inhibition via HLA-E (Brunetta et al., 2010). The mechanisms inducing NK cell redistribution are not completely understood, nonetheless one could imagine that dysfunctional $CD4^{+}$ T cells due to HIV-infection have a widespread influence on immune regulation and thus on NK cells as well.

Another possibility is that the pathologic redistribution of NK cell subpopulations is induced by HIV directly. It has been shown that a small subset of NK cells expresses CD4 and HIV co-receptors CCR5 and CXCR4, and that these cells can be infected by HIV. HIV does not replicated efficiently in NK cells; nevertheless the cells become completely dysfunctional due to the infection. It has been proposed that the infected NK cells form a reservoir for HIV particles and thus contribute to viral persistence (Valentin et al., 2002).

Ebola Virus

The Ebola Virus belongs to the *Filoviridae* and causes a hemorrhagic fever with mortality rates of up to 90%. The virus primarily infects endothelial cells leading to internal bleeding, hypotensive shock and eventually death. The Ebola virus is also able to infect hepatocytes and monocytes, such as DCs and macrophages. The virus replicates very rapidly, which induces a cytokine storm that impairs the endothelial barrier and dysregulates the immune response. Mortality of Ebola virus is correlated with increases in IL-2, IFN-α, IFN-γ, IL-10 and TNF-α in the periphery. On the other hand, a protective

effect for increased peripheral IFN-γ to Ebola infection has also been reported, indicating that either the cytokine profile determines infection outcome or that Ebola may actively regulate host immune responses (Sullivan et al., 2003).

Interferon interference

As previously mentioned, the Ebola virus is able to infect DCs, resulting in dysfunctional DC maturation, antigen presentation and interferon production. This abolishes their ability to induce the subsequent immune response and therefore the early host immune response is severely impaired, which enables the Ebola virus to expand rapidly (Bosio et al., 2003; Mahanty et al., 2003). In the infected DCs and endothelial cells, the viral protein VP35 has been shown to antagonize IFN-α/β production, and as a consequence the activation of NK cells and the Th1 response is reduced (Basler et al., 2000). It was found that VP35 induces end-capping of viral RNA, through which it cannot be recognized by viral RNA sensor molecules, like RIG-1, present in the cell (Leung et al., 2010). Ebola also expresses VP24, a protein that is able to inhibit interferon-induced apoptosis by binding to STAT1 downstream of the interferon receptor. As a consequence VP24 prevents the migration of STAT1 into the nucleus and activation of genes involved in apoptosis (Basler and Amarasinghe, 2009).

The early inhibition of innate immunity is crucial for Ebola pathogenesis, since it has been shown that an early innate immune response is positively correlated with survival from Ebola infection in humans (Leroy et al., 2000). This observation is supported by a study in a murine model, as Warfield et al. have shown that increased NK cell proliferation and trafficking early during infection is correlated with survival. In this model it was also demonstrated that vaccination with viral-like particles induces NK cell-dependent protection to Ebola with abundant production of INF-γ and increased cytotoxicity (Warfield et al., 2004).

Table 2 Strategies to escape NK cell surveillance by the Influenza virus, HCMV, MCMV, HBV, HCV, HIV and the Ebola virus

Strategy	Virus	Responsible protein / mechanism
Rapid replication	Influenza, Ebola	N/A
Changing protein modifications	Influenza, HIV	N/A
MHC-I mimicry / up-regulation	Influenza	?
	HCMV	UL18: MHC homologue
	MCMV	M144, m157: MHC homologue
Interferon interference	Influenza	NS1: inhibition of RIG-1 & PKR
	HCV	Cleavage prevention of RD from RIG-1
	HBV	?
	Ebola	VP24: prevention of nuclear entry of STAT1 VP35: end-capping of viral RNA strands
Infection of NK cells	Influenza, HIV	Dysfunctional NK cells
Redistribution of MHC molecules	HCMV	US2/11: ↑ HLA-E, ↓HLA-A/B UL40: stabilizes HLA-E
	MCMV	gp34: ↑ MHC-I, inhibitory complex formation
	HIV	Nef: ↑ HLA-E, ↓HLA-A/B
NKG2D ligand down-regulation	HCMV	UL16, UL142: Degradation of NKG2D ligands UL112: micro interfering RNA strand for MIC-B mRNA
	MCMV	gp40: retains NKG2D ligands in ER-Golgi m138 m145, m155: Degradation of NKG2D ligands
	HIV	Nef: ↓ MIC-A, ↓ ULBP1/2
	HBV	?
NCR interference	Influenza	HA: dissociation of CD3-ζ from NKp46
	HCMV	UL83: dissociation of CD3-ζ from NKp30
Antibody complex formation	HIV	Inhibition of NK cells, NK-mediated ADCC of DCs
Blockage of Ca^{2+} channels	HIV	Tat: binds L-type Ca^{2+} channels on NK cells → no degranulation
Integrin binding	HIV	gp120: binds α4β7 integrin → inhibits NK cells
Redistribution of NK cell subpopulations	HIV	?
CD81 cross-linking	HCV	E2: binds CD81 → unresponsive NK cells

HCMV: Human Cytomegalovirus, MCMV: Murine Cytomegalovirus, HIV: Human Immunodeficiency Virus, HBV: Hepatitis B Virus, HCV: Hepatitis C Virus, RNA: Ribonucleic Acid.

Conclusion

Previously, NK cells were considered to be bystander lymphocytes, merely reacting to cells with reduced MHC-I expression that could not be lysed by cytotoxic T cells. However, recent insights have demonstrated that NK cells occupy a more central, regulatory role within the immune system, spanning from the beginning to the very end of an immune response. NK cells are one of the earliest involved leukocytes in inflammation due to the ability to function without prior activation. These cells are shown to have interactions with most cells of the innate immune system, such as DCs, macrophages, mast cells and neutrophils, and these interactions are shown to initiate and shape the subsequent adaptive immune response.

During the initiation phase of an immune response, NK cells provide chemotactic substances and antigens for DCs, attributable to lysis of infected cells. NK cells support DCs during maturation via NK cell-mediated cytolysis of poorly matured DCs. The differentiated mDCs and NK cells maintain a positive feedback loop which sustains the function of these cells. It has been demonstrated that these NK:DC interactions are crucial for the Th1 immune response. A Th1 response is especially important for control viral infections, since it activates cytotoxic T cells, and induces B cells to differentiate into plasma cells that will produce virus-specific immunoglobulins. It has been shown that NK cells migrate to the lymph nodes to support DCs in the activation of naïve T cells via IFN-γ production (figure 2).

In the periphery, NK cells function in close collaboration with T cells. They uphold the Th1 milieu together with T helper cells and eradicate abnormal cells that are insensitive to cytotoxic T cell-mediated lysis. NK cells have a role in the termination of the immune response, by decreasing the expanded T cell population via cytotoxicity in a process called fratricide.

Due to the widespread responsibilities during inflammation, NK cells should be regarded as a potent target for therapeutic intervention. Recently, several studies are evaluating possibilities to therapeutically address NK cells with cytokines that enhance its functionality, like IL-2 and IL-15, for the treatment of cancer (reviewed by Egilmez et al., 2007). It is plausible that administration of NK cell-inducing cytokines is also beneficial as anti-viral therapy. In this report it was shown that resistance against HIV, Ebola virus and Influenza virus correlated with the magnitude of the NK cell response and it is likely that this is also valid for other viruses. IL-2, IL-12, IL-15 and/or IFN-α/β could be potent anti-viral agents due to their NK-cell stimulating capability. To be effective, cytokine therapy should be given during the acute infection phase, when NK cell immunity is most important. This phase is often asymptomatic and will therefore not receive medical attention, which complicates the application of cytokines for viral disease prevention. Nonetheless their potential should be evaluated, since cytokine administration might be beneficial for the treatment of more progressed viral infections.

Many viruses have developed strategies to counteract NK activity, which in itself is an argument for the importance of NK cells in control of viral infections. Therefore, a proper understanding of these strategies is crucial, since it reveals the essential mechanisms for NK cell functionality, and it could lead to new targets for therapeutic intervention. In this report, the inhibitory effect of several viruses on NK cell function has been discussed. This has put forward several strategies of which some are shared among viruses and others are more exclusive (Table 2). The majority of the described viruses utilize MHC-I mimicry/up-regulation/redistribution, down-regulation of NK activating ligands or interference with interferon signaling as NK cell inhibiting strategies. However, other viruses use more unique mechanisms, like calcium channel blockage and CD81 cross-linking. An important question remains: can there be targets identified within the mechanisms responsible for NK cell inhibition that can be therapeutically addressed to treat viral infections?

A commonly used NK cell escape mechanism is up-regulation or mimicry of MHC molecules, which has an inhibitory effect on NK cells. On the other hand, MHC up-regulation makes virally infected cells more susceptible for lysis by cytotoxic T cells. The influenza virus uses an unknown mechanism to upregulate host MHC molecules, thus influenza may be treated by enhancing cytotoxic T cell immunity, despite being protected from NK cells. This is beyond the scope of this report and will not be discussed in detail. CMV expresses MHC homologues to inhibit NK cells, UL18 in case of HMCV and m144/157 in case of MCMV. Antagonizing antibodies against MHC homologues could prove useful in NK cell-mediated eradication of virus-infected cells, since IgG-mediated CD16 cross-linking solitarily is sufficient to activate NK cells. Designing an efficient therapy based on antibodies is complicated, since; 1.) all polymorphism of the target protein must be known, and 2.) the antibodies must be specific for the viral protein, because of the homology with host MHC. Proteases that cleave virus-specific MHC homologues are useful as well, but should not interfere with host MHC molecules of healthy cells.

Many of viruses discussed in this report, down-modulate HLA molecules or NKG2D ligands via the lysosome-proteasome pathway. Responsible proteins - for instance: HIV's Nef; HCMV's UL2, UL11 and UL40; and MCMV's gp40 – are all intracellular proteins and therefore difficult to target with therapeutic agents. Reversible inhibitors of the proteasome are available which may be able to restore HLA and NKG2D ligand expression on infected cells. For instance, bortezomib is a proteasome inhibitor which has shown promising results in the treatment of different types of cancer. One of the reported effects of bortezomib is MHC up-regulation, through which the infected cells become more susceptible to cytotoxic T cell-mediated killing. It might as well be that the heightened MHC expression exceeds the threshold to activate NK cells via the activating low affinity receptors for HLA. In addition, proteasome inhibition may be able to restore NKG2D ligand expression and activate NK cells in that manner. On the other hand, proteasome inhibitors have inhibitory effects on antigen

presentation and maturation of DCs, and could therefore obstruct the immune response against the virus (reviewed by Einsele, 2010). Regardless this complication, proteasome inhibition could be a promising method to treat virus infections in immuno-compromised patients, who are definitely unable to counteract the infection.

NK cells are the main source of IFN-γ and dependent on class I interferon from monocytes and infected cells for enhanced functioning. Therefore it is not surprising that many of the discussed viruses interference with interferon signaling. Two types of interferon interference can be identified; 1.) interference with IFN synthesis, and 2.) interference in IFN signaling. Viruses that were found to interfere with IFN synthesis in infected cells are the influenza virus, HCV and Ebola. Although these viruses use different mechanism to block IFN production, all are via the inhibition of RIG-1, a protein that recognizes viral RNA and induces interferon production. RIG-I is a intracellular protein, which makes it difficult to target with a substance to restore its function within the infected cell. The net result of RIG-I activation is production of interferon, which could also be administered therapeutically. Administration of interferon is only useful when the concerned virus does not inhibit IFN signaling of infected cells, which the influenza virus and the Ebola virus are able to do, as was shown in this report. The mechanisms down-stream of the IFN receptor that are affected by these viruses are the JAK-STAT signaling pathway and PKR. In case of the Ebola virus, it was shown that its virulence can be significantly reduced by antagonizing VP35 in both *in vitro,* and murine *in vivo* models (review: Basler and Amarasinghe, 2009). This indicates how potent therapy that restores interferon sensitivity of infected cells could be and should therefore be investigated further.

It has been shown that some viruses actively inhibit the specialized NCRs of NK cells to overcome NK cell immunity. The NCR NKp46 is known to bind the influenza virus specific protein HA and induces NK cell activation as a result of this interaction. On the other hand, the HA protein is co-responsible for the virulence of the influenza virus and this may be due to the inhibitory effect of NK cells discussed in this report. Therefore therapeutically intervening with the HA:NKp46 interaction might enable NK cells once more to perform NKp46-mediated cytotoxicity on influenza virus-infected cells. However, the influenza virus mutates HA frequently and is able to change its protein modifications, which challenges the identification of an universal HA inhibitor. The ligand of NKp44 remains unclear, although it is known that it recognizes an influenza-specific antigen, as down-regulation of NKp44 results in reduced NK-mediated killing of influenza-infected cells. More research investigating the ligand of NKp44 may provide a target for NK-enhancing therapy of influenza. The role of NKp30 inhibition in HCMV infection should be investigated for that same reason, since the contribution of NKp30 inhibition to the pathogenesis of HCMV remains unknown.

An exclusive NK cell escape mechanisms is CD81 cross-linking as was demonstrated in HCV. CD81 is expressed on most hematopoietic and epithelial cells, and has shown to have anti-proliferative

effects when cross-linked. HCV infection can be asymptomatic for multiple years and cross-linking of CD81 on NK cells may very well be the main strategy responsible for the this effect. This immune suppression strategy affects not only NK cells, but the complete immune system and intervening in CD81 cross-linking may possibly restore immune surveillance in Hepatitis C patients. Noteworthy is that CD81 is considered a tumor suppressor gene, and since some viruses are associated with malignancies, it may be that these viruses address CD81 to repress the host immune system as well. Therefore, CD81 cross-linking should be investigated in viral infections, especially viruses with carcinogenic potential, since it could result in a novel type of therapeutics to treat viral infections or prevent the potential malignancies viruses may induce.

NK cell subpopulations in HIV-infected individuals are pathologically redistributed, resulting in dysfunctional NK cells. The responsible mechanism is currently undefined, however it is likely that the impaired $CD4^+$ T cell population may contribute to this phenomenon. More research is needed to elucidate the mechanism responsible for the pathologic redistribution of NK cell subpopulations and the role of $CD4^+$ T cells in establishing the impairment. Restoring NK cell immunity is of particular interest in HIV infection, since it was shown that NK cell activity and certain combinations of KIR and HLA-B alleles are associated with a prolonged symptom-free period during the progression to AIDS. Perhaps cytokine substitution could restore NK cell subpopulations, and in particular IL-21 because of its role in NK cell differentiation.

HIV has also demonstrated other NK cell escape mechanism, like Ca^{2+} channel blockage and integrin binding. A promising target for therapeutic intervention could be the secreted protein Tat, which is responsible for the blockage of calcium channels of NK cells during HIV infection. Tat is present in serum of HIV infected individuals and thus could have *in vivo* relevance. Neutralizing antibodies or substances against Tat may be able to restore NK cell immunity and prolong the symptom-free phase of HIV infection. It may be possible that other viruses actively block Ca^{2+} channels of NK cells as well, and therefore this should be evaluated as a therapeutic target for NK re-activation in viral infections other than HIV.

The ability of viruses to inhibit NK cells by means of integrin binding provides a promising field of research, because it is likely that other viruses use similar strategies to reduce NK cell surveillance. Integrins are commonly expressed by leukocytes and have widespread functions in the immune response, which make these molecules likely targets for immune escape. The identification of expressed or secreted viral proteins that are responsible for inhibition of NK cells via integrin binding may put forward potent treatment targets.

Due to the expression of CD16 by NK cells, antibody therapy could be a potent method to induce NK cell function. It was demonstrated that most patients have significant amounts of antibodies against viral proteins in their serum, also directed to those responsible for immune suppression,

however the immune system is not able to remove the virus in many cases. This puts forward a major difficulty for antibody therapy as NK cell activity enhancement; viruses use multiple mechanisms to escape the host's immune system and as a result treatment with virus-specific antibodies alone may not have a significant effect. This should be investigated for every virus individually, since the mechanisms used of immune escape differ between viruses.

In addition, designing a therapeutic agent that antagonizes a viral protein is very complex. It requires that the targeted protein has a domain that is specific for the virus, has no homolog sequence in the host, and is not subjected to frequent protein modifications. Various viral proteins resemble the natural ligand of their target receptor and thus are difficult to target, with for example blocking antibodies or substances, as it would also block the naturally occurring host protein. This could result in severe side effects that would be unethical to induce in harmless viral infections like influenza or CMV. On the other hand in incurable and lethal infections, such as HIV and Ebola, complications are of less importance and the usage of therapeutics with side-effects is more tolerated.

In viral infections where NK cells themselves can be infected, enhancing NK immunity is ineffective. Here it was discussed that influenza and HIV are able to infect NK cells. Although the viruses do not replicate efficiently in NK cells, infection renders the NK cells dysfunctional. Thus viruses must be studied in their ability to infect NK cells before a NK enhancing strategy can be defined. It may be possible that NK cell infection can be counteracted therapeutically, which perhaps results in recovery from infection. Clearly, this matter is important and should be investigated further.

Despite the difficulties, enhancement of NK cell function in viral infections by antagonizing NK cell escape mechanisms and/or stimulating NK cell activation represents a potent novel alternative for anti-viral therapy. Therapeutically addressing NK cells may result in relief of symptoms and perhaps even recovery, due to the principal role of NK cells in anti-viral immunity. It has been shown that vaccination results in increased NK cell activity (Horowitz et al. 2010; Scott-Algara et al., 2010) and that NK cells can acquire memory-like abilities. However, due to the escape strategies that viruses have evolved the NK cell-related functions may not be effective to its full potential. Therefore, the options to relief NK cells from the virus-induced inhibition should be investigated further to exploit the resourceful anti-viral properties that NK cells posses to the fullest.

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