Characterization of natural killer cell response to human cytomegalovirus infected dendritic cells

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To my family

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THESIS ABSTRACT

Suitable experimental conditions have been established to dissect the role of NK cell receptors (NKR) and cytokines in the NK cell response against autologous human cytomegalovirus (HCMV) infected monocyte derived dendritic cells (moDC). Our results reveal that NK cells are capable of responding to HCMV infected moDC that have down-regulated surface MHC class I molecules. In particular, we prove that HCMV infection decreases surface HLA-E expression on moDC, thus releasing $NKG2A^+$ NK cells from inhibition. We show that NKp46 and DNAM-1 NKR play a dominant role in the recognition of HCMV infected moDC and we provide evidences stressing the importance of the dynamics of viral immune evasion mechanisms in NK cell susceptibility. Finally, we find that type I interferons and IL-12 secreted in response to HCMV infection, beyond their participation in NK cell activation and IFN-γ secretion, transiently inhibit the expression and function of NKG2D in NK cells, thus providing a potential regulatory feedback mechanism to prevent NK cell reactivity against bystander healthy cells.

RESUM DE LA TESI

S'ha establert un sistema experimental autòleg per a poder estudiar la resposta de les cèl.lules *Natural Killer* (NK) contra les cèl.lules dendrítiques derivades de monòcits (moDC), infectades pel Cytomegalovirus humà (HCMV). Els nostres resultats mostren que les cèl.lules NK responen contra les moDC infectades per HCMV, que presenten una expressió de les molècules MHC de classe I a superficie reduïda. Específicament, demostrem que la infecció per HCMV disminueix l'expressió en superficie d'HLA-E en les moDC, alliberant així la inhibició de les cèl.lules NK NKG2A$^+$. Mostrem que els NKR anomenats NKp46 i DNAM-1 tenen un paper dominant en el reconeixement de les moDC infectades per HCMV i evidenciem la importància de la dinàmica dels mecanismes d'immunoevassió en la susceptibilitat a la resposta NK. Finalment, trobem que els interferons de tipus I i la IL-12 secretats en resposta a la infecció per HCMV, a més de participar en l'activació de la cèl.lula NK i en la secreció d'IFN-γ, inhibeixen l'expressió i la funció de NKG2D en les cèl.lules NK, com un mecanisme de regulació potencial per prevenir la reactivitat NK contra cèl.lules veïnes sanes.

PREFACE

Human natural killer (NK) cells are believed to participate in the early innate immune response to different infectious pathogens; in particular, substantial experimental evidence supports their role as a first line of defence against human cytomegalovirus (HCMV). HCMV is a β herpesvirus that establishes an asymptomatic life-long latent infection in immunocompetent hosts but causes severe congenital disease and important disorders in immunocompromised patients. Cells of the myeloid lineage are considered reservoirs for HCMV latency, and differentiation of myeloid progenitors to dendritic cells (DC) may reactivate the virus. Similarly to other herpesviruses, the ability of HCMV to persist in the host is associated to different immune evasion strategies. Yet, information on the nature of NK cell receptor-ligand interactions involved in the response to HCMV infected cells is scarce and most studies have been performed in infected fibroblasts. Our work provides novel insights on the way NK cells respond to HCMV-infected DC and how antiviral cytokines may regulate NK cell functions.

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PART I

INTRODUCTION AND AIMS

Chapter I

Introduction

1. INNATE IMMUNITY: AN OVERVIEW

In vertebrates, the immune system is divided into two major branches, the innate and adaptive immunity that are highly connected and reciprocally regulated. The adaptive immunity is required to provide long-lasting specific immunity and is mediated by T and B lymphocytes, while the innate immune system is the first line of defence against pathogens. From a phylogenetic standpoint, the innate immunity evolved long before the adaptive immune system, and homologous components can be found in invertebrates and plants. Although it might appear primitive, the innate immune system has recently been recognized to be more complex and sophisticated than previously thought and it is now increasingly clear that several aspects of the adaptive immune response are controlled by the innate response.

Besides the role in defense of epithelial barriers, the innate immune system includes cellular components that distinguish infectious agents and/or abnormal self from normal self molecules, using germline encoded receptors, which include Toll-like receptors (TLR), cell surface C-type lectin receptors, and the more newly defined families of NOD-like receptors and RIG-I helicase like receptors[1]. The cellular compartment is represented by granulocytes, mastocytes, monocytes, macrophages, dendritic cells (DC) and natural killer (NK) cells. Although each cell type has specific functions, they cooperate to orchestrate effective immune responses. They contribute to the direct elimination of infectious agents and regulate as well the development of adaptive immunity, influencing the quality and strength of the immune response.

2. IMMUNOBIOLOGY OF NATURAL KILLER CELLS

NK cells were originally described in 1975 as large granular lymphocytes with capacity to kill *in vitro* tumor target cells without previous sensitization[2,3]. At present, NK cells have gained recognition for their ability to secrete proinflammatory and antiviral cytokines, to recognize and

eliminate tumors, viral and parasitic infections as well as normal allogeneic cells and to have regulatory functions in the context of the adaptive immune response[4]. Recent data indicate that NK cells can also mount secondary responses, altering their functional capacities based on prior activation, a characteristic that was previously thought to be limited to adaptive immunity[5].

2.1 NK cell subsets distribution and characterization

NK cells originate in the bone marrow from $CD34^+$ haematopoietic progenitor cells and are widespread throughout lymphoid and nonlymphoid tissues. They constitute 5-25% of human peripheral blood mononuclear cells (PBMC) and up to 5% of the whole lymphocyte population in lymph nodes[6]. Additionally, NK cells can be found throughout most tissues including liver, spleen and lungs[7]. Several studies have also described the existence of NK cells in the uterine mucosa, where they promote vascularisation of implanting embryos during pregnancy[8]. A subset of cells expressing NK cell markers have been found in the mucosa-associated lymphoid tissue (MALT), where they are specialized in the production of interleukin (IL)-22[9,10], a member of IL-10 cytokine family involved in the homeostasis of epithelia and in the early host defense against microbial pathogens[11].

In humans, NK cells are conventionally defined as $CD56^+CD3^-$ lymphocytes. Based on the cell surface density of CD56, NK cells can be divided into two subsets that display distinct phenotypic and functional properties. $CD56^{dim}$ NK cells represent the vast majority in peripheral blood, accounting for up to 90% of all NK cells, but are rare in lymph nodes. They express high levels of FcγRIII (CD16) and are cytotoxic. $CD56^{bright}$ NK cells account for around 10% in peripheral blood and are the predominant subset found in secondary lymphoid tissues. $CD56^{bright}$ cells have a lower cytolytic capacity but efficiently produce cytokines. $CD56^{dim}$ and $CD56^{bright}$ subsets

also differ in the distribution of NK cell receptors (NKR) and in their proliferative response[6].

Interestingly, several lines of evidence indicate the existence in healthy individuals and the expansion during the course of HIV and HCV infection of a small subset of cells that are $CD3^-$ and $CD56^-$ but express CD16 and other NKR[12,13], suggesting that a definition of NK cells as $NKp46^+$ and $CD3^-$ might be appropriate to define this subset of lymphocytes[14].

2.2 NK cell effector functions

NK cells typically exert two major effector mechanisms: (1) recognition and killing of target cells through perforin and granzymes release, as well as expression of death receptor ligands and (2) activation and recruitment of other immune cell types through secretion of cytokines and chemokines[6].

The activation of NK cell mediated cytotoxicity occurs upon direct contact and recognition of transformed or infected cells, as well as via antibody dependent cellular cytotoxicity (ADCC) through engagement of FcγRIII receptor (CD16)[15].

The main cytotoxic pathway involves perforin and granzymes that are stored in granules inside NK cells and get released by exocytosis upon target cell recognition. According to the current view, perforin molecules form a pore in the membrane of the target cell which enables granzymes to enter into the cytoplasm and initiate the apoptotic process[16]. Further mechanisms include induction of apoptosis via TRAIL (TNF-related apoptosis-inducing ligand) or engagement of the Fas receptor on the target cell surface via the Fas ligand[17].

NK cells have also been shown to display regulatory capabilities, which are mediated by various cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-10, IL-13 and/or granulocyte-macrophage colony-stimulating factor (GM-CSF), released upon engagement of triggering NKR or signalling by other cytokines[18]. In particular, NK cell derived IFN-γ can

directly inhibit viral replication in infected cells[19] and promote the differentiation of naïve $CD4^+$ T cells into Th1 helper cells[20,21]. Following activation, NK cells have been shown to secrete chemokines, like MIP 1α, MIP 1β, IL-8 and RANTES, with the capacity to recruit T cells, B cells, neutrophils, and other activated NK cells at sites of inflammation[22].

The trafficking of NK cells is regulated by chemokine receptors whose expression differs among NK cell subsets and depends on their maturation state. $CD56^{dim}$ $CD16^+$ NK cells express high level of CXCR1, CX3CR1, and the recently identified chemerin receptor ChemR23 but not CC chemokine receptors. These receptors are likely to play a role during recruitment of NK cells into peripheral inflammatory tissues. CCR7 is expressed on $CD56^{bright}$ $CD16^-$ NK cells and is believed to regulate their homing to lymph nodes[23,24]. Recently it has been also reported that CCR7 de novo expression can be induced on $CD56^{dim}$ NK cells upon IL-18 stimulation[25].

The intensity and the quality of the NK cell response are finely regulated and depend on the cytokine microenvironment, as well as on interactions with other cells of the immune system that cooperate to promote NK cell "priming". Type I IFNs, IL-1, IL-12 and IL-18 are potent activators of NK cell effector functions, whereas IL-15 and IL-2 are important for NK cell maturation, proliferation, survival and, to some extent, cytotoxicity and cytokine secretion. On the other hand, NK cell function can be negatively regulated by transforming growth factor-β (TGF-β), and by regulatory T cells trough a TGF-β-dependent mechanism[4].

2.3 Target cell recognition

The mechanism that allows NK cells to discriminate target cells remained a mystery for a long time. In 1981 Klas Kärre made a key contribution postulating the "missing self hypothesis". This concept was based on the observation that NK cells kill target cells lacking self MHC class I molecules, whereas normal MHC class I surface expression levels

protects healthy autologous cells from lysis[26-28]. This hypothesis was further validated by the demonstration that NK cells destroy bone marrow cells obtained from gene targeted MHC class I deficient mice, otherwise genetically identical[29,30] and confirmed by the identification of several families of MHC class I binding receptors expressed by NK cells which engagement delivers inhibitory signals (inhibitory NKR)[31-35].

Tolerance to self is achieved during NK cells development and maturation through a process called "licensing" or "education". This process requires recognition by NK cell inhibitory receptors of self MHC class I molecules that are expressed in the steady-state condition. There is evidence that NK cells expressing self MHC-class-I-specific inhibitory receptors become functionally competent, whereas those lacking such a receptor remain functionally inert (unlicensed NK cells)[36]. Recently, it has been also shown that NK cell licensing may not be exclusively a process that occurs in bone marrow during development as "unlicensed" peripheral blood NK cells may become fully competent, changing the MHC environment[37].

Yet, in order to activate NK cells, other signals triggered by the engagement of specific activating NKR are required[38]. According to the current "dynamic equilibrium paradigm", the balance between inhibitory and stimulatory signals received by a NK cell determines the outcome of the interaction with the target cell. Healthy cells are protected from killing when signals delivered by stimulatory ligands are controlled by the engagement of inhibitory receptors. NK cell effector functions can be triggered when a target cell down-regulates expression of MHC class I molecules (as a result of transformation or infection) or when a pathological process up-regulates the expression of stimulatory ligands, engaging activating NKR (Figure 1). All these processes are also regulated by cytokines released in the milieu by neighbouring cells that can modulate the activation state of NK cells[4].

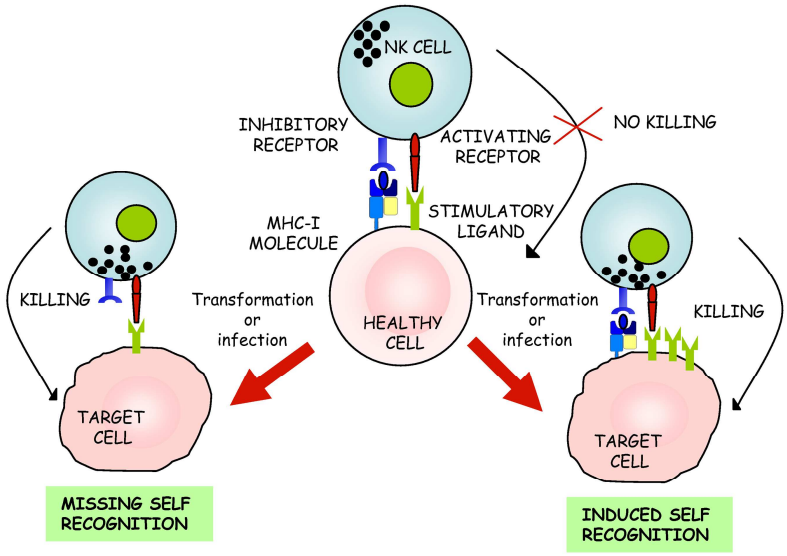

Figure 1. Regulation of NK cell function by inhibitory and activating NK cell receptors

2.4 Inhibitory and activating NK cell receptors

The first inhibitory NKR was described in mice and was named Ly49[35]. Since then, several other receptors have been identified in humans and rodents. The majority of inhibitory NKR are specific for MHC class I molecules, however some may bind non-MHC ligands. Although different in their extra-cellular region, these inhibitory receptors share a common signalling motif in their cytoplasmic region called immunoreceptor tyrosine-based inhibitory motif (ITIM). Phosphorylation of ITIM upon engagement of the receptor, e.g. via Src family kinase, allows docking of protein tyrosine phosphatases such SHP-1 and SHP-2, that mediate negative signalling by dephosphorylating different intracellular substrates, depending on the particular array of receptors engaged[39].

Besides inhibitory receptors, NK cells express a wide panel of activating and/or costimulatory molecules that cooperate to elicit cytolytic activity and cytokine secretion. Activating NKRs lack ITIM motifs and have charged

residues in their transmembrane domain that are necessary for association with adaptor proteins which have docking sites for downstream stimulatory signalling molecules. Most adaptors (FcεRγ, CD3ζ, and DAP12) contain immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic domains, which allow them to associate with ZAP70 and/or syk tyrosine kinases. Alternatively, human NKG2D associates to a different adaptor molecule, DAP10, that has an YxxM motif in its transmembrane domain which allows recruitment of phosphotidylinositol 3-kinase (PI3K)[40].
The following paragraphs briefly review the characteristics of the most important NKR.

2.4.1 NKR specific for MHC class I molecules

In humans, three different families of genes encoding inhibitory receptors specific for MHC class I have been identified: Killer Immunoglobulin-like Receptors (KIRs), Leukocyte Immunoglobulin-Like Receptors (LILRs) and CD94/NKG2 heterodimers. The CD94/NKG2 heterodimers are also found in rodents with functions similar to human receptors. In mice, instead of KIRs, NK cells express the highly polymorphic Ly49 C-type lectin receptors that are phylogenetically unrelated but functionally equivalent to KIRs, as an example of convergent evolution. All these inhibitory receptor families encode also for activating molecules whose physiological role remains unclear[41].
In general, each receptor exhibits a variegated expression pattern, leading to a complex combinatorial repertoire of NK cell specificities for MHC class I molecules. Expression of the inhibitory receptor repertoire among NK clones appears to be stochastic and, until recently, the general idea was that every NK cell expresses at least one inhibitory receptor specific for MHC class I in order to avoid autoreactivity[42]. Recent studies, however, revealed the existence of NK cells lacking expression of inhibitory receptors with diminished sensitivity to stimulatory ligands (hyporesponsive NK cells)[43,44].

2.4.1.1. KIRs

KIRs are polymorphic cell surface molecules present on NK cells and a small fraction of T cells. KIRs are encoded by genes located in the leukocyte receptor complex (LRC) region in chromosome 19q. In the LRC, KIR genes are organised in haplotypes, which have been defined in family segregation studies. The polymorphism of the KIR locus parallels that of the MHC and is associated with a growing number of human diseases[45,46].

KIRs generally recognize allelic determinants shared by distinct groups of HLA class I allotypes and can be classified into 15 different members, according to structural and functional differences. KIR receptors may contain two (KIR2D) or three (KIR3D) Ig-like extracellular domains; just like the murine Ly49 family, they can be equipped with different signalling domains: containing either inhibitory ITIM in the long cytoplasmic tail (e.g. KIR2DL or KIR3DL) or having short cytoplasmic tail lacking ITIM and bearing a charged transmembrane residue (Lys-Arg) which allows them to associate with an ITAM bearing adaptor molecule, DAP12, with activating potential (e.g. KIR2DS or KIR3DS); different KIR with similar overall organization but sequence divergence higher than 2% are generally numbered in series (e.g. KIR2DL1-3)[45,46] .

The ligands for several inhibitory KIRs have been defined, while the specificities of activating KIRs are so far mostly elusive, although some of them have been reported to recognize MHC class I molecules, with lower affinity than their inhibitory counterparts. Among the inhibitory KIRs, KIR2DLs recognize HLA-C allotypes, KIR3DL1 is the receptor for HLA-B allotypes with Bw4 motifs and KIR3DL2 is a receptor for HLA-A3/11[46-48]. It has been proposed that KIR2DL4, shared by all haplotypes, is able to bind the non-classical MHC class I molecule HLA-G1[49].

Beyond its function in recognition of HLA class I alleles, it has been suggested that KIR2DL2 may act as sensor of microbial products as it is able

to bind CpG-Oligodeoxynucleotides (ODNs) and shuttle them to NK cell endosomal compartments[50].

2.4.1.2 LILRs

LILRs (also known as Ig-like transcripts (ILT), LIR and CD85) are immune receptors encoded by genes located within the LRC, centromeric to the KIR cluster. The family consists in 11 genes and 2 pseudogenes encoding for Immunoglobulin superfamily (IgSF) proteins, expressed on both myeloid and lymphoid cells. Some members of the family contain cytoplasmic ITIM that recruit SHP tyrosine phosphatases and trigger inhibitory signals whereas others display a charged transmembrane residue (Arg) and associate to the FcεRγ chain, delivering activating signals[51,52].

Some LILRs act as receptors for HLA-class I molecules[51]. The best characterized is the inhibitory LILRB1 (ILT2/LIR1/CD85j), the only one that is detected on NK, T and B cells, besides myelomonocytic cells[53]. LILRB1 recognizes a wide range of classical HLA-class I allelic variants, as well as the non-classical molecules HLA- F[54] and –G[55], by binding to the conserved α3 domain[56]. It also recognizes with high affinity the human cytomegalovirus (HCMV)-encoded MHC class I homologue UL18[57] as it will be discussed later.

2.4.1.3 CD94/NKG2 receptors

CD94/NKG2 receptors are C-type lectin heterodimers, formed by the covalent association of CD94, a type II integral membrane glycoprotein with a short non-signaling cytoplasmic tail, with NKG2 molecules [58-60]. The NKG2 family includes: NKG2A (and its splice variant NKG2B), NKG2C, NKG2E (and its splice variant NKG2H), and NKG2F. The CD94 and NKG2 genes are all closely linked in the human NK gene complex at chromosome 12[61].

Among the CD94/NKG2 receptors, the best characterized are the inhibitory CD94/NKG2A and the activating CD94/NKG2C receptors. NKG2A contains a cytoplasmic ITIM that allows docking of SHP-1 tyrosine phosphatase, which mediates inhibitory signaling. CD94/NKG2C forms an activating receptor linked to KARAP/DAP12, an ITAM bearing adaptor molecule which triggers a tyrosine kinase dependent pathway[62-65](Figure 2).

Both receptors are constitutively displayed by different subsets of human NK cells, γδ and αβ T lymphocytes and a minor proportion of NK and T cells from adult blood donors may co-express both receptors. It has also been shown that NKG2A may be transiently displayed by CD94/NKG2C$^+$ NK cell clones under the influence of IL-12, providing a potential negative regulatory feedback mechanism[66].

The natural ligand for the CD94/NKG2A and CD94/NKG2C complex, as well as presumably for the activating receptor CD94/NKG2E, is the non-classical MHC class I molecule, HLA-E[67-69] in humans and its homologue Qa1 in mice [70,71]. HLA-E is a class Ib molecule that is highly homologous to classical MHC class I proteins and is widely expressed in different tissues. This molecule binds to hydrophobic peptides derived from residues 3 to 11 of the leader sequence of some class Ia HLA and HLA-G molecules. The loading of nonamers into HLA-E is dependent on the transporter associated with antigen processing (TAP) which is responsible of transporting the processed leader peptides from the cytosol to the lumen of the endoplasmic reticulum (ER)[72,73]. Accordingly, the levels of HLA-E surface expression allow NK cells to monitor through the CD94/NKG2A inhibitory receptor the normal biosynthesis of MHC class I molecules.

Compared to MHC class Ia molecules, HLA-E polymorphism is rather limited and only eight alleles encoding three different proteins were described so far[74]. Two allelic variants of HLA-E are found at high frequency in humans, maintained through balancing selection; they are distinguished by a single sequence dimorphism at position 107: glycine

(E*0101; HLA-E^{G}) or arginine (E*0103; HLA-E^{R}). Biophysical studies have shown that HLA-E^{G} has a slightly lower affinity for leader peptides compared to HLA-E^{R}. Interestingly, the marginal decrease in the affinity of HLA-E^{G} appears to result in a lower level of cell surface expression[75].

According to distinct crystal structure analyses, the HLA-E peptide binding groove is highly restrictive for peptide binding and residues at position P2, P3, P6 and P9 of nonamers are critical[76]. In particular, functional data indicate that HLA-E shows very low affinity for peptides derived from several HLA alleles that contain a threonine (Thr) in P2, instead of metionine (Met)[77].

Structural analysis of the CD94/NKG2-HLA-E complex revealed that the CD94 subunit almost completely dominated the binding with HLA-E[78]. Although NKG2A and NKG2C show ~ 92% identity in the extracellular region, functional studies have revealed that CD94/NKG2C has approximately eightfold lower affinity for HLA-E than CD94/NKG2A[79], probably resulting from conformational changes in CD94[76].

The affinity of the interaction between HLA-E and CD94/NKG2 is also dependent on the peptide bound to HLA-E. In particular, it has been shown that HLA-E bound to the leader sequence peptide of HLA-G has a higher affinity for CD94/NKG2 receptors than complexes with nonamers derived from classical HLA molecules[79,80]. Moreover, several studies revealed that some HLA unrelated peptides, able to stabilize HLA-E on the surface of the cells, form complexes displaying a very low affinity for CD94/NKG2A molecules [81,82]. On the other hand, peptides derived from viral proteins, with sequences identical or different from canonical leader nonamers, have been shown to bind to HLA-E and interact with CD94/NKG2A[83-85].

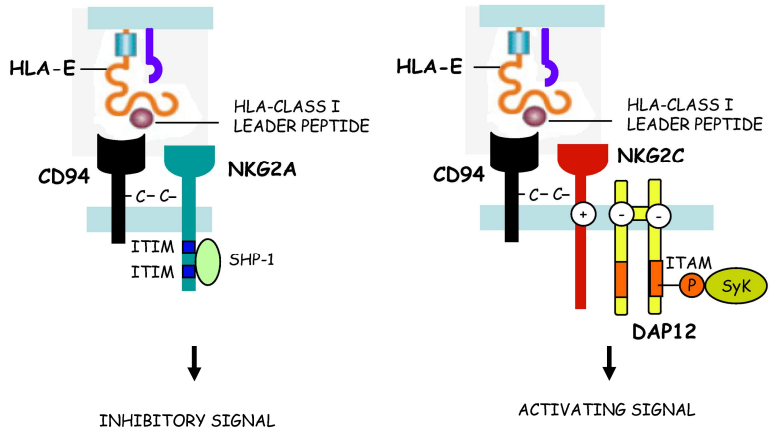

Figure 2. CD94/NKG2A inhibitory and CD94/NKG2C activating receptors

2.4.2 NKR specific for non MHC class I molecules

The large majority of NK cell activating receptors and some inhibitory receptors do not recognize MHC class I molecules and for some of them the biological role is not well understood, as the corresponding ligands have not been identified so far.

In humans, the group of NK cell activating or co-stimulatory receptors that do not recognize MHC class I molecules includes NKG2D, the natural cytotoxicity receptors (NKp46, NKp30 and NKp44), DNAM-1, NKp80, NKp65 and members of the SLAM family (NTB-A, 2B4 and CRACC), among others. Moreover, several receptors with inhibitory features (TIGIT, CEACAM1, SIGLEC-7) that are not specific for MHC class I molecules have been also characterized and will be discussed later on.

2.4.2.1 NKG2D

NKG2D is a well characterized activating receptor involved in innate and adaptive immune responses to infection, as well in tumor immunosurveillance and autoimmunity. This receptor belongs to the subfamily of C type lectin-like receptors and is expressed as a disulfide-linked

homodimer on the cell surface[86]. The gene encoding for NKG2D, named KLRK1, is highly conserved and is located in human chromosome 12, within the NK gene complex, and in a syntenic region of mouse chromosome 6[86]. In humans, NKG2D is constitutively expressed by all NK cells, most γδ T cells and all αβ $CD8^+$ T cells[87,88]. The expression of NKG2D in a small subset of $CD4^+$ T cells has been also documented in healthy blood donors[89] and expansion of this subset is observed in patients with rheumatoid arthritis (RA)[90], in late-stage cancer patients[91], as well as in patients with juvenile-onset systemic lupus erythematosus[92]. It has been shown by our group that *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) from healthy seropositive individuals with HCMV promotes variable expansion of $CD4^+NKG2D^+$ T cells specific for viral antigens[89].

Human NKG2D, as other activating receptors, has short cytoplasmic tail and the signaling activity is mediated through its association with the adaptor protein DAP10. Upon engagement of NKG2D, the cytoplasmic YxxM motif of DAP10 is tyrosine phosphorylated and can recruit phosphotidylinositol 3-kinase (PI3K) and the adaptor Grb2. The association of one NKG2D homodimer with two DAP10 homodimers forms a stable hexameric complex on the cell surface. By contrast murine NKG2D is expressed in two isoforms which are generated by alternative splicing: a long form (NKG2D-L) that has a 13-amino-acid extension at the amino terminus and associates only with DAP10, and a short form (NKG2D-S) that can associate with DAP10 or DAP12/KARAP[40] (Figure 3).

In NK cells, NKG2D was reported to function as a primary activating receptor in redirecting killing assays and in experiments with soluble recombinant NKG2D ligands[93,94]. However other studies have shown that the exclusive engagement of NKG2D fails to induce NK cell activation but is necessary to co-stimulate with other activating molecules cytotoxicity and cytokine secretion[95,96].

In resting $CD8^+$ T cells NKG2D has a co-stimulatory rather than a primary activation function[87]. It has also been shown that $CD8^+$ T cells, upon stimulation with IL-2 or IL-15 can acquire the ability to kill NKG2D ligand-bearing target cells in a TCR independent fashion[97]. The role of NKG2D has been studied in HCMV specific $NKG2D^+CD4^+$ T cells where it functions as a prototypic costimulatory receptor, synergizing with TCR[89].

NKG2D has been described as a sensor of cellular stress, as its ligands are cellular proteins generally induced in response to a variety of stimuli that perturb the normal cellular function, including genotoxic and oxidative stress, heat shock, viral infections and tumor transformation[98].

NKG2D ligands (NKG2DL) are distantly related homologues of MHC class I molecules, and in human are divided into two families: the MHC class I polypeptide related chain (MIC) protein family that includes MICA and MICB and the cytomegalovirus UL16-binding proteins (ULBP; also known as retinoic acid early transcript 1 (RAET1) proteins family, which consists of six members (ULBP1-4, RAET1G or ULBP5 and the recently identified RAET1L or ULBP6). MICA and MICB are transmembrane proteins with three immunoglobulin-like domains and short cytoplasmic tails. ULBP/RAET1 proteins have two α domains and, of the six molecules, four (ULBP1, ULBP2, ULBP3, and ULBP6), are GPI-anchored proteins (Figure 3). In mice, cellular ligands for NKG2D receptor are the murine UL-16 binding protein-like transcript (MULT-1), the minor histocompatibility protein H60 and five members of the retinoic acid early transcript 1 (RAE1) family (Figure 3)[98].

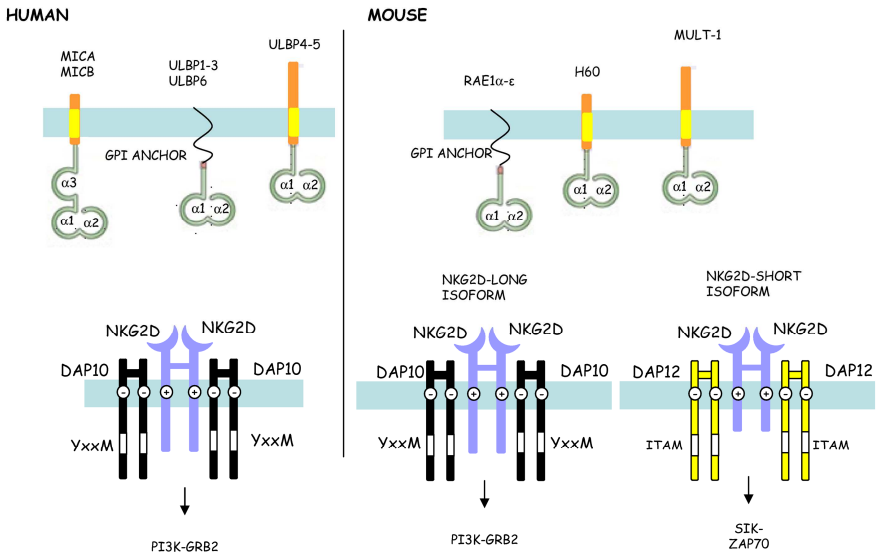

Figure 3. NKG2D and its MHC-I like ligands in human and mice

All the ligands share an MHC class I α1α2 domain that binds to NKG2D in a fashion remarkably similar, despite the low homology between them[98]. NKG2DL are also highly polymorphic; particularly MICA and MICB genes for which 70 and 31 alleles have been described, respectively. It has been suggested that such high degree of polymorphism as well the diversity of NKG2DL is the result of an evolutionary pressure principally driven by pathogens [99].

The expression of NKG2DL in humans and mice is tightly regulated at the transcriptional, post-transcriptional and post-translational levels, thus preventing NK cell mediated attack of normal healthy cells and providing a rapid induction of NKG2DL upon stress[98]. Indeed, it has been recently demonstrated that transcripts of human NKG2DL are widely distributed, however the proteins have been detected constitutively only in few epithelial tissues, while in others their expression is prevented by post transcriptional mechanisms, as endogenous cellular miRNAs[100]. A post translational regulation mechanism was also recently demonstrated for the mouse NKG2D ligand MULT-1. It was shown that the ubiquitination of MULT-1

in normal cells leads to its lysosomal degradation and prevents its expression at the cell surface[101]. It is possible that similar mechanisms also regulate protein expression of human NKG2DL.

The induction of NKG2DL expression on the cell surface has been shown upon tumor transformation, as a consequence of the DNA damage response[102]. Several tumor cell lines and freshly extracted primary tumors express NKG2D ligands constitutively and are susceptible to NK cell killing *in vivo* and *in vitro*[103,104]. The role of NKG2D in tumor immunosurveillance has been also confirmed recently in NKG2D deficient mice which show impaired NK cell mediated killing of certain tumors *in vitro* and have an increased susceptibility to primary tumorigenesis *in vivo*[105].

It is also well known that viral infection induces the expression of several NKG2D ligands, either by directly promoting their expression or indirectly through the induction of interferons[98]. In particular, the role of NKG2D as an antiviral defense mechanism has been extensively studied in the context of cytomegalovirus infection as it will be discussed later. NKG2DL expression has also been shown to be upregulated in $CD4^+$ T cells upon human immunodeficiency virus (HIV) infection as a consequence of the action of HIV vpr gene product [106], in human DC upon infection with influenza virus [107], as well as in response to Epstein-Barr virus[108], mousepox [109], and mouse hepatitis virus[110].

Beyond a role of NKG2D in the immune response to tumor cells and pathogens, the participation of NKG2D in the cross-talk between immune cells has been recently described. Expression of NKG2DL, potentially inducing NKG2D mediated activation of NK cells, has been reported in activated T cells [111,112], type I interferon stimulated DC[113], LPS activated macrophages [114] and DC treated with TLR agonists[115].

It is of note that NKG2DL and cytokines can reciprocally affect the surface expression of NKG2D in NK cells and T cells. Sustained expression of NKG2DL, as well as the shedding of soluble forms can induce the

internalization and degradation of NKG2D in NK [116] and T cells[117], thus impairing NKG2D-mediated immune recognition. This phenomenon has been reported in advanced stages of tumor growth or upon *in vitro* antigen stimulation of total PBMC. Moreover, NKG2D surface expression has been also shown to be modulated by the action of different cytokines or catabolites produced upon macrophage activation. In particular IL-2, IL-15 and IL-7 have been described to increase NKG2D transcripts, whereas TGF-β, IFN-γ, IL-21, reactive oxygen species and L-kineurine decrease NKG2D expression[118-121].

2.4.2.2 Natural cytotoxicity receptors (NCRs)

Natural cytotoxicity receptors (NCRs) are defined by their preferential expression on NK cells and their ability to induce NK cell mediated cytotoxicity upon cross-linking by specific monoclonal antibodies (mAbs) in a redirected killing assays[122]. The NCR family includes three type 1 glycoproteins, belonging to the Ig-SF: NKp46 (NCR1, CD335)[123,124], NKp44 (NCR2, CD336)[125] and NKp30 (NCR3, CD337)[126]. Only NKp46 has a functional homologue in mouse, named Ncr1[127]. NCRs have different extracellular structures and associate with different transmembrane-anchored polypeptides bearing ITAM, including CD3ζ, FcεRIγ or DAP12, as shown in Figure 4. Despite that they signal via different adaptor molecules, it was suggested that a functional cross-talk exists between them and, remarkably, the engagement of one NCR leads to activation of the signalling cascades associated with the others, possibly resulting in the amplification of the activating signals[128].

NCRs have been mainly involved in the recognition and killing of various tumors, including carcinomas, melanomas, neuroblastomas, myeloid and lymphoblastic leukaemia, and multiple myeloma[129]. This has become evident by in vitro killing assays using specific blocking mAbs and also as a consequence of the strict correlation that exist between the density of NCRs

expression on NK cells and their ability to kill targets cells[129]. Despite their main role in the killing of tumor cell targets, increasing evidences suggest the participation of these receptors in several other processes as the recognition of infected cells, the cross-talk between immune cells and their involvement in autoimmune processes.

NKp46 was the first NCR to be identified and is probably a major activating NKR. It is encoded by the NCR1 gene, which maps in the telomeric region of LRC. The extracellular domain of NKp46 is formed by two C2-type Ig-like domains and the signalling is dependent on CD3ζ or FcεRγ ITAM bearing adaptor proteins. Cross-linking of NKp46 with specific mAbs leads to calcium mobilization, cytotoxicity and cytokine release[123,124].

The cellular ligands of NKp46 are still not defined. *In vivo* studies have shown that NKp46 is important for melanoma and lymphoma eradication [130,131]and it has been recently demonstrated that NKp46 recognizes unknown ligands on pancreatic beta cells and has a critical role in type 1 diabetes progression[132]. Soluble NKp46-Fc fusion proteins bind to several human and mouse tumor cells and it was suggested that membrane-associated heparan sulphate proteoglycans (HSPGs) might be involved in the recognition[133], however, since heparan sulphate is also found on normal cells that are not killed by NK cells, it is not likely that it represents the specific activating ligand but probably its recognition facilitates the interaction with a still unknown molecular structure[134].

Beyond a role in tumor immunoediting and autoimmunity, NKp46 has been described to directly recognize viral hemagglutinin (HA) proteins of various influenza strains and to mediate killing of virally infected cells[135]. Particularly, dendritic cells infected with influenza virus have been shown to activate IFN-γ secretion by NK cells through engagement of NKp46 and NKG2D and this activation was partially blocked by antibodies specific for NKG2DL and HA[107]. Moreover, Gazit and colleagues have shown that influenza infection is lethal in NKp46 knockout mice, strongly supporting a role of

NKp46 in the triggering of specific immune response[136]. On the other hand, NKp46 has been also reported to contribute to NK cell activation induced by mycobacterium tuberculosis infected macrophages, suggesting a wider role of NKp46 in the control of infectious diseases [137].

NKp44 was the second NCR identified on human cells and, unlike the other NCRs, is not constitutively expressed on peripheral blood NK cells but is induced upon IL-2 activation[125]. NKp44 displays a single Ig V like domain and both expression and activation depend on the adaptor molecule DAP12[125,138]. In a similar fashion to NKp46, NKp44 has been shown to be activated by influenza virus hemagglutinin H1 and H5N1[139,140]. Recently, NKp44 has also been involved in the pathogenesis of human immunodeficiency virus (HIV) infection. Vieillard and colleagues found that the ligand for NKp44 is upregulated on $CD4^+$ T cells upon HIV infection and this effect is mediated by a gp41 derived peptide[141].

The third NCR identified was NKp30[126]. This receptor has one V-type Ig like domain and is coupled with the adaptor molecule CD3ζ. Similar to NKp46, NKp30 is preferentially expressed on resting NK cells and is up-regulated upon activation[142].

NKp30 has been involved in NK cell mediated killing of several tumor cells and also in the cross-talk between NK cells and dendritic cells. As it will be discussed later, NKp30 was shown to recognize an unknown ligand on DC and to mediate the killing and/or maturation of dendritic cells[143,144]. Recently, it has been suggested that the nuclear factor HLA-B-Associated Transcript-3 (BAT3) is released on exosomes from tumor cells and immature DC engaging NKp30 receptor on natural killer cells[145,146]. Besides BAT3, the B7 family member B7-H6 was found to be a tumor cell ligand of NKp30[147]; however, the fact that it is not expressed on dendritic cells suggests that other molecules are recognized by this NCR. While the cellular ligand of NKp30 is still not well defined it has been proposed that the HCMV

tegument protein pp65 functionally interacts with NKp30 and inhibits NK cell activation, by altering signal transduction[148].

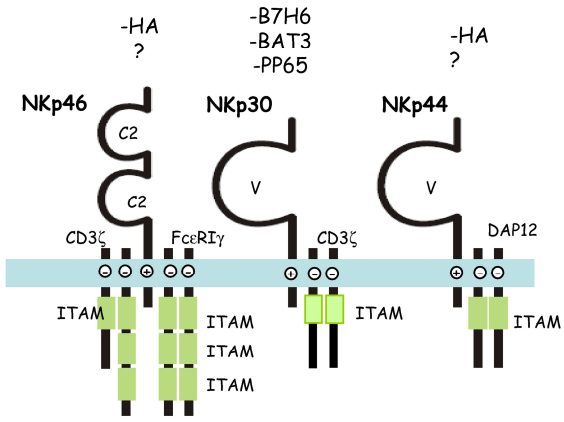

Figure 4. Human natural cytotoxicity receptors

2.4.2.3 Other NK cell associated receptors

Additional surface molecules that are not exclusively expressed on NK cells (NKp80, NKp65, DNAM-1, TIGIT, CEACAM, SIGLEC7, 2B4, NTB-4 and CRACC) have been shown to cooperate in modulating NK cell mediated cytotoxicity and NK/target adhesion.

NKp80 is a homodimeric NKC-encoded activating NKR expressed by NK cells and a small subset of $CD56^+$ T cells. Unlike other NKR, NKp80 does not couple to any adaptor molecule and the signal transduction pathway is not well defined[149].

Recently NKp80 has been shown to bind activation-induced C-type lectin (AICL). AICL is preferentially expressed on the surface of myeloid cells and is upregulated upon TLR stimulation. AICL-NKp80 interaction promotes NK cell-mediated cytolysis of malignant myeloid cells and, during the cross-talk between NK cells and monocytes, stimulates the release of proinflammatory cytokines from both cell types [150].

NKp65 is an NKC-encoded killer cell lectin-like receptor, distant relative of NKp80 NK cell receptor. NKp65 is expressed on several human NK cell

lines, (such as NK92 and its derivative, NK92MI) but not on blood NK cells. Recently it has been shown that it binds with high affinity CLEC2A, a keratinocyte-associated C-type lectin (like) molecule. This association promotes NK cell cytotoxicity and cytokine secretion, suggesting an involvement of this receptor in the NK cell mediated recognition of human keratinocytes[151].

DNAX accessory molecule 1 (DNAM-1, CD226) is a 65-kDa glycoprotein belonging to the Ig-SF expressed by virtually all human NK cells, T lymphocytes and monocytes[152]. It physically associates with the integrin LFA-1 and the protein tyrosine kinase Fyn [153,154] and specifically recognizes two closely related molecules belonging to the Nectin family, poliovirus receptor (PVR; CD155) and Nectin-2 (CD112) [155]. In monocytes, and possibly also in other immune cells, DNAM-1 plays a role in transendothelial migration, where it facilitates the adherence to endothelial cells, that express DNAM-1 ligands (DNAM-1L), and migration between cell junctions [156]. In NK cells, DNAM-1 triggers NK cell lysis of several tumor cells expressing CD155 and CD112 [131,157-160] and cooperates with NKp30 in the NK-mediated killing of both immature and mature DC[161]. Moreover, DNAM-1 promotes LFA-1-mediated costimulation of naive $CD4^+$ and $CD8^+$ T cells[162,163].

Recently it has been shown that PVR and Nectin-2 are also recognized by an ITIM bearing inhibitory receptor, named TIGIT, that is expressed by NK cells and all T cell subsets (including Treg and memory T cells), excepting naïve $CD4^+$ T cells[164]. TIGIT suppresses T cell activation in an indirect manner, by promoting the generation of mature immunoregulatory dendritic cells that secrete IL-10[164]. In NK cells it has been shown that the interaction of TIGIT with PVR and Nectin-2 inhibits NK mediated killing of tumor cells and protects normal cells from NK-mediated cytotoxicity[165].

Other inhibitory receptors that do not recognize MHC-class I molecules have been described in humans; among them, the carcinoembryogenic

antigen cellular adhesion molecule 1 (CEACAM-1) has been shown to function as an MHC-class-I-independent inhibitory receptor in NK cells when ligated homophilically by CEACAM-1 on target cells[166]. Another non MHC class I binding NK inhibitory receptor that has been identified is SIGLEC7 that is known to bind sialic acid residues[167]. Cross-linking of SIGLEC7 using specific antibodies leads to inhibition of target cell lysis[168].

2B4, NTB-A and CRACC belong to the signalling lymphocytic activation molecule (SLAM) family[169]. NTB-A and CRACC establish homophilic interactions, whereas 2B4 binds to CD48, a member of the CD2 family of receptors that is expressed on most hematopoietic and endothelial cells. In human blood NK cells, these receptors generally trigger activating signals when stimulated with specific mAbs in redirected killing assays. Upon tyrosine phosphorylation of immunoreceptor tyrosine-based switch motif (ITSM) they associate to a small cytoplasmic protein termed SAP. Subsequent recruitment of Fyn activates protein tyrosine phosphorylation of different components of the activating cascade. Notably, these receptors can also bind SHP-1 and, depending on the association with SAP or SHP-1, they mediate opposing functions, activating or inhibitory, respectively. It has been suggested that SAP may compete with phosphates in the interaction with the receptor thus preventing the generation of an inhibitory signal[169]. Indeed, in X-linked lymphoproliferative (XLP) patients, SAP gene is mutated and 2B4 and NTB-A have been found to display inhibitory rather than activating activity[170]. Moreover, an inhibitory role of 2B4 in NK cells at an early stage of differentiation [171] and in NK cells isolated from human decidua [172], has been reported.

3. IMMUNOBIOLOGY OF DENDRITIC CELLS

Dendritic cells (DC) are specialized antigen presenting cells that orchestrate innate and adaptive immune responses. They are capable of inducing activation of naïve T lymphocytes and hence play a central role in initiating primary immune response against pathogens[173]. In addition, DC play a critical role in the induction of immunological tolerance, through cross talk with T and B cells and modulate innate immune responses through cross-talk with NK cells.

3.1 Dendritic cell subsets

DC constitute a very heterogeneous cell type with distinct morphology, location and functions. Humans and mice display two major DC types: myeloid DC (mDC, also called conventional or classical DC) and plasmacytoid DC (pDC).

In mice, there are three different subsets: two are considered conventional DC which express high level of CD11c and differentially express CD8α and CD11b. $CD11c^+$ $CD11b^+$ $CD8\alpha^-$ cells are predominantly found into the marginal zones surrounding the white pulp areas of the spleen, whereas $CD11c^+$ $CD11b^{low/-}$ $CD8\alpha^+$ segregate to the T cell area of both spleen and lymph nodes. $CD8\alpha^+$ DC are able to produce large amounts of IL-12 and polarize naive $CD4^+$ T cells toward the Th1 cell phenotype, whereas $CD8\alpha^-$ DC preferentially induce Th2 cell responses. The third subset is the plasmacytoid DC which are also called natural interferon producing cells since thay produce large amount of type I interferon in response to viral infection[174].

In humans two main DC subsets have been identified: myeloid DC (mDC), derived from myeloid $CD34^+$ progenitors, and plasmacytoid DC (pDC), which are mainly of lymphoid origins. The myeloid DC subset expresses, among other cell markers, CD11c, CD11b and CD4. This subset includes

peripheral tissue resident DC (such as Langerhans cells (LC) and dermal interstitial DC), secondary lymphoid resident DC and circulating blood myeloid DC[174]. Cells resembling human dermal myeloid DC can be also generated *in vitro* from peripheral blood monocytes by culturing them in the presence of exogenous GM-CSF and IL-4[175].

Plasmacytoid DC is a rare subtype of circulating dendritic cells found in the blood as well as in peripheral lymphoid organs. Human pDC express CD123, but do not bear CD11c or CD14. This subset is considered a main source of type I interferon in response to viral infection.

Besides differences in their ontogeny and surface markers, mDC and pDC differ also in the repertoire of pattern recognition receptors (PRRs) expressed and in the type of cytokines secreted. In particular, mDC express all TLRs except TLR7 and TLR9, which are selectively expressed by pDC and, once activated, secrete high levels of interleukin-12 (IL-12). On the other hand, pDC sense viruses through TLR7, TLR8, TLR9 and two cytosolic sensors for viral genome, RIG-I and MDA5, and upon activation produce mainly IFN-α[176]. Thus, in contrast to other cells, pDC do not have to be infected to produce type I interferons. IFN-α can inhibit viral replication in infected cells and play a major role in antiviral defence. Moreover, this antiviral cytokine can enhance cytotoxic activity of NK cells and macrophages and can promote T cells survival as well as antibody production by B cells.

3.2 Maturation of dendritic cells

Generally, two stages of DC development can be distinguished. Immature DC reside in both peripheral tissues and lymphoid organs and, under steady-state conditions, act as "sentinels" either by directly recognizing microbial components, using germline encoded pattern recognition receptors (PRRs), or by receiving signals from other cells of the innate immune system that are exposed to pathogens. At that stage, immature DC have a potent endocytic

and phagocytic capacity, but express low surface expression of MHC class I and class II and costimulatory molecules. Upon encounter with microbial products or tissue damage, when a stimulatory threshold is reached, DC initiate a complex process of maturation that transforms them into an antigen presenting cells (APC) able to mediate T-cell priming [173]. The T cell-mediated immune responses induced can vary depending on the specific DC lineage and maturation stage in addition to the activation signals received from the microenvironment. Indeed, cytokines secreted by other cells of the innate immune compartment have been shown to influence DC functions[177].

In vitro maturation of monocyte derived DC can be achieved in the presence of various pathogen-derived products, such as LPS, (ds)RNA and polyinosinic: polycytidylic acid (poly-IC) as well as cytokines (primarily TNF) or signals provided by interaction with T cells (such as through CD40-CD40L interactions)[173].

Upon maturation, DC increase CD83 expression, which is one of the best markers of maturation and undergo to a chemokine receptor switch, down-regulating inflammatory chemokine receptors and up-regulating the lymph node homing receptor CCR7. Mature DC also translocate MHC molecules to the cell surface and upregulate costimulatory molecules, including CD40, CD58, CD80 and CD86 that are involved in the subsequent interactions with naïve T cells[174].

Both mature pDC and mDC secrete sets of redundant chemokines in three waves, which correspond to the three stages of the immune response to microbes. The first chemokines allow attraction of NK and neutrophils, following by secretion of chemokines aimed to attract T cells and monocytes. Finally when mature DC land in the draining secondary lymphoid organs they attract B cell and follicular helper T cells[174].

DC also secrete several cytokines that dictate the polarization of $CD4^+$ T cells towards Th1, Th2, Th17 or regulatory T cells (Tregs). IL-12, produced mainly by activated myeloid DC, plays a pivotal role in the differentiation

and expansion of Th1 cells, whereas the presence of IL-4 in the milieu is crucial for Th2 differentiation. Dendritic cells that are conditioned in environments rich in TGFβ, such as the intestine, present pathogen-derived antigens to induce the differentiation of naive $CD4^+$ T cells to regulatory T cells or T helper 17 cells, depending on the dominance of retinoic acid or IL-6, respectively[178].

In addition to T cell stimulation, DC contribute to the stimulation of B cells, both in the lymph nodes area and in germinal centers. Moreover DC can also modulate the function of other cells of the innate immune compartment, such as NK cells.

4. NK CELL/DENDRITIC CELL INTERACTIONS

In the last decade several studies have emphasized the crucial role of DC/NK cell interactions in the regulation of the innate and adaptive immune response to pathogens and tumors [179]. The original description of NK–DC cross-talk was published in a report by Fernandez *et al.* in 1999[180], in which the anti-tumor response of mouse NK cells was shown to be enhanced by DC *in vivo*. Subsequently, NK cell activation by DC was found to be required for an efficient innate immune response against a variety of pathogenic challenges in mouse models [181-183]. Human myeloid[143,184-186] and plasmacytoid DC[185] have also been reported to mediate NK cell priming through direct cell contact and cytokine secretion. In particular, myeloid DC-derived IL-12 and IL-18, produced upon PRR engagement, were mainly implicated in NK cell mediated IFN-γ secretion, while type I interferons, mainly secreted by pDC, preferentially induce NK cell cytotoxicity[184,185,187-189]. In addition, IL-2 produced by DC stimulated with microbial products[190] and IL-15 presented on DC via binding to IL15Rα[181,191,192] might also contribute to NK cell activation. Optimal NK cell activation by DC requires synapse formation to promote polarised secretion of cytokines towards NK cells [187,191] and NK cell receptor-ligand interactions (Figure 5)[113].

The interaction between DC and NK cells is not uni-directional and could also result in NK cell induced DC maturation or DC killing, depending on the activation status of both players, thereby influencing the adaptive immune response[193]. NK cell-mediated maturation of DC primarily depends on TNF-α and IFN-γ[184,186] and requires triggering of NKp30 on NK cells [144] (Figure 5). This process appears important for the initiation of adaptive immune responses when DC are not directly activated; accordingly, it has been shown that NK cells activated by MHC class I deficient target cells can prime DC to produce IL-12 and to stimulate CD8+ T cells *in vivo* (314)[194].

In addition to their support in adaptive immunity through DC maturation and T cell polarization, NK cells are also able to kill autologous myeloid immature DC but not mature DC, through engagement of two NK cell activating receptors, NKp30 and DNAM-1, thereby influencing DC homeostasis (Figure 5)[143,161]. It has been shown that a subset of NK cells expressing the inhibitory CD94/NKG2A receptor but lacking KIR is mainly responsible of this process, suggesting that the level of expression of HLA-E is critical in the response of NK cells to immature DC[195].

The NK cell mediated editing of myeloid DC may have clinical relevance. Indeed, this process has been proposed to be involved in the prevention of graft-versus-host disease (GVHD) in allogenic hemopoietic stem cell transplantation[196] and graft rejection in solid organ transplantation[197,198].

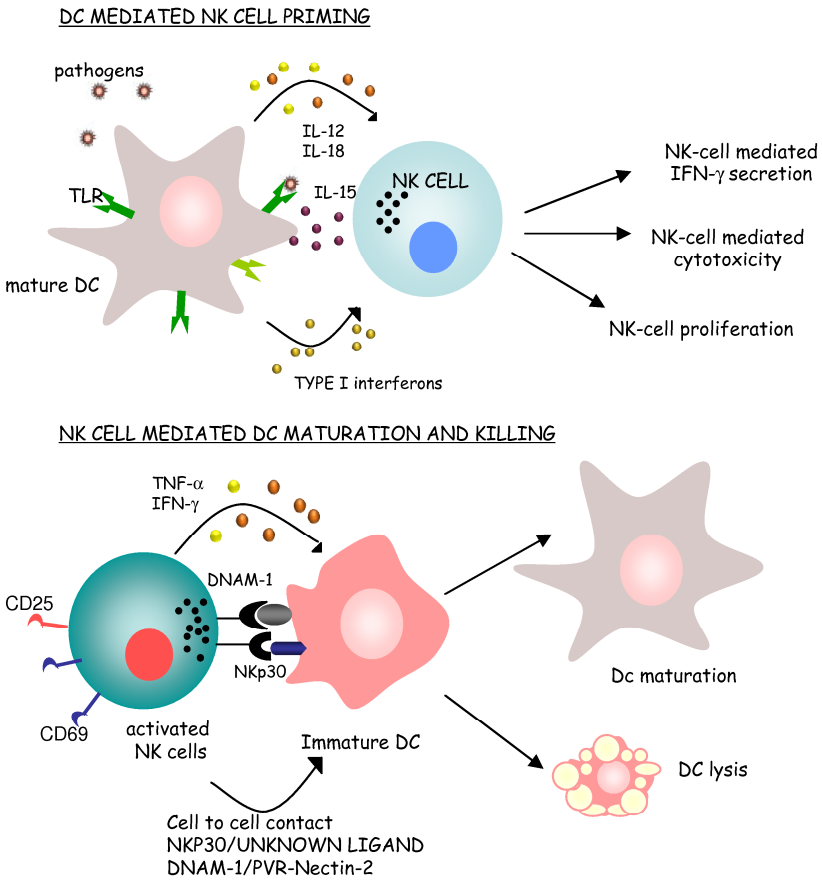

Figure 5. NK cell/Dendritic Cell cross-talk

5. IMMUNE RESPONSE TO HCMV

5.1 Human cytomegalovirus

The Human cytomegalovirus (HCMV), or *human herpesvirus 5* (HHV-5), is a member of the *Herpesviridae* family, which includes, among others, Herpes simplex virus 1 and 2 (HSV-1 and 2), Epstein-Barr virus (EBV), Varicella-zoster virus (VZV) and Kaposi's sarcoma-associated herpesvirus (KSHV). HCMV is the prototypic member of the *Betaherpesvirinae* subfamily and is a highly species-specific pathogen that causes widespread persistent infection in humans[199].

The seroprevalence of HCMV ranges between 40 to 90% depending on the socioeconomic status of the population and it increases with age. Primary infections occur often during childhood by transmission through breast-milk or saliva and are normally asymptomatic in immunocompetent hosts. Upon infection, the virus persists in a life-long latent state which is interrupted by phases of reactivation that allow virus shedding and transmission to new hosts[199].

Although no HCMV disease is observed in healthy carriers, due to effective immune control, severe clinical symptoms associated to HCMV primary infection or reactivation are observed in individuals with immature or compromised immune systems, such as premature newborns, transplant recipients or AIDS patients. Transplacental transmission during pregnancy can lead to neurological lesions and HCMV is considered as the main viral cause of congenital disorders[200]. In transplant recipients, HCMV infection can lead to severe complications such as pneumonia, hepatitis, enteritis, encephalitis and retinitis, and is considered a leading cause of graft rejection following solid-organ allograft transplantation. In AIDS patients with low $CD4^+$ T cells counts, HCMV commonly causes retinitis[201].

HCMV has been also associated with chronic conditions, such as coronary artery disease and diabetes, where it is still not clearly determined whether

the virus is an etiological agent or an opportunistic pathogen contributing to the exacerbation of the disease[202].

To date, no vaccine to prevent HCMV infection or disease is available and the current therapy for active HCMV infection in immunocompromised patients relies upon several antiviral chemotherapeutic drugs that have severe side effects[203].

5.1.2 HCMV biology

HCMV is the largest human herpes virus with a double stranded linear DNA genome of ~240Kb encoding for at least 170 open reading frames (ORF) that are likely to be functional. Mutagenesis studies have demonstrated that only around 50 ORFs are essential for virus replication and virion assembly in tissue cultures[204]. Thus the majority of the viral genome encodes for proteins that are involved in the pathogenesis of the HCMV infection and in the interaction with the host. Moreover, 14 HCMV microRNAs (miRNA) have been recently discovered. MiRNA are small noncoding RNA that regulate gene expression post-transcriptionally. To date, only few viral and cellular targets have been identified for these miRNAs, thus their functions are still largely unknown[205].

The genome of HCMV is organized in unique long (UL) and unique short (US) regions, each flanked by inverted repeats. Conventionally, the ORFs in the UL domains at the left end of the map are named RL1-14, and they are followed by UL1-151, IRS1, US1-34 and TRS1. Comparing to HCMV clinical isolates, laboratory strains that have serially passaged *in vitro* have undergone several deletions and concomitant duplication and most of them have deleted a sequence of 15Kb (UL/*b'*) from the right end of the UL region that contains more than 19 ORF, many of them involved in HCMV pathogenesis[206] (Figure 6A). The HCMV genome is enclosed by capsid-proteins and further surrounded by tegument proteins. A lipid bilayer

envelope that contains both host and viral glycoproteins forms the external layer[199] (Figure 6B).

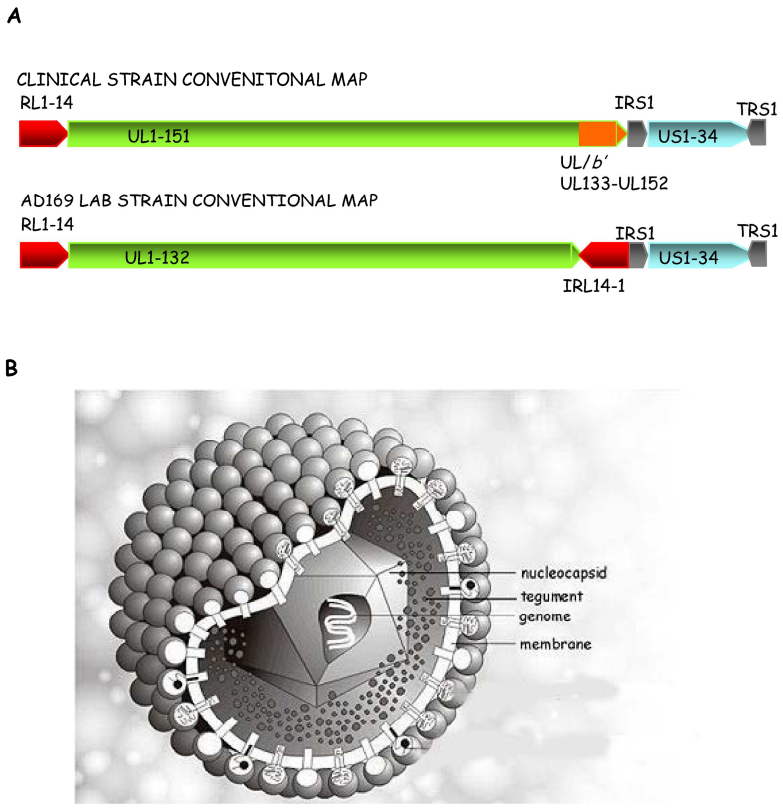

Figure 6. (A) HCMV ORF organization. (B) HCMV virion structure.

In vivo HCMV is capable of infecting different cell types, including fibroblasts, epithelial cells, endothelial cells, smooth muscle cells and hematopoietic cells[207]. The transcriptional programme accompanying HCMV lytic infection starts with immediate early (IE) genes (0 to 2h), followed by early (E) (< 24h) and then late genes (>24h), which ultimately lead to replication, virion assembly and shedding. The IE proteins, of which IE72 (IE1) and IE86 (IE2) are the major forms, are potent transactivators of gene expression and their synthesis is absolutely critical for the expression of E and L genes and the activation of lytic infection programme[208].

In the absence of IE gene expression, productive infection is effectively suppressed and infection may result in establishment of latency. In particular, cells of early myeloid lineage have been reported to be the main reservoir of HCMV *in vivo*. In these cells, the repression of IE genes expression occurs as a consequence of specific post translational modification of histones associated with viral major immediate early promoter [209].

Several mechanisms have been proposed to be responsible for HCMV reactivation, including inflammation through TNF-α and proinflammatory prostaglandins[201]. Recently, it has been reported that the reactivation of HCMV from latency in $CD34^+$ myeloid precursors may occur upon differentiation into DC or macrophages as result of changes in chromatin structure associated to viral major immediate early promoter, allowing transcription of IE genes and activation of lytic infection programme[209,210]. Indeed, terminally differentiated myeloid DC and macrophages are believed to be the source of HCMV viral particles during reactivation *in vivo* thereby representing more informative and appropriate experimental cell systems to analyze the immune response to HCMV than primary fibroblasts.

5.2 HCMV infection of dendritic cells

Not all HCMV viral strains can infect cells of the myeloid lineage. Studies *in vitro* have shown that the tropism for endothelial cells and myelomonocytic cells mostly depends on alterations in the *UL128-131* gene region, which encodes components of the virion particle involved in viral entry to the cell[211,212]. Indeed, the extensively used laboratory strain AD169, which lacks a functional *UL131* protein, is unable to grow in myeloid, endothelial or epithelial cells. In contrast, HCMV clinical isolates and endothelial adapted (EC) HCMV viral strains, such as TB40/E, efficiently infect DC and macrophages[213,214].

HCMV infection of monocyte derived DC was shown to be productive and lytic[214], and partially dependent on viral binding to DC-SIGN[215]. High

efficiency levels of infection were dependent on the use of large viral doses and expression of viral immediate early, early and late genes was shown to be slightly delayed in comparison to infected fibroblasts[214].

The infection of myeloid DC by HCMV has been described as an immunoevasion mechanism aimed to avoid T cell response. Indeed, HCMV infection causes an impairment of DC function, leading to inefficient T-cell activation. Particularly, HCMV infection of DC has been reported to impair expression of costimulatory molecules and chemokine receptors, to reduce antigen presentation by down-regulating MHC class I and class II surface expression, to prevent up-regulation of the lymph node homing receptor CCR7 and to inhibit maturation upon LPS stimulation[216-222]. Soluble CD83 molecules secreted by HCMV infected DC[223] as well as induced expression of CD95L (FasL) and TRAIL on infected cells[224] have been also proposed to be responsible of related reduction of T cell responses. Yet, in a recent publication, the activation of freshly isolated blood DC ($CD11c^+$ DC and plasmacytoid DC) has been reported to be not compromised by the virus and both populations secrete high levels of IFN-α and retain full T cell-stimulatory capacity when exposed to HCMV[225].

5.3 Control of CMV by the immune system

Experimental *in vivo* mouse model using murine cytomegalovirus (MCMV), that shares many features with its human counterpart in terms of infection and pathogenesis indicates that both adaptive and innate immune responses are important in limiting reactivation at local sites and preventing dissemination. Furthermore, HCMV disease occurs only upon immunosuppression confirming that, also in humans, a constant immune surveillance is required to keep persistent infection under control[226]. On the other side, CMV has developed several strategies to escape immune recognition and to persist in the host[227].

5.3.1 T cell response to CMV and related immune evasion mechanisms

Although HCMV elicits a broad adaptive immune response including B cells producing neutralizing antibodies, HCMV specific $CD8^+$ and $CD4^+$ T cells, play a major role in controlling and restricting viral replication during persistent infection [226].

A key role for T cell immunity in the control of CMV infection was firstly recognized in studies using MCMV models in which T cell depletion was coincident with CMV reactivation and dissemination, whereas adoptive transfer of CMV specific $CD8^+$ cytotoxic lymphocytes (CTL) conferred protection[228,229]. In humans, several evidences suggest that both $CD4^+$ and $CD8^+$ T cell compartments are necessary to prevent HCMV disease[230,231]. Particularly, $CD8^+$ T cells seem to be responsible for clearing infection whilst $CD4^+$ helper T cells are needed to provide growth factors and cytokines necessary for the activation and differentiation of memory $CD8^+$ cells into effector cells[232].

The importance of T cell mediated immunity is also reflected by the remarkably high frequency of HCMV specific T cells in peripheral blood of healthy carriers, particularly in the elderly. These cells show a clonally restricted TCR repertoire as well as low expression of CCR7, CD27 and CD28, consistent with an effector-memory phenotype. It has been suggested that clonal expansions may be a result of repeated exposure to antigen during reactivation and may lead to impaired responses against other pathogens[233,234].

In order to persist in the host, HCMV has developed sophisticated mechanisms to evade the T cell response interfering with MHC class I and II antigen presentation pathways[227]. Moreover, it has been recently demonstrated in CMV-infected rhesus macaques that MHC class I interference and consequent evasion of $CD8^+$ T cells response is crucial for establishment of persistent secondary infection[235]. To date, four different viral genes, encoded by a gene cluster located in the unique short (US) region

of the HCMV genome, have been described to interfere with MHC class I (and II) pathways. US3 is expressed at IE time points and retains class I molecules in the endoplasmic reticulum (ER) by inhibiting optimal peptide loading. US2 and US11 are expressed at early-late stages and cause translocation of MHC heavy chains from the ER to the cytosol for proteasomal degradation. US6 is expressed late in the viral cycle and inhibits peptide loading through binding to the transporter associated with antigen presentation (TAP)[227]. Remarkably, US2 and US3 have been also described to interfere with MHC class II presentation[236]. Besides these immunovasins, the tegument protein pp65 has been reported to prevent processing of the viral major IE antigen IE1 for MHC presentation[237]. It is of note that, among them, US6, but not US2 or US11, has been shown to down-regulate the expression of the non classical MHC class I molecule HLA-E[238], whereas HLA-G has been recently described to be the target of US10, however the functional consequences of this observation have not been elucidated so far[239].

5.3.2 Innate immune response to CMV

Innate immunity acts as the first line of host defence against CMV infection. It has been described that upon MCMV exposure, the engagement of PRRs, such as TLR-3 and TLR-9, activates signal transduction pathways that lead to the production of type I interferons and proinflammatory cytokines. Moreover, it has been shown that HCMV, through the interaction of viral glycoproteins gB and gH with TLR-2, elicits a strong innate response, characterized by NF-κB activation, inflammatory cytokine secretion, and induction of the interferon pathway. Recently two cytosolic DNA sensors, DAI and AIM2 have been shown to recognize CMV DNA, inducing respectively type I interferon response and inflammasome assembly, the latter leading to the activation of caspase 1 and secretion of mature IL-1β and IL-18[240].

An important outcome of the early cytokine response is the activation of NK cells. Indeed, NK cells are an integral part of the innate immune response to CMV. They are involved in the clearance of experimental MCMV[241] and the adoptive transfer of NK cells has been shown to provide protection[242]. Moreover, studies performed with different mouse strains have revealed that certain strains of mice are more resistant to MCMV due to the presence of the cmv-1 genetic locus, which encode for the Ly49H NK cell receptor complex[243,244]. This receptor was proved to directly bind to m157, a viral protein with structural homology to non classical MHC molecules that is expressed on MCMV infected cells. The m157 protein was also shown to bind the inhibitory receptor Ly49L. Thus, the effect of m157 expression depends on Ly49 receptor expression, which varies in mouse strains. In MCMV resistant mouse strains the interaction between Ly49H and m157 results in Ly49H$^+$ NK cell expansion during the late phase of infection and MCMV clearance[245,246].

The NK cell response, in MCMV infected mice, has been also shown to be principally dependent on DC. Following MCMV infection, plasmacytoid DC are a source of IFN-α, IL-12, and TNF-α and are capable of activating NK cells cytotoxicity and IFN-γ secretion[247,248]. CD8α$^+$ splenic DC have been reported to be required for the late expansion of Ly49$^+$ NK cells, while conversely NK cells are necessary for the maintenance of this DC subset and for the development of T cell responses[183]. Conventional CD11b$^+$ DC are a target for MCMV and infection results in their functional impairment [249], as reported for HCMV infected DC. However, early after infection, CD11b$^+$ DC have been shown to initiate an early anti viral response, contributing to the *in vivo* clearance of the virus by interacting with NK cells. IFN-α and NKG2D-NKG2DL interactions contribute to the activation of NK cell cytotoxicity, whereas IL-12 and IL-18 released by infected cells promote the secretion of IFN-γ[189]. Altogether these observations highlight a crucial role of DC/NK cross-talk in the response to MCMV.

How the interaction of DC and NK cells influences the pathogenesis and the immune response during HCMV infection is still not well defined; however, the important role of NK cells in the early control of infection has been already suggested in humans, based on clinical observations. Individuals with defects in NK cell function often exhibit enhanced susceptibility to severe primary herpes virus infection, including HCMV disease[250]. In a recent case report, a patient lacking functional T cells was shown to reduce the viremia during primary HCMV infection through the involvement of an NK cell mediated response[251]. Moreover, in transplant patients, NK cell activity was shown to increase during both primary and recurrent infection and correlate with the recovery[201].

Besides clinical data, *in vitro* experiments using HCMV infected fibroblasts suggest that the loss of MHC class I molecules that occurs in infected cells to avoid T cell recognition may impair the engagement of NK cell inhibitory receptors promoting the activation of NK cell effector functions. Reciprocally, the virus, due to the strong selective pressure exerted by NK cells, has developed different strategies to escape innate cell surveillance, preventing the expression of ligands for several activating receptors or selectively maintaining inhibitory receptors for HLA class I molecules engaged.

5.3.3 NK cell receptors involved in the response to HCMV

Several NKR have been involved in the innate immune response to HCMV. However, most of the evidences are essentially indirect and based on the identification of viral immune evasion molecules expressed by HCMV targeting these receptors. Moreover, *in vitro* studies have reported variously both inhibition and enhancement of NK cell killing of HCMV infected cells[252-255]. Differences in experimental conditions, such as timing of infection, viral strains and cell targets may account for this discrepancy. Figure 7 and the following paragraphs summarize the data highlighting the

putative roles of NKR in the response to HCMV and viral mechanisms to counteract NK cell activation.

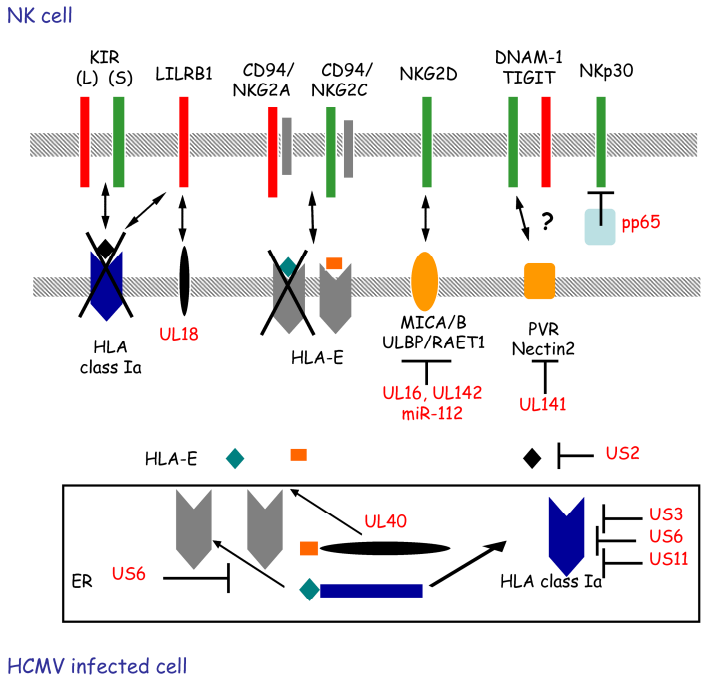

Figure 7. NK cell receptor-ligand interactions described and HCMV immune evasion strategies (modified from López-Botet et al[256])

5.3.3.1 LILRB1

One strategy used by CMV to escape NK cell function is to express decoy MHC class I like molecules that trigger inhibitory receptors. The HCMV UL18 is a highly glycosylated protein expressed late during the infection that shows homology with MHC class I molecules, associates with β2-microglobulin and binds endogenous peptides. It has been described that UL18 may function as an immunoevasin by engaging the inhibitory receptor LILRB1 on NK cells[57].

As it was reported before, LILRB1 is the only receptor belonging to the LILR family that is expressed also on subsets of NK and T cells[53]. It recognizes broadly MHC class I molecules and the engagement by specific cross-linking Abs or by HLA class I molecules, delivers negative signals to

NK and T lymphocytes [53,257-259]. LILRB1 also binds UL18 with 1000-fold higher affinity than its physiological ligands [260,261] but the outcome of this interaction during infection is still matter of debate, as well as the availability of UL18 on infected-cell membrane[262]. It has been reported that the binding of UL18 to LILRB1 may act as a viral escape mechanism from NK-cell-mediated lysis[263] or alternatively leads to the killing of HCMV-infected cells by NK cells[253]. Prod'homme et al. showed that UL18 inhibited LILRB1$^+$ NK cells but stimulated LILRB1$^-$ cells by a still undefined mechanism[264]. With respect to T lymphocytes, it has been demonstrated that UL18 has a stimulatory function but there are conflicting results regarding the role of the UL18/LILRB1 interaction in this outcome[265,266]. Interestingly, LILRB1 is highly expressed on HCMV specific CD8$^+$ T[267] and the proportions of T lymphocytes[268] and NK cells (Romo et al., unpublished results) expressing LILRB1 are raised in healthy HCMV$^+$ individuals. Altogether these results suggest a possible role of this receptor in the immune response to HCMV.

5.3.3.2 CD94/NKG2A

The inhibitory receptor CD94/NKG2A recognizes the non classical MHC class I molecule HLA-E bound to peptides derived from the leader sequences of classical MHC-I molecules, allowing NK cells to monitor global MHC class I expression. It is of note that class I leader peptides are loaded onto HLA-E by a TAP-dependent pathway. Consequently, conventional HLA-E surface expression is blocked by HCMV US6, an inhibitor of TAP function, whilst US2 and US11 have been shown to preserve HLA-E expression[238]. The effect of US3 or US10 on HLA-E surface expression has not been reported.

Remarkably, the UL40 glycoprotein of AD169 HCMV contains a hydrophobic nonamer (VMAPRTIL) in its leader sequence that is identical to that of some classical HLA molecules and can be presented by HLA-E[83]. Adenoviral expression of UL40 in HLA-E transfected fibroblasts induced

TAP-independent up-regulation of HLA-E surface expression and elicited protection against lysis by NK cells. It is of note that the HLA-E stabilization by the UL40 derived peptide appears also resistant to the action of US6 and may contribute to maintain HLA-E expression during infection[83]. In another study, Cerboni et al. showed that transfection of UL40 in different cell lines (293T, 721.221, K562) did not consistently confer protection from NK cell lysis except when transfection was combined with treatment with IFN-γ, suggesting a more complex scenario[269].

In the context of HCMV infection the relevance for NK cell recognition of UL40 mediated immune evasion is controversial. In an allogeneic infection system, wild type HCMV infected fibroblasts, in the late phase of infection, were resistant to NK cell lysis, whereas cells infected with a HCMV lacking UL40 were killed via an HLA-E- and CD94/NKG2A-dependent mechanism[254]. On the other hand, Leong et al. reported that blocking CD94/NKG2A inhibitory receptor did not augment NK cell lysis of HCMV infected fibroblasts[253] and in another study Falk and colleagues found that NK cell activity during HCMV infection is dominated by US2–11-mediated HLA class I down-regulation, regardless of the presence or absence of UL40[252]. Besides that, it must be taken in account that direct evidence proving that the peptide bound to HLA-E in HCMV infected cells is derived from UL40 has not been provided. Furthermore, the role of UL40 in an *in vitro* infection system other than AD169 infected fibroblasts, such as myeloid DC remains unexplored.

5.3.3.3 CD94/NKG2C

The activating receptor complex CD94/NKG2C is expressed in a subset of NK cells and T cells and recognizes, as well, HLA-E, though with lower affinity than to CD94/NKG2A. In healthy adults[268] and children[270], in aviremic HIV$^+$ infected patients[271], as well as in patients with B-cell chronic lymphocytic leukemia (B-CLL)[272], HCMV seropositivity has been shown to

correlate with increased proportions of NK and T cells expressing CD94/NKG2C. As observed for Ly49H expression in MCMV infection, HCMV might shape the NKR repertoire, resulting in an expanded NKG2C$^+$ compartment. In line with this hypothesis, CD94/NKG2C$^+$ cells were observed to expand *in vitro* upon co-culture of PBMC from seropositive individuals with HCMV-infected fibroblasts and this expansion was blocked by an anti CD94 mAb[273]. These data suggest that a cognate interaction between the activating CD94/NKG2C receptor and a ligand expressed by HCMV-infected cells could be driving the process. Kuijpers and colleagues[251] recently reported an NKG2C$^+$ NK cell lymphocytosis during an acute HCMV infection in a patient with a primary T-cell immunodeficiency (SCID). The patient achieved a 3 log reduction of viremia despite the absence of T cells, thus suggesting that NKG2C$^+$ NK cells may actively participate in the control of HCMV infection. Interestingly, CD94/NKG2C$^+$ cells were described to include high proportion of inhibitory receptors (inhibitory KIR and LILRB1) and lower levels of NCR compared to NKG2A$^+$ subsets[268]. Thus, it is conceivable that the impact of HCMV infection on the NKR repertoire may influence the quality of the NK cell-mediated response. Yet, the molecular basis underlying the expansion of CD94/NKG2C compartment in HCMV seropositive individuals still remains unsolved and no viral ligand for CD94/NKG2C has been found thus far.

5.3.3.4 NKG2D

NKG2D is an activating NKR that recognizes stress inducible molecules, such as MICA, MICB and ULBP1-6. Infection with CMV leads to the upregulation of NKG2D ligand transcripts[274,275] directly through viral proteins, such as HCMV IE1 and IE2, or indirectly through the induction of cytokines[98]. Human fibroblasts infected with HCMV have been described to stimulate NK cells and CD8$^+$ T cells in a partially NKG2D-dependent

manner[274,275]. On the other hand, CMV employs multiples strategies to interfere with NKG2D ligand expression, reflecting the strong evolutionary pressure of this recognition system in immune response to virus. The UL16 protein of HCMV inhibits surface expression of MICB, ULBP1, ULBP2 and ULBP6 through sequestration of these ligands in the ER [274-279]. The viral protein UL142, contained in the UL/b' region, binds to MICA and ULBP3 preventing their expression via retention in the *cis*-Golgi[280-282]. Interestingly, UL142 does not bind to the frequent found *MICA*008* allele, which lacks a cytoplasmic tail, thereby making it resistant to the action of UL142[280]. In addition to a direct action on NKGD2L proteins, HCMV has been shown to express an array of small non coding microRNA, and one of them (miR-UL112) was predicted to target a specific site in the *MICB* 3' untranslated region (3'-UTR) and was shown to suppress cell surface expression of MICB[283]. Several viral proteins have been also recognized to be involved in the down-modulation of NKG2D ligands expression in mice. In particular m145, m152 and m155 retain MULT-1, RAE-1 family members and H60 respectively. In addition m138 down-modulates MULT-1, H60 and RAE-1ε and, consistently, mutant viruses lacking either of these immunoevasins are attenuated *in vivo* during the early days post-infection[284].

5.3.3.5 DNAM-1

A role of DNAM-1 in the response to HCMV infected cells was proposed upon identification of the immune evasion function of the viral glycoprotein UL141[285]. UL141 gene is located in the UL/b' region of HCMV genome, known to encode for viral proteins that are responsible for NK cell evasion. Indeed, laboratory strains, such as Towne and AD169, that have lost UL/b' region are less effective in promoting protection against NK cell attack than low passage isolates.

UL141 was shown by Tomasec et al. to act by sequestering in the ER the ligand of DNAM-1 and TACTILE (CD96), CD155 (PVR)[285]. It has been

recently reported that HCMV targets also Nectin-2 for proteasome-mediated degradation, thus removing both activating ligands for DNAM-1 from the cell surface during productive infection, impairing NK cell mediated killing of infected fibroblasts. Significantly, cell surface expression of both CD112 and CD155 was restored after infection with a HCMV bearing a deletion of UL141. However, while UL141 alone is sufficient to mediate retention of CD155, UL141 requires assistance from additional unknown HCMV-encoded functions to suppress expression of CD112[286].

5.3.3.7 NKp30

NCRs have been mainly involved in the recognition and killing of various tumors, but increasing evidence suggest an important role of these activating receptors in the innate immune response to viruses. In the context of HCMV infection, it has been described that the tegument protein pp65 (UL83) is able to bind NKp30. Paradoxically, the functional outcome of this interaction is not the NK cell activation but is a generalized inhibition of NK cell-mediated lysis. Indeed, pp65 was described to induce dissociation of the adaptor molecule CD3ζ from NKp30. This dissociation was reported to result in a decreased NK-cell activation in response to a wide range of target cells, including immature DC. Even though is not clear when and where this interaction occurs *in vivo* this mechanism could represent an additional HCMV mediated immune evasion strategy[148].

Chapter II

Aims

This project has been developed in the context of the study of the role of natural killer (NK) cells in the immune response to human cytomegalovirus (HCMV). The main objectives of the project were:

- To set up an autologous system to characterize the NK cell response to human cytomegalovirus infected dendritic cells

- To analyse the role of the NK cell receptor-ligand interactions and antiviral cytokines in the response to HCMV infected cells

PART II

RESULTS

Chapter III

NKp46 and DNAM-1 NK cell receptors drive the response to human cytomegalovirus-infected myeloid dendritic cells overcoming viral immune evasion strategies

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Giuliana Magri, Aura Muntasell, Neus Romo, Andrea Saez-Borderias, Daniela Pende, Daniel E Geraghty, Hartmut Hengel, Ana Angulo, Alessandro Moretta, and Miguel López-Botet

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NKp46 and DNAM-1 NK cell receptors drive the response to human cytomegalovirus-infected myeloid dendritic cells overcoming viral immune evasion strategies

Running Title: NK cell response to HCMV-infected DC

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ABSTRACT

Information on natural killer (NK)–cell receptor-ligand interactions involved in the response to human cytomegalovirus (HCMV) is limited and essentially based on the study of infected fibroblasts. Experimental conditions were set up to characterize the NK response to HCMV-infected myeloid dendritic cells (DCs). Monocyte-derived DCs (moDCs) infected by the TB40/E HCMV strain down-regulated the expression of HLA class I molecules and specifically activated autologous NK-cell populations. NKG2D ligands appeared virtually undetectable in infected moDCs, reflecting the efficiency of immune evasion mechanisms, and explained the lack of antagonistic effects of NKG2D-specific monoclonal antibody. By contrast, DNAM-1 and DNAM-1 ligands (DNAM-1L) specific monoclonal antibodies inhibited the NK response at 48 hours after infection, although the impact of HCMV-dependent down-regulation of DNAM-1L in infected moDCs was perceived at later stages. MoDCs constitutively expressed ligands for NKp46 and NKp30 natural cytotoxicity receptors, which were partially reduced on HCMV infection; yet, only NKp46 appeared involved in the NK response. In contrast to previous reports in fibroblasts, HLA-E expression was not preserved in HCMV-infected moDCs, which triggered CD94/NKG2A$^+$ NK-cell activation. The results provide an insight on key receptor-ligand interactions involved in the NK-cell response against HCMV-infected moDCs, stressing the importance of the dynamics of viral immune evasion mechanisms.

INTRODUCTION

Human cytomegalovirus (HCMV) infection is highly prevalent in healthy persons and the virus remains in a lifelong latent state, undergoing occasional reactivation and causing an important morbidity in immunocompromised patients[1]. An effective defense against CMV infection requires the participation of both T and NK cells[2]. To escape T cell-mediated recognition, CMV interferes with the expression of major histocompatibility complex (MHC) molecules and antigen presentation[3]. The loss of HLA class I molecules in infected cells impairs the engagement of inhibitory receptors thus promoting the activation of NK cell effector functions. Reciprocally, the virus has developed different strategies to escape NK cell surveillance, preventing the expression of ligands for some activating receptors (i.e. NKG2D, DNAM-1)[4-9] or selectively maintaining inhibitory receptors for HLA class I molecules engaged. The UL18 glycoprotein binds with high affinity to the LILRB1 inhibitory receptor expressed in different leukocytes[10]; yet, formal experimental evidence for its function in immune evasion remains elusive[11]. In the same line, a leader signal peptide of the UL40 HCMV protein was shown to stabilize the surface expression of HLA-E, thus repressing NK cell activation by engagement of the inhibitory CD94/NKG2A receptor[12,13]. On the other hand, the NK-cell subset bearing the CD94/NKG2C triggering molecule tends to expand in peripheral blood from HCMV$^+$ persons[14] and upon in vitro co-culture of peripheral blood mononuclear cells (PBMCs) with HCMV-infected fibroblasts[15]. A CD94/NKG2C$^+$ NK lymphocytosis was detected in a patient with a selective T-cell deficiency, coinciding with an acute HCMV infection and associated with a reduction of viremia[16]. Altogether these results suggest that the CD94/NKG2C$^+$ NK-cell subset may play an active role in the response to HCMV.

Information on the nature of NK-cell receptor (NKR)-ligand interactions involved in the response against HCMV infected cells is scarce, and most

studies have been performed in infected fibroblasts, not fully representative of the different cell types susceptible to *in vivo* infection. Cells of the myeloid lineage are considered reservoirs for HCMV latency, and differentiation of myeloid progenitors to dendritic cells (DCs) may reactivate the virus[17]. Furthermore, monocyte-derived DCs (moDCs) appear susceptible to *in vitro* HCMV infection by endothelial cell (EC)-adapted viral strains[18]. Infection of moDCs has been reported to impair their maturation, inhibiting surface expression of MHC class I and II, co-stimulatory molecules, and chemokine receptors (i.e CCR1 and CCR5), thus interfering with the development of virus-specific T-cell responses[19-21].

Beyond their key role in antigen presentation, DCs may establish a cross-talk with NK cells that reciprocally regulates their functions. Myeloid DCs can induce NK-cell functions and activated NK cells may alternatively kill immature DC or promote their maturation. These processes are dependent on activating NKR (i.e. NKp30 and DNAM-1) and cytokine secretion[22-25]. A subset of NK cells expressing the inhibitory CD94/NKG2A receptor but lacking KIR is mainly responsible for mediating the response to autologous immature moDCs[26]. HCMV infection of plasmacytoid DC has been shown to alter plasmacytoid DC-mediated activation of NK cells, inducing CD69 surface expression and interferon-γ (IFN-γ) secretion but decreasing perforin levels and cytotoxicity against the K562 tumor cell line[27]; yet, lysis/degranulation in response to infected plasmacytoid DC was not assessed, nor were the NK cell receptors participating in the response characterized.

In the present study, suitable experimental conditions were set up to characterize the NK-cell response against autologous HCMV-infected moDCs. Our results demonstrate that NK cells efficiently react against HCMV-infected moDCs, overcoming viral immune evasion strategies, and furthermore provide novel insights on the role played by different activating NK-cell receptor-ligand interactions, as well as on the influence of viral immune evasion mechanisms in this complex process.

MATERIALS AND METHODS

Cell isolation and generation of moDCs

PBMCs were obtained from heparinized blood samples by separation on Ficoll-Hypaque gradient (Lymphoprep; Axis-Shield PoC AS). Samples were obtained with the informed consent of the subjects in accordance with the Declaration of Helsinki, and the study protocol was approved by the institutional Ethics Committee. Standard clinical diagnostic tests were used to analyze serum samples for circulating IgG antibodies against HCMV (Abbott Laboratories).

MoDCs were generated as described previously[28]. Briefly, monocytes were obtained by positive selection, using anti-CD14 microbeads (Miltenyi Biotec), and cultured for 6 days in RPMI 1640 medium supplemented with 10% fetal calf serum, interleukin-4 (IL-4; 25 ng/ml, R&D Systems) and granulocyte-macrophage colony-stimulating factor (50 ng/ml, PeproTech). After 6 days, cells were $CD14^{-}CD1a^{+}$ and $CD83^{-}$ as assessed by immunofluorescence staining.

NK-cell enrichment was performed by negative selection using EasySep Human NK Cell Enrichment kit (StemCell Technologies) according to the manufacturer's recommendations, obtaining more than 98% $CD3^{-}$ $CD56^{+}$ populations. In some assays, NK cells were activated culturing PBMCs either overnight or for 7 days, with 40 U/ml or 10 U/ml recombinant IL2 (Proleukin; Chiron) added every 2 days, respectively.

Antibodies, immunofluorescence and flow cytometry analysis

Flow cytometry analysis was performed using monoclonal antibodies specific for the following surface molecules: CD14-Phycoerythrin (PE), CD3-PE, CD83-PE, CD1a-PE, CD56-PE (BD Biosciences Pharmingen), CD69-PE, CD25-PE and HLA-ABC-Fluorescein isothiocyanate (FITC) (Immunotools), CD94/NKG2C-PE (R&D Systems) and CD94/NKG2A-PE (Beckman

Coulter). HP-1F7 anti-HLA class I was generated in our laboratory[28] and D1.12 anti-HLA class II was kindly provided by Dr. Roberto Accolla (Università of Insubria, Varese). The HLA-E-specific 3D12 monoclonal antibody (mAb)[29], anti-MICA (clone BAM195, IgG1) mAb[30], as well as L95 (IgG1, anti-PVR) and L14 (IgG2a, anti-Nectin-2) mAbs[25] were previously described. MICB (clone 236511, IgG2b), ULBP-1 (clone 170818, IgG2a), ULBP-2 (clone 165903, IgG2a), and ULBP-3 (clone 166510, IgG2a) specific mAbs were purchased from R&D Systems; anti-ULBP-4 mAb (clone M475, IgG1) was kindly provided by Amgen (Thousand Oaks, CA). Control IgG2a-PE was from BD Biosciences; FITC or PE-conjugated F (ab')$_2$ rabbit anti-mouse Ig were from DAKO Denmark.

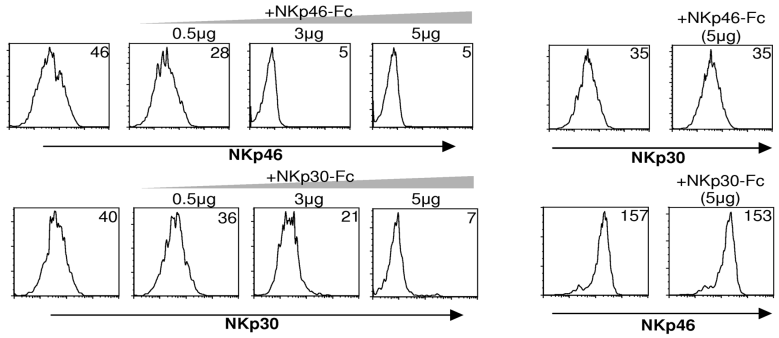
Cells were pretreated with human aggregated IgG (10μg/ml) to block Fc receptors, and subsequently labeled with specific antibodies. For indirect immunostaining, samples were incubated with unlabeled Abs followed, after washing, by FITC or PE-conjugated F(ab')$_2$ polyclonal rabbit anti-mouse Ig. Flow cytometric analysis was performed as previously described[28]. Cell viability was measured using the Annexin-V-FLUOS Staining Kit (Roche Diagnostics) according to the manufacturer's instructions.

For blocking experiments the following mAbs were used at saturating concentrations: KL247 (IgM, anti-NKp46), F252 (IgM, anti-NKp30), F5 (IgM, anti-DNAM-1), L95 (IgG1, anti-PVR), L14 (IgG2a, anti-Nectin-2), BAT221 (IgG1, anti-NKG2D); a neutralizing mAb for human IL-12 (clone 20C2, IgG1) was obtained from the ATCC and blocking antibody against IFN receptor chain 2 (IFNAR; clone MMHAR-2, IgG2a) was from Calbiochem. An anti-myc mAb (9E10, IgG1) was used as negative control.

Expression of NCR-Fc chimeric construct and immunofluorescence

The plasmids for expression of NKp30 and NKp46 as fusion proteins with the Fc portion of human IgG1 have been described[31] and were generously provided by Dr Ofer Mandelboim (Hebrew University-Hadassah Medical

School, Jerusalem, Israel). Recombinant proteins were expressed by transfection of HEK 293T cells using the calcium phosphate method; 18 hours later, cells were washed twice and cultured on serum-free medium (EX-cell ACF CHO Medium, Sigma-Aldrich). At day 3 and 6 the supernatants were recovered and the soluble molecules purified by affinity chromatography with Protein A Sepharose CL-4B (GE Healthcare) and analysed by SDS-PAGE and Coomassie blue staining. The specificity of NCR-Fc chimeric proteins was checked using a competition assay by staining $CD3^{-}CD56^{+}$ NK cells with NKp46 (BAB281) or NKp30 (AZ20) mAbs in the absence or presence of increasing concentrations of NKp46-Fc or NKp30-Fc fusion proteins (Supplemental Figure 1).



Supplemental Figure 1. Specificity of NCR-Fc chimeric proteins. $CD3^{-}CD56^{+}$ NK cells were stained with mAbs specific for NKp46 (BAB281) or NKp30 (AZ20) in the absence or presence of increasing concentrations of NKp46-Fc or NKp30-Fc fusion proteins. Inserted numbers correspond to fluorescence intensity values (geometric mean).

Cells were pretreated with rabbit serum (30µl) to block Fc receptors, and human IgG1 (2 µg) was used as a negative control. Cells were incubated with NKp30-Fc or NKp46-Fc (3 µg) for 45 min at 4°C followed, after washing, by PE-conjugated anti–human Ig (Jackson ImmunoResearch Laboratories). 4',6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) was added to exclude dead cells from the analysis.

HCMV preparation, sequencing of UL40 gene and infection of moDCs

Stocks of the TB40/E HCMV strain[32] (kindly provided by Christian Sinzger, Institute for Medical Virology, University of Tübingen, Germany) and the clinical isolate UL1271[33] were prepared as described previously[30]. Purified stocks were resuspended in serum-free Dulbecco minimal essential medium, stored at –80°C, and titrated by standard plaque assays on MRC-5 cells. Inactivation of viral stocks was achieved by UV light using an UV-crosslinker (Bio-Rad GS genelinker UV chamber).

The sequence of the *UL40* gene in HCMV isolates TB40/E and UL1271 was analysed by direct sequencing of agarose purified PCR products which were amplified from HCMV virions using primers: 5'-TCC TCC CTG GTA CCC GAT AAC AG -3' and 5'-CGG GCC AGG ACT TTT TAA TGG CC-3'[34].

moDCs were incubated overnight either alone (mock), with TB40/E (multiplicity of infection (MOI): 50-100) or with the same concentration of UV-inactivated virus. Thereafter, cells were washed twice, counted and resuspended in RPMI 10% Fetal calf serum. At 48 hours after infection moDC were harvested washed and cytospin preparations were stained by indirect immunofluorescence with a mouse anti-CMV IE-1/IE-2 monoclonal antibody (clone mab810, Chemicon) followed by FITC-conjugated goat anti-mouse Ig and examined as previously described[30]. Briefly, slides were examined with a Leica DM6000B fluorescence microscope (HC PL fluotar 10X/0.30, dry) in Dako fluorescence mounting medium and images were taken with a Leica DFC300 FX digital camera and were analyzed with the Lieca FW4000 Fluorescence Workstation software.

NK cell functional assays

Purified fresh or IL-2 activated NK cells were resuspended in complete medium and cocultured for 24 or 48 hours with autologous moDCs (uninfected, TB40/E infected or treated with UV inactivated virus) in 96-well flat-bottom plates at different moDCs/NK ratios. Surface expression of

CD69 and CD25 on $CD56^+$ cells was analyzed by flow cytometry. Culture supernatants were harvested at 24 and 48 hours , and IFN γ concentration was measured by enzyme-linked immunoassorbent assay (ELISA, Human IFNγ Module Set; Bender MedSystems) as recommended by the manufacturer; all experiments were performed in triplicate.

Activation of NK-cell cytotoxic function was tested using the CD107a mobilization assay. PBMCs were stimulated either overnight or for 7 days with IL-2, and NK cells were subsequently purified by negative selection. Next, NK cells were incubated for 5 hours at 37ºC in the presence of monensin (5ng/ml, Sigma-Aldrich), anti CD107a FITC (BD Biosciences Pharmingen) together with autologous moDCs uninfected (mock), TB40/E infected, or treated with UV-inactivated TB40/E; assays were performed at 48 hours or, when specified, at 72 hours after DC exposure to the virus. The HLA class I-defective erythroleukemia K562 cell line was used as a positive control for degranulation. Cells were then washed in PBS supplemented with 2mM EDTA, stained for 30 minutes at 4°C with anti-CD56 PE and analyzed by flow cytometry. In some experiments, NK cell functional assays were carried in the presence of saturating concentrations of a panel of NK-cell receptor-specific mAbs.

Statistical analysis

Statistical analysis was performed by the Mann-Whitney U test, using the SPSS, version 9.0 software. Results were considered significant at the 2-sided P level of .05.

RESULTS

NK cell activation against autologous HCMV-infected moDC

Suitable experimental conditions were established to characterize the NK-cell receptor-ligand interactions involved in the response to HCMV-infected myeloid DCs. To this end, immature moDCs were incubated with medium (mock), the TB40/E HCMV strain (MOI 50-100), or UV-inactivated TB40/E (UV-TB40/E). Cells were stained 48 hours later by indirect immunofluorescence with a mAb specific for the HCMV IE-1/IE-2 antigen (Figure 1A). Based on the percentage of IE-1/IE-2$^+$ cells, the infection rate of moDC varied from 40% to 90% in different experiments; nuclear IE-1/IE-2 staining was undetectable in mock and UV-TB40/E-treated cultures, though few IE-1/IE-2$^+$ cells were occasionally stained in the latter (Figure 1B).

Compared with UV-TB40/E-treated moDCs, the expression of HLA class I and class II molecules decreased in HCMV-infected moDCs (Figure 1C), in agreement with previous reports[19,20]. Inhibition of HLA class I expression is known to be mediated by US2, US3, US6 and US11[3], whereas the mechanisms underlying the different pattern of HLA class II down-regulation are more complex[19]. The proportion of cells displaying a reduced expression of HLA class I molecules correlated with the number of IE-1/IE-2$^+$ cells in all experiments, and time course analysis revealed that the inhibition of HLA-I expression was detectable at 48 hours (data not shown). The noninfected subset in TB40/E treated cultures, as well as cells incubated with UV-inactivated TB40/E, expressed higher levels of HLA class I molecules than mock moDCs, an effect attributed to the endogenous production of type I IFN, according to previous reports[35]. No changes in the surface levels of CD83 were observed (Figure 1C) indicating that HCMV did not induce moDCs maturation, as previously described[20].

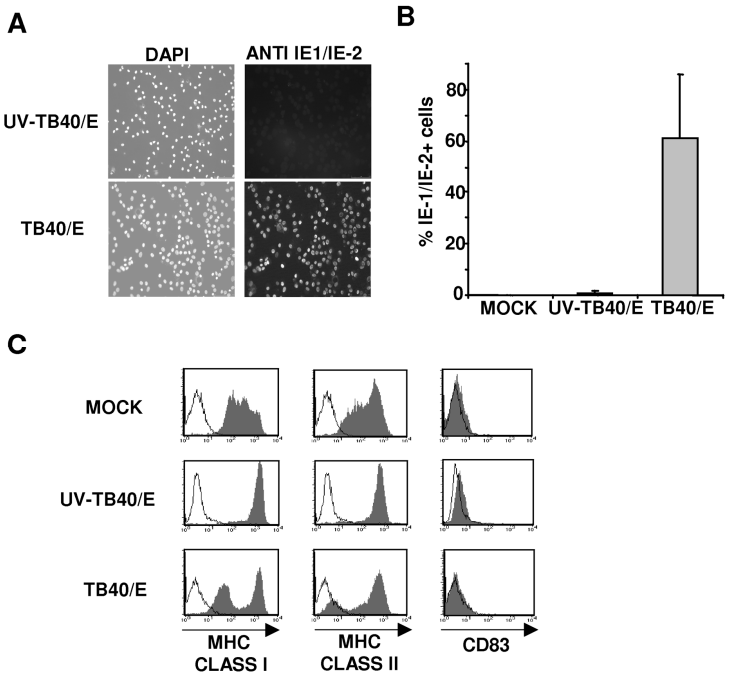
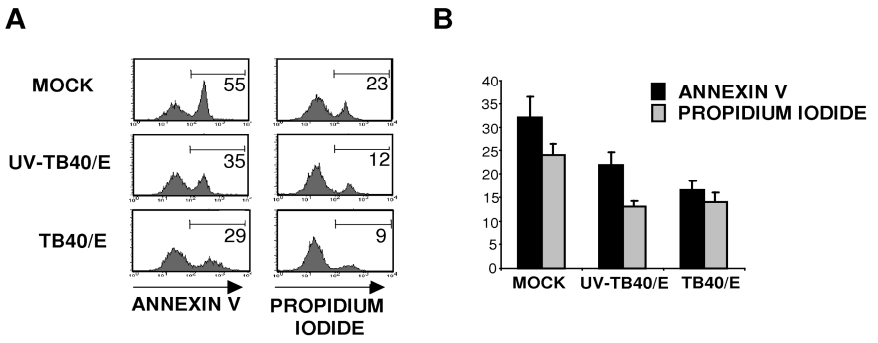


Figure 1. moDCs infected by the endothelial cell adapted TB40/E HCMV strain down-regulate MHC class I and II expression. Immature moDCs were incubated overnight with complete medium (MOCK), HCMV (TB40/E) or UV inactivated HCMV (UV-TB40/E) **(A)** Cells were stained 48 hours after infection with a mouse anti-CMV IE-1/IE-2 monoclonal antibody (right panels, FITC) and DAPI nuclear staining (left panel). Images were analyzed as described in "HCMV preparation, sequencing of UL40 gene, and infection of moDCs". **(B)** Histograms represent the percentages of IE-1/IE-2$^+$ cells detected in 8 independent experiments. (Mean ± SD). **(C)** Flow cytometry performed at 72 hours after infection. moDCs were surface labelled by indirect immunofluorescence with mAbs specific for HLA class I, class II (HLA-DR) or CD83 mAbs (open histograms, isotype control; filled histograms specific staining). Results of a representative experiment (60% IE-1/IE-2$^+$ cells) of 4 performed are shown.

The viability at 72 hours after TB40/E infection, assessed by staining with annexin V and propidium iodide, was comparable to that of control moDCs treated with UV-TB40/E, and higher than that of mock-treated cells (Supplemental Figure 2); it is of note that, as described in "HCMV preparation, sequencing of UL40 gene, and infection of moDCs", in these

experiments cells were deprived of cytokines employed to induce moDCs differentiation.



Supplemental Figure 2. Viability of HCMV infected moDCs (A) Cell viability was determined by flow cytometry at 72h post virus exposure assessing Annexin V-FITC and propidium iodide (PI) staining. **(B)** Results correspond to the percentages of annexin V^+ and PI^+ cells detected in three independent experiments (mean ± SEM)

NK-cell populations from HCMV seronegative donors were cultured alone or in the presence of autologous moDCs untreated (mock), incubated with UV-TB40/E or infected with TB40/E. NK cells up-regulated the surface expression of activation markers (i.e. CD25 and CD69) and secreted high concentrations of IFN-γ (Figure 2A-B) in the presence of HCMV-infected moDCs, displaying a marginal or undetectable response to mock or UV-TB40/E-treated moDCs.

To investigate whether HCMV-infected moDCs triggered NK cell-mediated cytotoxicity, degranulation was assessed using the CD107a mobilization assay. As shown in Figure 2C-D, a significant increase in the percentage of $CD107a^+$ $CD56^+$ NK cells was specifically detected in response to TB40/E-infected moDCs, but not upon incubation with mock or UV-TB40/E-treated moDCs.

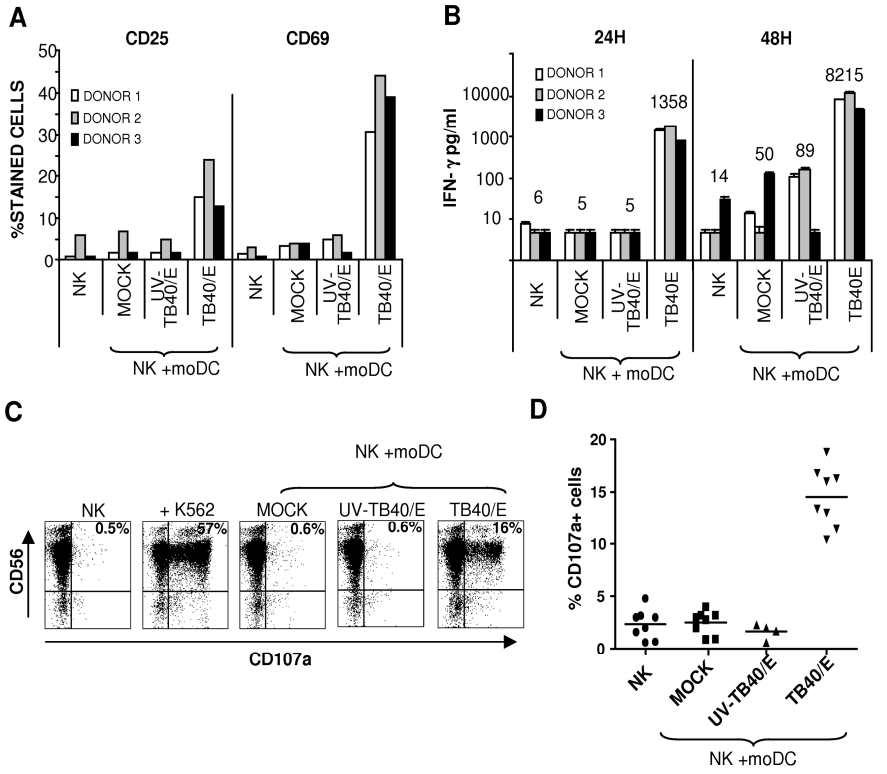
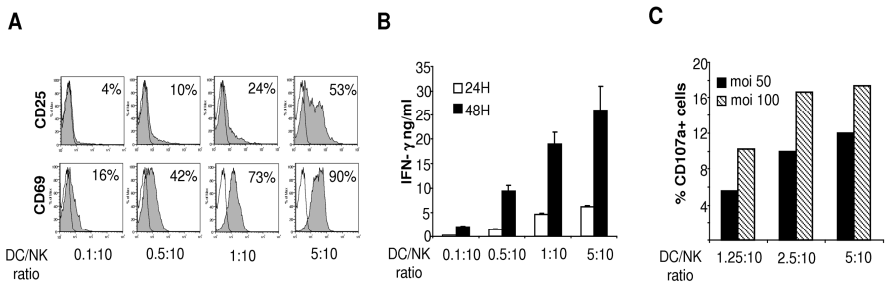


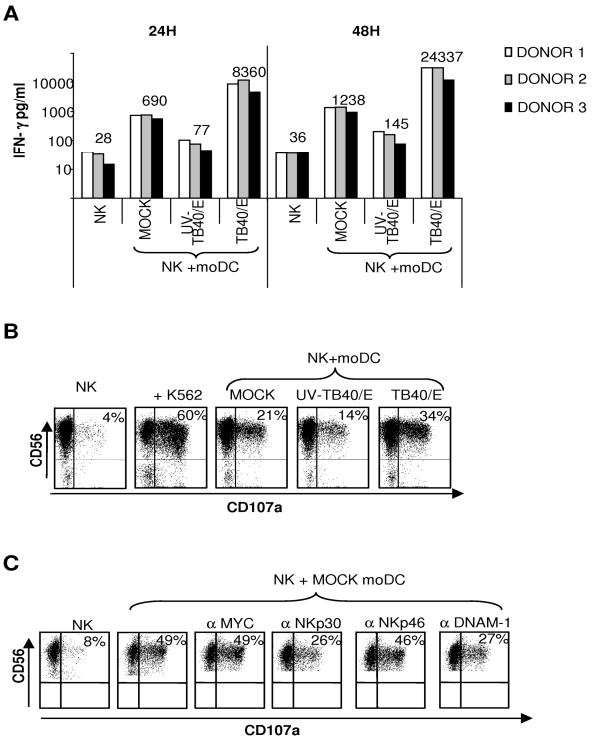
Figure 2. Specific NK cell activation in response to autologous HCMV-infected moDCs. (A, B) Freshly purified NK cells were cultured for 48 hours either alone, or with mock moDCs, UV-TB40/E-treated moDCs or TB40/E-infected moDCs in the presence of 10U/ml of IL-2 (moDC/NK= 1:20). **(A)** CD25 and CD69 expression was analyzed by flow cytometry. **(B)** IFN-γ production was detected in culture supernatants by ELISA. Results from 3 representative experiments performed with different donors are shown. Numbers correspond to mean value of IFN-γ production. **(C-D)** NK cells purified by negative selection from PBMCs stimulated overnight with IL-2 were co-cultured for 5 hours with target cells as described in "NK-cell functional assays". Surface CD107a expression in $CD56^+$ cells was analysed by flow cytometry. **(C)** Dot plots from a representative experiment of 8 performed are shown (moDC/NK = 1:4), including the K562 cell line as a control. The percentage of $CD107a^+$ cells is included in each dot blot (TB40/E moDC: 65% 1E-1/IE-2^+cells) **(D)** Scatter plots displaying the percentage of $CD107a^+$ cells from 8 different experiments performed.

Raising the moDC/NK ratio and the MOI enhanced NK-cell activation (Supplemental Figure 3).



Supplemental Figure 3. Effect of moDC/NK ratio on NK cell activation. Analysis of CD25, CD69 expression **(A)** and IFN-γ production (mean ± SD of triplicates) **(B)** in fresh NK cells incubated with increasing numbers of TB40/E moDCs in the presence of 10U/ml of IL-2. Results of a representative experiment of 3 performed are shown.**(C)** Bar graphs from a representative experiments representing the percentage of CD107a+ NK cells cultured for 5 hours with increasing numbers of TB40/E moDCs (moi 100: 65% 1E-1/IE-2+cells; moi 50: 40% IE-1/IE-2+cells).

NK cell populations cultured for 7 days in the presence of IL-2 reacted as well preferentially against HCMV-infected moDCs by secreting IFN-γ and mobilizing CD107a. Nevertheless, in these conditions, a substantial IFN-γ production and an increase of $CD107a^+$ cells (Supplemental figure 4A-B) were also detected in response to mock uninfected moDCs. This effect was reduced when IL-2 activated NK cells were incubated with moDCs treated with UV-TB40/E, which up-regulated HLA class I expression (Figure 1C). These results are in agreement with the reported ability of activated NK cells to react with immature moDCs, and consistent with the protective role of HLA class I molecules in mature moDCs[36]. As previously reported[23,25], NK-cell degranulation in response to mock moDCs was partially inhibited in the presence of mAbs specific for the NKp30 NCR and DNAM-1 but not by an anti NKp46 mAb (Supplemental Figure 4C).



Supplemental Figure 4. NK cells pre-activated for 7 days with IL-2 respond to both mock and HCMV infected moDCs.PBMCs were stimulated for 7 days with IL-2 (10 U/ml) and NK cells were subsequently purified by negative selection. **(A)** IL-2 activated NK cells were cultured for 48 hours alone, with mock moDCs, UV-TB40/E moDCs, or TB40/E moDCs in the presence of 10U/ml of IL-2. IFN- γ production was detected by ELISA in culture supernatants. Numbers correspond to mean value of IFN-γ production. **(B, C)** IL-2 activated NK cells were cultured for 5 hours with target cells as described in methods. Surface CD107a expression in $CD56^+$ cells was analysed by flow cytometry. **(B)** Dot plots from a representative experiment out of three performed are shown (moDC/NK = 1:4), including the K562 cell line as a positive control. The percentage of $CD107a^+$ cells is included in each dot plot. **(C)** IL-2 activated NK cells were co-cultured for 5 hours either alone or with autologous immature moDCs. In parallel, the effect of mAbs specific for NKp30, NKp46, DNAM-1 and a control mAb (anti-myc) was tested. Dot plots from a representative experiment out of three performed are shown.

Studies in murine cytomegalovirus infection have revealed the important regulatory role exerted on the NK-cell response by type I IFN and IL-12, secreted in response to the virus challenge[37]. In our experimental system,

neutralizing IL-12 markedly inhibited IFN-γ secretion (Figure 3A) while blocking type I interferon receptor (IFNAR) partially reduced CD69 expression on the surface of NK cells (Figure 3B), both measured 24 hours after coculture of NK cells with moDCs. By contrast, NK cell-mediated cytotoxicity triggered by HCMV-infected moDCs at 5 hours was not significantly affected (Figure 3C).

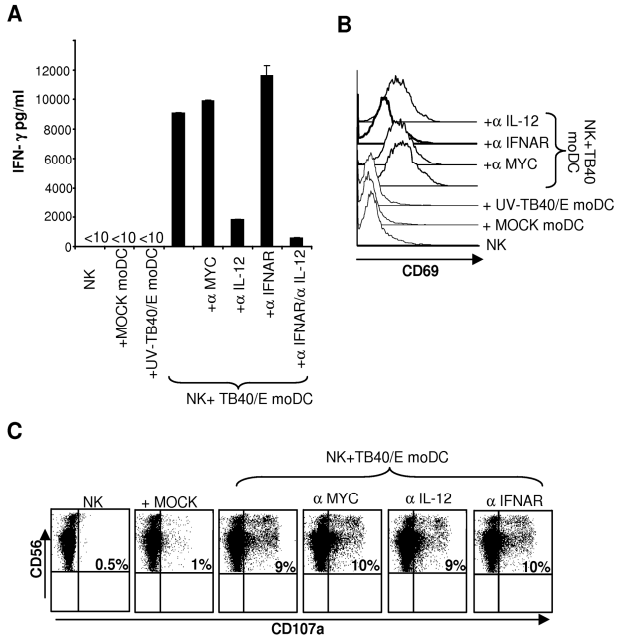


Figure 3. IL-12 and IFN-alpha contribute to NK cell activation induced by HCMV-infected moDCs. Freshly purified NK cells were cultured with target cells for 24 hours as described in figure 2A-B (moDC/NK= 1:4; TB40/E moDC: 80% IE-1/IE-2$^+$ cells). In parallel, the effect of IL-12 specific, IFNAR specific and control (myc) mAbs was tested. **(A)** IFN-γ production was measured by ELISA (mean ± SD of triplicates). **(B)** CD69 expression on NK cells was assessed by Flow cytometry. **(C)** NK cells purified by negative selection from PBMC stimulated overnight with IL-2 were cocultured for 5 hours with target cells as described in "NK-cell functional assays". In parallel, the effect of IL-12 specific, IFNAR specific and control (myc) mAbs was tested. Surface CD107a expression in CD56$^+$ cells was analysed by flow cytometry. The percentage of CD56$^+$CD107a$^+$ is included in each dot plot Results of a representative experiment of 3 performed are shown.

The original studies described in this section were essential to establish reliable experimental conditions required to dissect the role played by NKR-ligand interactions with infected moDCs. In that way an overlapping response against non-infected moDCs was avoided, allowing in short-term assays to discriminate NKR involvement from the effects of cytokines.

Role of activating receptors in the NK-cell response to HCMV-infected moDCs

To elucidate the nature of activating receptors involved in the response to HCMV infected moDCs, the antagonistic effect of NKR and NCR-specific mAbs (i.e. anti NKp46, NKp30, DNAM-1, NKG2D) was assessed in NK-cell degranulation assays (Figure 4A) and on the production of IFN-γ, analyzed in culture supernatants 6 hours after incubation with infected moDCs (Figure 4B). NKp30 and NKG2D-specific mAbs did not alter CD107a NK cell expression and IFN-γ secretion which were significantly inhibited in the presence of anti-NKp46 and DNAM-1 mAbs, thus indirectly supporting that both receptors participate in the NK-cell response against HCMV infected moDCs (Figure 4A-B).

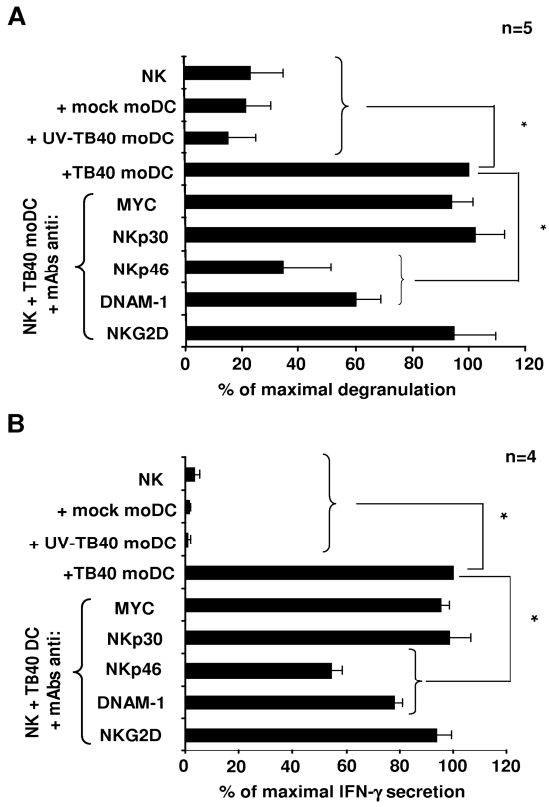
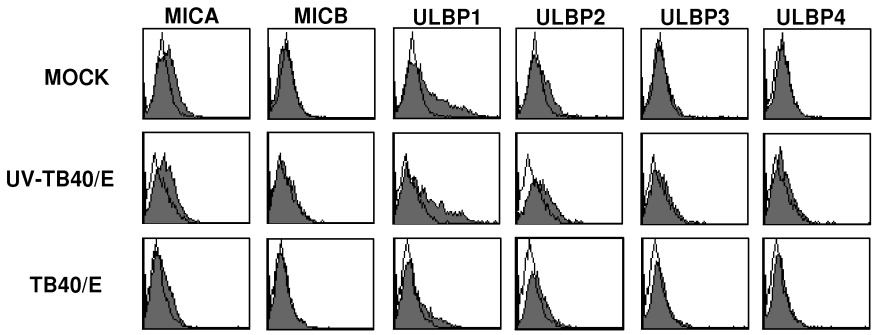


Figure 4. NK cell degranulation and IFN-γ secretion in response to TB40/E-infected moDCs involves NKp46 and DNAM-1 activating receptors. (A) NK-cell degranulation against moDCs (moDC/NK= 1:4) was measured by the CD107a mobilization assay in the presence of blocking mAbs as described in "NK-cell functional assays". Assays were performed 48 hour after DC exposure to the virus. For each experiment, data were normalized to the response of NK cells incubated with HCMV-infected moDCs in the absence of mAbs (100%); in these conditions the numbers of $CD107a^+$ cells ranged from 11.4% to 18.8%. **(B)** The same experimental conditions used for degranulation assays were applied. At 6 hours supernatants were harvested and assayed for the presence of IFN-γ by ELISA. Data were normalized to the IFN-γ levels detected in supernatants of NK cells incubated with TB40/E moDCs in the absence of mAbs (100%); in these conditions the absolute concentrations of IFN-γ ranged from 164 to 915 pg/ml. Data correspond to mean ± SEM (*$p<0.05$).

Expression of different NKG2D ligands (NKG2DL; i.e. MICA/B and ULPB1-4) was low or undetectable in uninfected moDCs and, remarkably, was not up-regulated upon HCMV infection at 48 and 72 hours after infection

(Supplemental Figure 5; data not shown). In the case of ULBP1, a significant decrease in the expression was detected compared with control moDCs. Altogether, the data are consistent with the efficient ability of HCMV to interfere with the surface expression of NKG2DL, thus explaining the lack of antagonistic effect of anti NKG2D mAb.



Supplemental Figure 5. NKG2D ligands expression on moDCs. MoDCs were labelled at 48 hours after virus exposure by indirect immunofluorescence with mAbs specific for MICA, MICB, ULBP1, ULBP2, ULBP3, ULBP4 (open histograms, isotype control; shaded histograms, specific staining). Results of a representative experiment (50% IE-1/IE-2 $^{+}$ cells) of 3 performed are shown.

NK-cell degranulation and, to a lesser extent, IFN-γ secretion were significantly decreased in the presence of anti-DNAM-1 mAbs (Figure 4). DNAM-1 participates in NK-cell mediated-recognition of immature moDCs which express both DNAM-1 ligands (DNAM-1L), PVR and Nectin-2[25]. The UL141 HCMV protein has been reported to inhibit PVR[9] and Nectin-2[38] expression in infected fibroblasts, contributing to immune evasion; thus, we analysed the expression of DNAM-1L in HCMV infected moDCs. Time-course analysis revealed that at 48 hours after infection the expression of both DNAM-1L on moDCs was minimally altered compared with a marked down-regulation detected at 72 hours in infected cells (Figure 5A), identified by the loss of HLA class I expression (Figure 5B). To assess the impact of the inhibition of DNAM-1L expression on the NK-cell response, the antagonistic effects of DNAM-1 or a combination of PVR and Nectin-2 specific mAbs on

the response to TB40/E-infected moDCs were compared at different time-points after infection. As shown in figure 5C, HCMV infected moDCs triggered NK-cell mediated cytotoxicity, and degranulation was inhibited in the presence of blocking mAbs at 48 hours. By contrast, at 72h after infection a reduction in the percentages of CD107a$^+$ cells was observed, remaining unaltered in the presence of the DNAM-1 specific mAb whereas, paradoxically, anti DNAM-1L mAbs enhanced degranulation (Figure 5D).

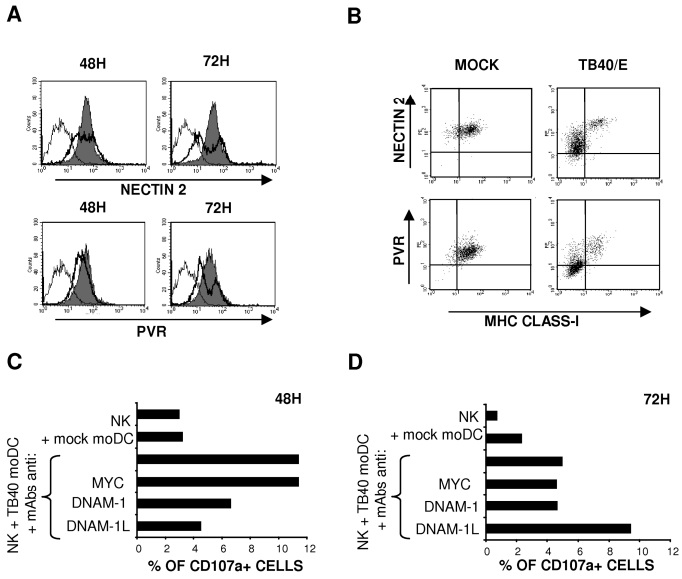


Figure 5. HCMV infection down-regulates PVR and Nectin-2 expression in moDC: influence on the NK cell mediated response at different post-infection stages. (A) Mock MoDCs (filled histograms) and TB40/E moDCs (bold line, open histograms) were surface labelled at 48 hours and 72 hours post-infection by indirect immunofluorescence with Nectin-2 and PVR-specific mAbs. Staining with isotype control is included (thin line, open histograms). Results of a representative experiment (65 % IE-1/IE-2$^+$ cells) of 3 performed are shown. **(B)** Mock moDCs and TB40/E moDCs at 72h post- infection were also co-stained with FITC-conjugated HLA class I specific mAbs. Results of a representative experiment (70% IE-1/IE2$^+$ cells) of 3 performed are shown. **(C, D)** NK-cell degranulation against moDCs was measured by the CD107a mobilization assay in the presence of blocking mAbs as described in "NK-cell functional assays". Assays were performed at 48 hours **(C)** and 72 hours **(D)** after DCs exposure to the virus. Results of a representative experiment out of three performed are shown for each condition.

The data indicate that the DNAM-1 receptor plays a relevant role in the NK-cell response at early stages of HCMV infection, whereas the effects of HCMV-dependent down-regulation of DNAM-1L are perceived at later stages, thus stressing the importance of the kinetics of expression of immune evasion mechanisms.

Based on the antagonistic effect of NCR-specific mAbs (Figure 4) NKp46, but not NKp30, appeared involved in the recognition of TB40/E infected moDCs. To assess the expression of NKp30 and NKp46 ligands in moDCs, we analysed by flow cytometry the binding of NCR-Fc fusion proteins. As shown in Figure 6A, both NKp30-Fc and NKp46-Fc clearly stained moDCs thus providing, to our knowledge, the first evidence that these cells constitutively express surface ligands for both NCRs. At 48 hours after infection, when functional assays were performed, staining of infected moDCs by soluble NCR was partially reduced. By contrast, a marked decrease of NKp30-Fc and, especially, of NKp46-Fc specific binding to moDCs was observed at 72 hours after infection (Figure 6B). It is of note that, as compared with the selective loss of HLA class I and DNAM-1L in infected cells (bimodal distributions in Figures 1C and 5A), down-regulation of NCR ligands homogeneously affected all TB40/E-treated moDCs (Figure 6B), including both infected and noninfected cells.

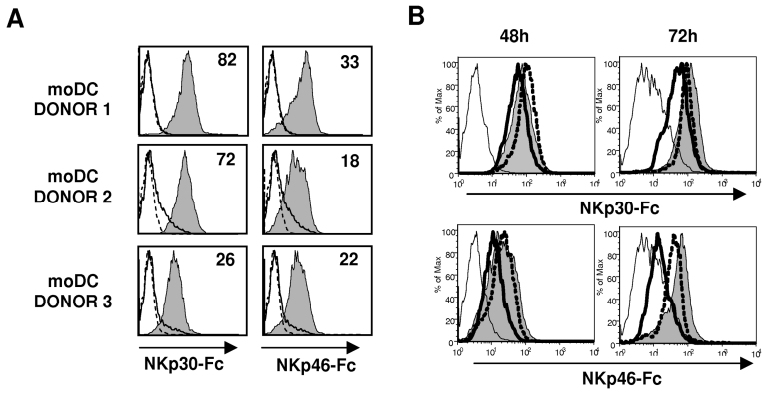


Figure 6. NKp30 and NKp46 ligands are constitutively expressed on moDCs and are down-regulated at late stages of HCMV infection. (A) NKp30 and NKp46 ligands expression on moDCs was assessed by indirect immunofluorescence and flow cytometry using soluble recombinant NKp30-Fc and NKp46-Fc fusion proteins as described in "expression of NCR-Fc chimeric construct and immunofluorescence". Staining with NKp30-Fc and NKp46-Fc (filled histograms) was compared to control human IgG1 (thin line, open histograms) and with secondary antibodies alone (dotted line, open histograms). Inserted numbers correspond to geometric means. **(B)** Mock moDCs (filled histograms), UV-TB40/E moDCs (dotted line, open histograms) and TB40/E moDCs (bold line, open histograms) were surface labelled at 48 hours and 72 hours after virus exposure by indirect immunofluorescence with NKp30-Fc and NKp46-Fc. Staining with a human IgG1 is included as a control (thin open histograms). Results of a representative experiment (70 % IE-1/IE-2$^+$ cells) of 4 performed are shown.

CD94/NKG2A+ NK cells efficiently respond to HCMV-infected moDCs which down-regulate HLA-E expression

A leader signal peptide from the UL-40 HCMV protein has been reported to stabilize the surface expression of HLA-E in fibroblasts, thus repressing NK cells bearing the CD94/NKG2A inhibitory receptor[12,13]. On the other hand, indirect evidence has been obtained supporting that CD94/NKG2C$^+$ NK cells may be involved in the response to HCMV[15,16].

Thus, we comparatively assessed the response of CD94/NKG2A$^+$ and CD94/NKG2C$^+$ NK cells from selected HCMV+ blood donors against HCMV infected moDCs employing the CD107 mobilization assay. Remarkably, CD94/NKG2A$^+$ NK cells degranulated against HCMV infected

immature moDCs more efficiently than the NKG2C$^+$ subset, whereas both comparably reacted against the HLA class I negative K562 leukemia cell line (Figure 7A).

A reduced surface expression of HLA-E was detected in TB40/E infected moDCs thus providing an explanation for these unexpected observations (Figure 7B). By contrast, the noninfected moDCs subset up-regulated HLA-E in parallel to total HLA class I, comparable to cells treated with UV-inactivated TB40/E, consistent with their response to endogenous type I IFN as discussed for Figure 1. An obvious question was whether the UL40 leader signal peptide from TB40/E displayed the canonical sequence reported to stabilize HLA-E (VMAPRTLIL). Although this issue was not addressed in a detailed report on TB40/E[39], the annotated sequence (GenBank accession number EF999921) included a mutation in p2, a key anchor residue, where Met was substituted by Val; sequencing our TB40/E batch confirmed the mutation. Thus we considered to what extent down-regulation of HLA-E expression in TB40/E infected moDCs might be attributed to this structural change. The possibility to generate a revertant employing the conventional approach based on the use of a TB40/E BAC was ruled out, as this mutant lacks the *US2, US3* and *US6* genes, thus preserving in infected cells high surface levels of all HLA class I molecules, including HLA-E (data not shown). To circumvent this drawback, we tested a different HCMV clinical isolate (UL1271)[33] capable of infecting moDCs and bearing the UL40 peptide canonical sequence (Figure 7C). HLA-E was also clearly down-regulated in moDCs infected with this HCMV clinical isolate (Figure 7D), as compared to its expression levels in mock moDCs. On the other hand, similarly to TB40/E, UL1271 treatment also enhanced total HLA class I and HLA-E expression in the residual non-infected moDC subset (Figure 7D). These observations point out that UL40 is inefficient to protect infected moDCs against NKG2A$^+$ NK cells, in contrast to its role in immune evasion reported in fibroblasts.

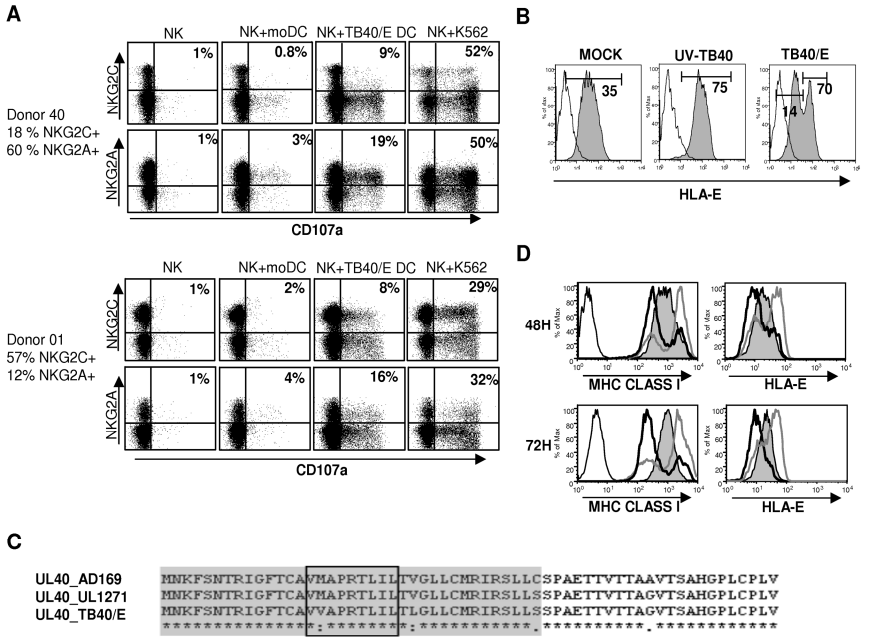


Figure 7. HCMV-infected moDCs trigger a preferential response of CD94/NKG2A$^+$ NK cells associated to a down-regulation of HLA-E expression. (A) NK cells purified by negative selection from PBMC stimulated overnight with IL-2 were co-cultured for 5h with target cells as described in methods. Surface CD107a expression on CD94/NKG2A$^+$ and CD94/NKG2C$^+$ NK cells was analysed by flow cytometry. Dot plots from 2 representative donors out of six analysed are shown. The proportions of CD107a$^+$ cells referred to total NKG2A$^+$ or NKG2C$^+$ cells are specified in bold. **(B)** MoDCs were surface labelled 48h post virus exposure by indirect immunofluorescence with an HLA-E specific mAb (3D12) (open histograms, isotype control; filled histograms specific staining). Fluorescence intensity (geometric mean) of selected populations is included. Results of a representative experiment (60 % IE-1/IE-2$^+$ cells) out of six performed are shown. **(C)** Alignment of part of gpUL40 amino acid sequences from AD169, TB40/E and the HCMV clinical isolate UL1271; predicted leader sequences are shaded, and HLA-E binding peptides are boxed. **(D)** MoDCs were mock treated (filled histograms) or infected with the HCMV clinical isolate UL1271 (moi 25: grey line; moi 100: black line, open histograms) and surface labelled at 48h and 72h post virus exposure by indirect immunofluorescence with mAbs specific for HLA-E (3D12) and HLA class I molecules (HP-1F7). Results of a representative experiment (moi 25: 25% IE-1/IE-2+ cells; moi 100: 85% IE-1/IE-2+cells) out of three performed are shown.

DISCUSSION

A vigorous specific NK-cell response to autologous HCMV-infected moDCs was detected by the expression of activation markers, IFN-γ production and NK-cell degranulation. The marginal response to mock-infected or UV-TB40/E treated moDCs supported that NK-cell activation was triggered by the fraction of infected cells in which HLA class I molecules were down-regulated. In line with previous studies in influenza-infected moDCs[40], type I IFN and IL-12 contributed to the response against HCMV-infected cells, complementing the NKR/NCR-dependent signals. In this regard, our results revealed a dominant role of NKp46 and DNAM-1 in NK cell recognition of HCMV-infected moDCs, without a detectable involvement of NKp30 and NKG2D. These observations pointed out clear differences with NK-cell response to non-infected moDCs, where anti DNAM-1 and NKp30, but not anti NKp46 mAbs, inhibited NK activation.

NKG2D has been reported to be involved in the response to MCMV infected DCs[37], and is thought to participate as well in the defence against HCMV[41]. In this case, the evidence is essentially indirect and based on the identification of viral immune evasion molecules in HCMV (i.e. UL16, UL142 and miR–UL112)[4-7] that selectively interfere with the surface expression of NKG2D ligands (NKG2DL), similar to those employed by MCMV (m138, m145, m152, m155)[8]. The existence of multiple CMV strategies targeting the expression of NKG2DL in different species is interpreted as an evidence for the strong evolutionary pressure exerted by the lectin-like receptor in anti viral defence. The expression of NKG2DL appeared virtually undetectable in TB40/E infected moDCs, reflecting the effectiveness of immune evasion mechanisms and consistent with the lack of antagonistic effects of anti-NKG2D mAbs in the response of NK cells. In contrast, NKG2DL were shown to be up-regulated in influenza-infected moDCs, contributing to trigger NK-cell activation[40].

DNAM-1 was reported to participate in the response of activated NK cells against autologous immature moDCs, which express PVR and Nectin-2[25]. Functional studies also supported the importance of this receptor-ligand system in the NK-cell response to HCMV-infected moDCs. The UL141 HCMV molecule was originally reported to inhibit the surface expression of PVR, interfering with NK cell mediated recognition of infected fibroblasts[9]. We confirmed that this DNAM-1 ligand was down-regulated in TB40/E infected moDCs and, in agreement with a recent report[38], we detected a similar effect on the expression of Nectin-2. Time-course analysis indicated that inhibition of DNAM-1L was limited at 48 hours after infection, when the NK cell response was assessed, becoming marked at 72 hours. Blocking DNAM-1 or DNAM-1L with specific mAbs comparably inhibited NK activation at 48 hours, whereas anti DNAM-1L mAbs enhanced degranulation at 72 hours. This paradoxical effect might be explained by the involvement of TIGIT, a recently identified inhibitory receptor expressed by T and NK cells which binds PVR with higher affinity compared with DNAM-1[42,43]. The partial loss of PVR and, particularly, of Nectin-2 expression in infected cells below a critical threshold might impair activation via DNAM-1, while maintaining TIGIT-dependent negative signalling; further studies are required to directly verify this hypothesis. Thus, DNAM-1 plays a relevant role in NK-cell recognition of HCMV-infected moDCs early during infection, whereas the effect of viral-mediated down-regulation of DNAM-1L prevails at later stages, thus illustrating the importance of the kinetics of immune evasion mechanisms.

Based on the antagonistic effect of NCR-specific mAbs, the response to HCMV- infected moDCs was dependent on NKp46, in contrast to the dominant role played by NKp30 in the response of IL-2-activated NK cells to immature non-infected moDCs[23,24]. NKp46 was originally reported to interact with influenza hemagglutinin (HA)[31], contributing with NKG2D to trigger the NK cell response against influenza infected moDCs[40]. By contrast, no HCMV

molecules interacting with this NCR have been identified and the nature of its cellular ligands remains unknown. It is of note that the molecular basis for NKp30-mediated recognition of non-infected moDCs is also uncertain, as they do not display the B7-H6 ligand[44].

Taking advantage of the availability of soluble NCR-Fc fusion proteins, we observed that both NKp30-Fc and NKp46-Fc specifically bound to the surface of non-infected moDCs. To our knowledge, this provides the first unequivocal evidence that ligands for both NCR are constitutively expressed by this cell type. Time-course analysis during HCMV infection revealed that binding of NCR fusion proteins did not increase at 48h post infection, when the NK cell response was assayed, but appeared reduced at later stages. Down-regulation of NCR ligands became more evident for NKp46 and, moreover, had an impact on the NK cell response that was not anymore antagonized by anti NKp46 mAbs at 72 hours (data not shown). Hence, the dominant role of NKp46 played in the response could not be simply explained by an induction of NCR ligand expression in HCMV-infected moDCs. It is conceivable that NKp46 may trigger NK activation simply as a result of the down-regulation of HLA class I expression in infected cells resulting in the loss of inhibitory NKR signalling, in agreement with the missing self hypothesis, as proposed[45]. Alternatively, the possibility that qualitative changes in the conformation/structure of NKp46 ligand might take place during infection, increasing the affinity for the receptor, cannot be ruled out.

On the other hand, in line with previous functional studies[22-24], our data support the expression of an NKp30L in moDCs, different from the B7H6 molecule. Binding of NKp30-Fc to infected moDCs remained essentially unaltered at 48 hours, and the basis for the apparent lack of involvement of NKp30 in the response to HCMV infected moDCs is uncertain. By contrast, NKp30 participated in the response of 7-day IL-2 activated NK cells, but not of freshly isolated NK cells, against noninfected moDCs which display

NKp30 ligand(s) and normal levels of HLA class I molecules. The data suggest that the function of this NCR may depend on the metabolic status of the NK cell. In this regard, NKp30 surface expression levels were up-regulated after 7-day stimulation with IL-2 (data not shown). On the other hand, the pp65 (UL83) tegument HCMV protein was reported to interact with NKp30 and to interfere with signalling, apparently uncoupling the receptor from its adaptor molecule by a still undefined mechanism[46]. Thus, the possibility that pp65 released by infected cells might selectively impair NKp30-mediated activation should be also considered.

Further studies are required to characterize the molecular nature of the NKp30 and NKp46 ligands expressed by moDCs that were decreased at late stages post-infection (72 hours). It is of note that the loss of NCR ligand expression homogeneously affected all cells in TB40/E-treated cultures, including the noninfected cell fraction (IE1/2-negative) where class I expression was preserved. The mechanism underlying this effect, and in particular the putative role played by soluble factors produced by HCMV-infected cells, is currently being investigated.

NKG2A$^+$ NK cells degranulated more efficiently than the NKG2C$^+$ subset in response to infected moDCs, in which surface HLA-E expression was down-regulated. A mutation in p2 (Met/Val) of the TB40/E UL40 nonamer binding to HLA-E was detected, though did not account for its inability to preserve the class Ib molecule levels in HCMV infected moDCs, which was confirmed on infection with a clinical isolate bearing the canonical UL40 signal peptide sequence (VMAPRTLIL). Together, the data support that the ability of UL40 to stabilize HLA-E expression, as originally described in fibroblasts[13], is inefficient to preserve the class Ib molecule surface levels in moDCs and to prevent NKG2A$^+$ NK cell activation. Further studies are required to evaluate the impact that the UL40 mutation may have on HLA-E expression by HCMV-infected fibroblasts, as well as on the response of NK cells and of the CTL subset reported to specifically recognize the class Ib molecule through

the TcR[47,48] ; eventually, this might allow to understand the basis for the selection of the mutation in TB40/E.

Compared with NKG2A$^+$ cells, the NKG2C$^+$ NK cell subset includes higher proportions of KIR$^+$ and LILRB1$^+$ cells, and bears lower surface levels of NKp30 and NKp46 NCR[14]. This might explain the lower response of CD94/NKG2C$^+$ cells against HCMV-infected moDCs, also reported in fibroblasts[49], that appears contradictory with their putative involvement in the response to the virus. Though the mechanism(s) underlying the late expansion of the NKG2C$^+$ NK cell subset in response to HCMV infection[15] remain unknown, the phenomenon is reminiscent of the expansion of circulating virus-specific specific CTL displaying a terminally-differentiated phenotype, often associated to NKR expression, which exhibit reduced effector functions against virus-infected cells[50].

In conclusion, our results support that human NK cells are capable of effectively counteracting viral immune evasion strategies and responding to infected moDCs that have impaired their antigen presenting functions, thus indirectly favouring the development of adaptive immune responses to viral antigens cross-presented by healthy DCs.

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AUTHORSHIP CONTRIBUTION

GM designed and performed experiments, analyzed results, and wrote the manuscript; ML-B designed the research, analyzed results and wrote the manuscript; AMu and AA analysed and discussed results; NR and AS-B helped in the experimental work; DP, AMo, DEG and HH provided essential reagents and scientific advice.

CONFLICT OF INTEREST DISCLOSURE

AMo is founder and shareholder of Innate-Pharma. The remaining authors declare no competing financial interests.

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Chapter IV

Supplemental results

This section contains supplemental unpublished results that will be further commented in the discussion

1. The down-regulation of PVR and Nectin-2 in HCMV infected DC is abrogated when UL141 is missing

In order to clarify the role of HCMV UL141 glycoprotein in the down-modulation of DNAM-1L in HCMV infected moDC, a ΔUL141 TB40/E deletion mutant was used. This viral mutant (kindly provided by Christian Sinzger, Institute of Medical Virology, University of Tubingen, Germany, named from here on TB40/E-B) derives from the TB40/E bacterial artificial chromosome (BAC) that contains, besides the engineered deletion in the US1-US7 gene region, a spontaneous frameshift insertion in the UL141 gene, thus representing a naturally generated UL141 deletion mutant as it does not produce a functional gpUL141[287]. Immature moDC were incubated overnight either with complete medium (mock) or TB40/E-B at MOI 50 or 100. 72 hours later cells were surface labeled by indirect immunofluorescence with mAb specific for Nectin-2 and PVR. The rate of infection was determined by indirect immunofluorescence with mAbs specific for the HCMV IE-1/IE-2. As shown in Figure 1, in the absence of UL141, PVR and Nectin-2 were not anymore downregulated in infected cells, and their surface expression was even increased, compared to mock moDC.

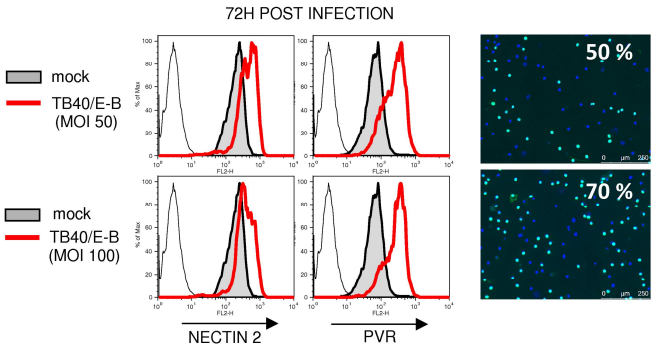

Figure 1. UL141 is necessary for DNAM-1L downregulation in moDC. Mock moDC (filled histograms) and TB40/E-B moDC (red line, open histograms) were surface labeled at 72h post infection by indirect immunofluorescence with Nectin-2 and PVR-specific mAbs. Staining with an isotype control is included (thin line, open histograms) and micrographs of corresponding IE-1/IE-2 staining in HCMV infected cells are reported (green, IE1-IE2; blue DAPI). In bold the % of IE-1/IE-2+ cells detected.

2. HCMV infection down-regulates DNAM-1 expression in moDC

DNAM-1 is reported to be expressed on cells of myeloid origin, including monocytes and moDC[152]. In order to verify if DNAM-1 surface expression is modulated during HCMV infection, immature moDC were incubated overnight with complete medium (mock) or UV TB40/E or TB40/E at MOI 100. 72 hours later cells were surface labeled by indirect immunofluorescence with mAb specific for MHC class I (clone HP-1F7) and DNAM-1 (clone F5). The rate of infection was determined by indirect immunofluorescence with mAb specific for the HCMV IE-1/IE-2. Our results indicate that, similarly to DNAM-1L, the expression of DNAM-1 is strongly down-regulated in HCMV infected moDC, identified by the loss of HLA-class I expression (Figure 2).

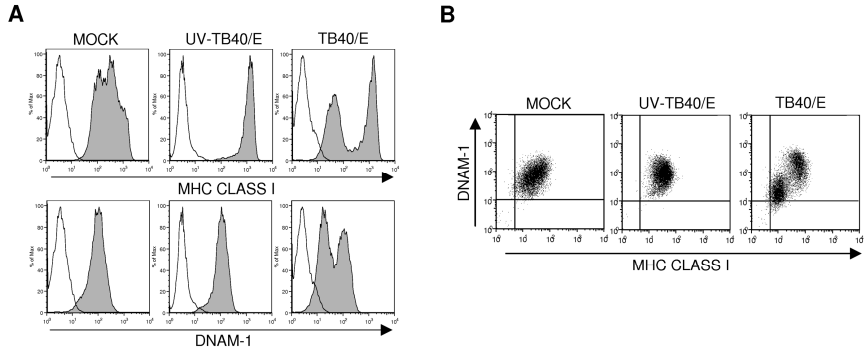

Figure 2. HCMV infection down-regulates DNAM-1 expression in moDC. A) MOCK moDC, UV-TB40/E moDC and TB40/E moDC. were surface labelled at 72h post infection by indirect immunofluorescence with mAbs specific for HLA-class I, or DNAM-1 (open histograms represent isotype control, and filled histograms, specific staining). Results of a representative experiment (60% IE-1/IE-2^+ cells) of 3 performed. **B)** mock moDC, UV-TB40/E moDC and TB40/E moDC at 72h post infection were double stained for FITC conjugated MHC class I and DNAM-1. Results of one representative donor are shown (60% IE-1/IE-2^+ cells)

3. The NK cell response against HCMV infected moDC measured 72 hours after infection is not antagonized by NKp30 and NKp46 mAbs

The surface expression of the ligand/s for NKp30 and NKp46 in moDC is decreased 72 hours after infection in TB40/E treated moDC cultures (Figure 6, Chapter III). In order to assess if the down-regulation of NCR ligands has an impact on the NK cell response, degranulation against infected moDC was measured by the CD107a mobilization assay, 72 hours after DC exposure to the virus, in the presence of NKp30 and NKp46 blocking mAbs as described previously. Our results indicated that an increase in the percentage of $CD107a^+$ NK cells was specifically detected in response to TB40/E infected moDC (mean: 5.6% $CD107a^+$ cells), compared to NK cells alone (mean: 0.5% $CD107a^+$ cells) and NK cells incubated with MOCK moDC (mean: 2.5% $CD107a^+$ cells). On the other hand, the NK cell response to infected moDC measured 72 hours after viral infection was significantly lower, compared to assays performed 48 hours post viral exposure (mean 14.6% CD107a+ cells, Figure 2D, Chapter III) and was not anymore antagonized by anti NKp46 mAbs.

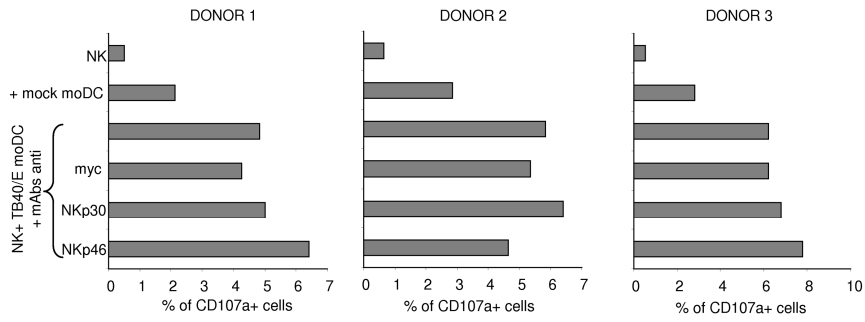

Figure 3. NK cell response against HCMV infected moDC 72 hours post viral exposure is not antagonized by NKp30 or NKp46 mAbs. NK cell degranulation against moDC was measured by the CD107 mobilization assay in the presence of blocking mAbs as described previously. Assays were performed 72 hours after DC exposure to the virus. Results of three experiments performed are shown.

4. NKp30 and NKp46 ligands are constitutively expressed on fibroblasts and are down-regulated at late stages of infection

To assess the expression of NCR ligands in HCMV-infected fibroblasts, the infection of the fetal human lung fibroblast cell line MRC-5 was established. MRC-5 cells were mock treated or infected with HCMV AD169 or TB40/E (MOI 1), and surface expression of NKp30L and NKp46L was analyzed 72 hours post virus exposure by indirect immunofluoresce and flow cytometry using soluble recombinant NKp30-Fc and NKp46-Fc fusion proteins as described in the "Material and methods" section, in chapter III. As shown in Figure 3, NKp30 and NKp46 ligands are constitutively expressed on the surface of MRC5 and their expression is markedly down-regulated upon HCMV infection, regardless of the viral strain used.

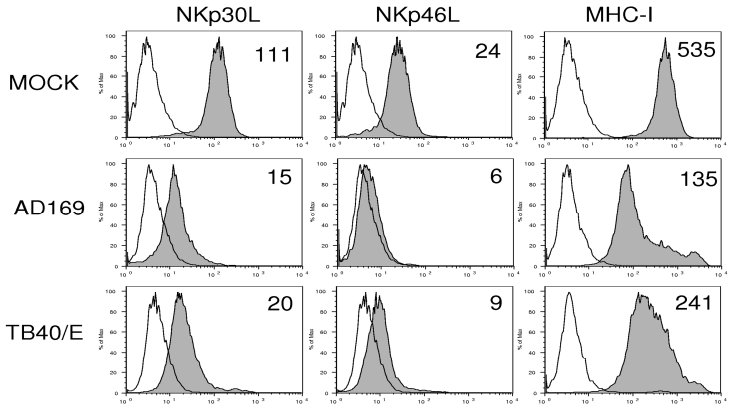

Figure 4. NKp30 and NKp46 ligands are constitutively expressed on MRC-5 cells and are down-regulated at late stages of HCMV infection. MRC-5 cells (ATCC CCL-171) grown in complete DMEM in 6-well plates at 70% of confluence were mock treated or infected with HCMV AD169 or TB40/E (MOI 1). Surface expression of NKp30L and NKp46L was analyzed 72 hours post virus exposure. Parallel staining with anti MHC-class I specific mAb is shown. Results of a representative experiment (100% IE1/IE2$^+$ cells) of 4 performed are shown.

5. Surface expression of NKp30 and NKp46 ligands in moDC decreases upon IFNβ1 treatment

To investigate whether type I interferons can affect NKp30 and NKp46 ligands expression in moDC, cells were left untreated or stimulated for 72 hours with 1200U/ml of rIFNβ1(Merck Serono). NKR ligands surface expression on moDC was analysed by indirect immunofluorescence and flow citometry using soluble recombinant NKp30-Fc and NKp46-Fc fusion proteins as described previously. As shown in Figure 4, treatment with IFNβ1 decreased NKR ligand surface expression, as observed upon HCMV infection.

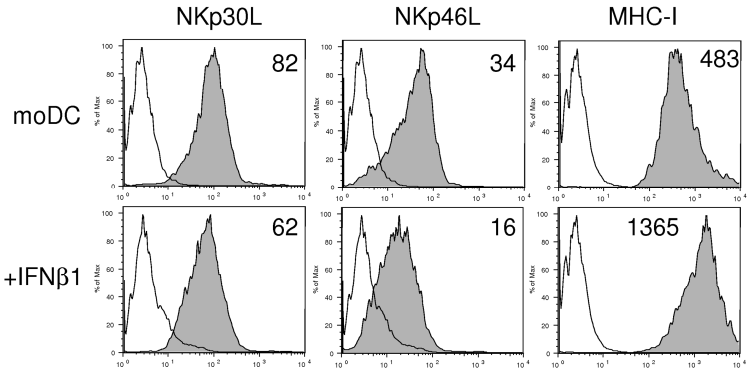

Figure 5. NKp30L and NKp46L surface expression on moDC decreases upon IFNβ1 treatment. NKp30 and NKp46 ligand expression on moDC left untreated or stimulated for 72h with 1200U/ml of IFNβ1 was assessed by indirect immunofluorescence and flow cytometry using soluble recombinant NKp30-Fc and NKp46-Fc fusion proteins. Parallel staining with anti MHC-class I specific mAb is shown. Results of an experiment of 2 performed are shown.

6. HLA-E expression is preserved in HCMV infected fibroblasts

We investigated whether HLA-E expression is down-regulated in HCMV infected fibroblasts along the viral cycle. MRC-5 cells were mock-treated or infected with HCMV AD169, TB40/E, or the UL1721 clinical isolate (MOI 1). At 48 hours and 72 hours after infection, HLA-E and total HLA class I surface expression was analysed by indirect immunofluoresce and flow cytometry using 3D12 and HP-1F7 mAbs, respectively. Interestingly, while MHC class I molecules were down-regulated, HLA-E surface expression did not decrease in fibroblasts infected with HCMV AD169, UL1271 or TB40/E. Indeed, a slight increase in HLA-E surface expression was observed in AD169 or UL1271, but not in TB40/E infected fibroblasts.

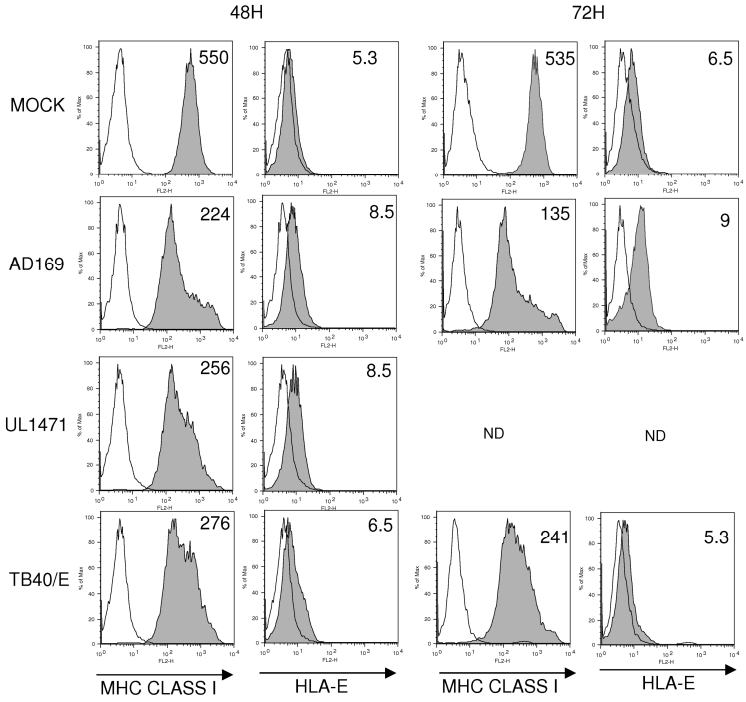

Figure 6. HLA-E expression in HCMV infected MRC-5. MHC-I and HLA-E expression on MRC-5 was assessed by indirect immunofluoresce and flow cytometry using specific mAbs as described previously. MRC-5 cells were either mock-treated or infected with HCMV AD169, TB40/E strains or the UL1271 clinical isolate. Surface expression of MHC class I and HLA-E was analyzed at 48 and 72 hours post virus exposure. Results of one representative experiment out of 3 performed are shown.

7. DC infected with the TB40/E-B mutant preserve MHC class I expression and are less sensitive to NK cells

Since the viral mutant derived from TB40/E BAC (TB40/E-B) lacks 3 genes responsible for MHC class I down-regulation (U*S2*, *US3* and *US6*)[287] it can be used to assess the involvement of inhibitory receptors in our system. MoDC were either mock-treated or infected with TB40/E or TB40/E-B (ΔUS1-US7). 48 hours post-infection cells were surface labeled by indirect immunofluorescence with MHC class I and HLA-E specific mAbs. As shown in Figure 6A, moDC infected with TB40/E-B preserved high surface levels of all HLA class I molecules, including HLA-E. To investigate whether moDC infected with US1-US7 deletion mutant are still able to trigger an NK cell response, the CD107a mobilization assay was employed. As shown in Figure 6B, the percentage of NK cells that degranulate against moDC infected with the viral mutant lacking *US2*, *US3* and *US6* genes is lower compared to that of NK cells responding to TB40/E infected moDC, despite their similar infection rate.

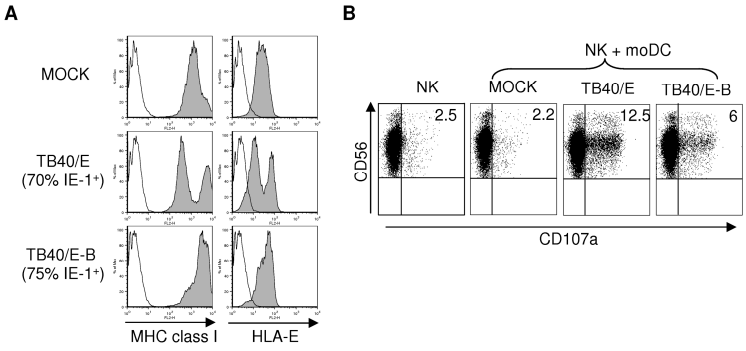

Figure 7. DC infected with TB40/E-B viral mutant preserve MHC class I molecules and are less sensitive to NK cells. A) Mock moDC, TB40/E and TB40/E-B infected moDC were surface labeled at 48 hours post infection by indirect immunofluorescence with MHC-I and HLA-E specific mAbs. Staining with isotype control is included (thin line, open histograms). **B)** NK cells purified by negative selection from PBMC stimulated overnight with IL-2 were co-cultured for 5 hours with autologous target cells. Surface CD107a expression in $CD56^+$ cells was analyzed by flow cytometry. Results of a representative experiment out of 3 performed.

Chapter V

Inhibition of NKG2D expression in NK cells by cytokines secreted in response to human cytomegalovirus infection

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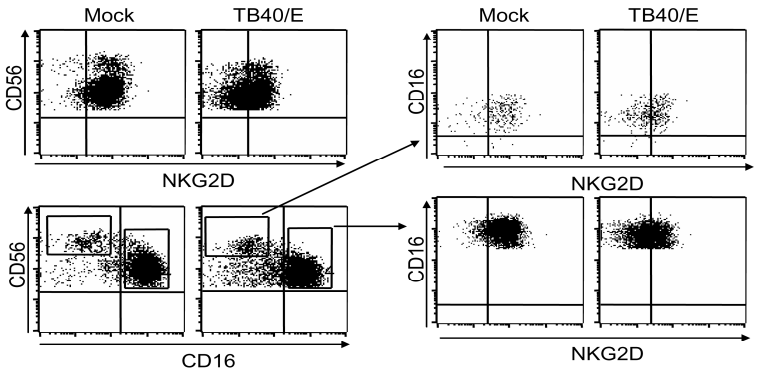
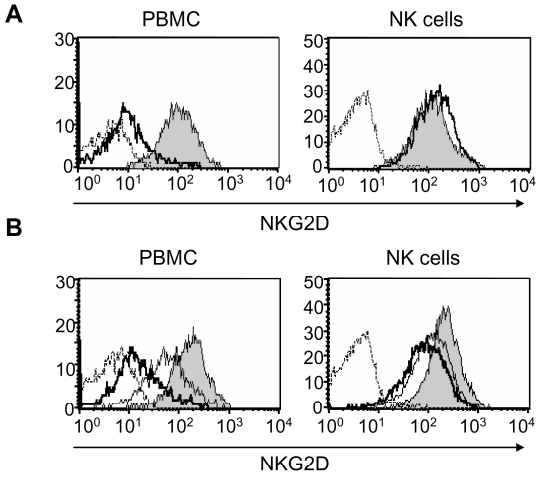
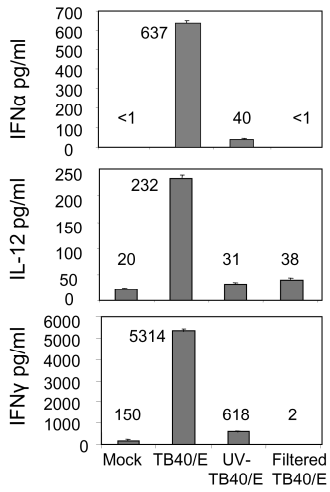


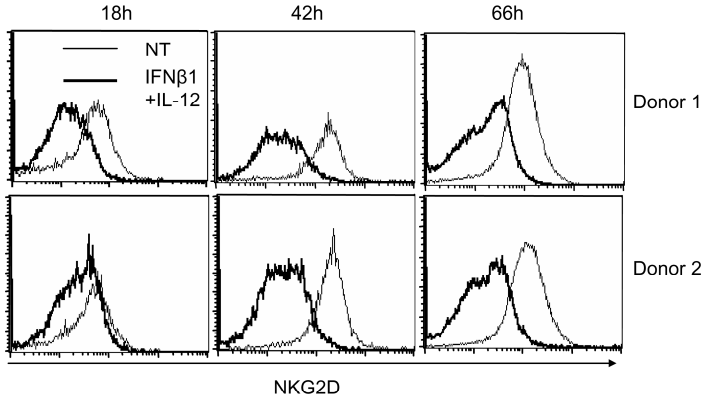
Supplementary Figure 1: NKG2D expression in CD56 bright and CD56 dull NK cells after exposure to TB40/E. PBMCs were cultured in the presence or absence of TB40/E for 3 days and NKG2D (1D11) and CD16 expression was monitored in a four color staining with specific mAbs in combination with CD3 and CD56 by flow cytometry. Data from one representative donor out of two tested is shown



Supplementary Figure 2:Soluble factors from HCMV-infected PBMC cultures induce NKG2D down-regulation. (A) PBMC or isolated NK cells from the same donor were incubated with TB40/E and surface NKG2D expression on CD56+CD3- cells was evaluated by FACS at 72h. Histograms correspond to the isotype controls (discontinuous lines), mock-treated (grey profiles) and TB40/E infected-samples (bold lines). Results corresponding to a sample from a representative donor out of four tested are shown. **(B)** Freshly isolated PBMC or purified NK cells from the same donor were incubated with either filtered or non-filtered supernatants obtained from PBMC co-cultured with HCMV for 2 days. NKG2D expression was assessed on CD56+CD3- cells after 72h. Histograms correspond to the isotype controls (discontinuous lines), NKG2D expression in samples treated with supernatants from mock-treated PBMC (grey profiles) and in samples treated with crude or filtered supernatants from TB40/E infected PBMC (bold lines and thin lines, respectively). Results correspond to a sample from a representative donor out of three tested.



Supplementary Figure 3: IFNα, IL-12 and IFNγ production in PBMC cultures treated with TB40/E. PBMC were cultured with TB40/E, UV-TB40/E, or the filtered TB40/E stock and the production of different cytokines was measured at 72h in the supernatants by ELISA. Results obtained in one representative donor out of three tested are shown.



Supplementary Figure 4: Kinetics of NKG2D down-regulation upon cytokine exposure Isolated NK cells were incubated in the presence of IL-12 (30ng/ml) and IFNβ1(1200U/ml) for the indicated time periods and surface NKG2D expression (1D11) on CD56+CD3- cells was monitored by FACS. Data from two independent donors is shown.

PART III
DISCUSSION AND CONCLUSIONS

Chapter VI

Discussion

The results presented in the first part of this thesis demonstrate that NK cells can efficiently react against autologous HCMV infected dendritic cells (DC), overcoming viral immune evasion mechanisms, and provide evidences that cytokines as well as cognate NK cell receptor-ligand interactions are involved in the activation of NK cell response.

Different strategies employed by CMV to escape NK cell surveillance have been characterized[227]. Yet, the nature of surface NK cell receptors involved in the response to infected cells in humans remains ill defined. Moreover, the majority of *in vitro* studies have been carried out with HCMV laboratory strains which have suffered a number of genetic changes and using infected fibroblasts that are not fully representative of the different cell types susceptible to *in vivo* infection. Among them, myeloid DC are believed to play a central role in the biology of HCMV infection. DC originate from $CD34^+$ myeloid precursors, which constitute a main site for viral latency, and are themselves susceptible to direct infection[210]. In addition, *in vivo* studies using mouse models have demonstrated that MCMV infected myeloid DC might regulate the innate immune response to the virus establishing a cross-talk with NK cells[189].

In order to characterize the NK cell response to HCMV infected DC an in vitro experimental system was established. To this end, monocyte derived immature DC (moDC) were infected with the endothelial tropic HCMV viral strain TB40/E and the response of autologous NK cells was analyzed at different levels. Compared to previous *in vitro* studies where allogeneic fibroblasts were used, our experimental setting allows to evaluate more precisely the contribution of the "missing self" in the NK cell response to infected cells.

Our results show that NK cell activation, detected by the induction of activation markers, IFN-γ production and NK cell degranulation was specifically triggered by HCMV infected moDC, in which HLA-class I

molecules were down-regulated, whereas a marginal or undetectable response was observed to mock or UV-TB40/E treated moDC.

Furthermore, our results indicate that activation of NK cell cytotoxicity was dependent on the phase of the viral cycle and more evident at early stages (48 hours post viral exposure); by contrast, partial protection from NK cell attack was observed during the late phase of infection (72 hours post infection), despite the persistent down-regulation of MHC class I molecules. These data highlight the importance of the kinetics of HCMV immune evasion strategies in the balance between NK cell recognition and HCMV escape and support that NK cells may respond to infected cells before the release of infectious virus that occurs in dendritic cells starting from day three post infection[223]. In this scenario NK cells would limit viral spread once natural infection is established, presumably controlling as well reactivation from latency in infected DC.

NKp46 and DNAM-1 were shown to play a dominant role in the recognition of HCMV infected moDC, without a detectable participation of NKp30 and NKG2D. Similarly, NKp46 and DNAM-1 also participate in the response to HCMV infected macrophages (Neus Romo, unpublished), pointing out the similarities in the NK cell response to infected cells of myeloid origin.

One of the principal targets of HCMV immune evasion is the NKG2D lectin-like receptor. HCMV infection is reported to lead to the up-regulation of NKG2D ligand (NKG2DL) transcripts, directly through viral proteins, such as HCMV IE-1 and IE-2, or indirectly through the induction of cytokines[98]. In order to avoid NKG2D mediated killing, several strategies are employed by HCMV to prevent surface expression of NKG2DL on infected cells (i.e. UL16, UL142, and miR UL112)[288]. Yet, expression of NKG2DL, particularly MICA and ULBP3, was reported on the surface of AD169 infected fibroblasts[274,275] and NKG2D-mediated NK and $CD8^+$ T cell responses have been described[274], thus suggesting a role of NKG2D in the

response to infected cells. In our experimental system, NKG2D was not involved in the recognition of infected cells. Indeed, surface expression of different NKG2DL, including MICA and ULBP3, was virtually undetectable on infected cells, along the different phases of the viral cycle, reflecting the effectiveness of the immune evasion strategies targeting NKG2DL in dendritic cells. The discrepancy between our and previous results is presumably due to the fact that HCMV clinical isolates as well as TB40/E, but not AD169, encode for an additional immunoevasin, UL142, involved in intracellular sequestration of MICA[280,281] and ULBP3[282]. Accordingly, TB40/E infected moDC would express all known viral molecules that have been shown to prevent NKG2DL surface expression, thus explaining the lack of involvement of this lectin-like receptor in our experimental system. On the other hand, differences in NKG2DL modulation between HCMV infected fibroblasts and DC should be also considered. Further studies using HCMV deletion mutants may help to clarify the specific role of the different viral proteins in the regulation of NKG2DL surface expression in HCMV infected DC.

Based on the antagonistic effect of a specific mAb, DNAM-1 appeared involved in the recognition of HCMV infected DC. The ligands of DNAM-1, PVR and Nectin-2, are widely expressed on normal cells including neuronal, epithelial, endothelial, myeloid and fibroblastic cells[155,161]. It has been reported that the infection of fibroblasts with HCMV viral strains, bearing an intact UL/b' region, decreased the expression of DNAM-1 ligands, thus interfering with NK cell mediated recognition. The early-late glycoprotein UL141, encoded in this region, has been reported to sequester PVR in the endoplasmic reticulum, preventing its surface expression[285]. In a recent paper, it has been shown that UL141 is also necessary for the retention of Nectin-2 in infected fibroblasts, however additional unknown viral mechanisms were required to prevent its expression[286].

Flow cytometry analysis of TB40/E infected moDC confirmed that at 72 hours post infection, but not at 48 hours, PVR and, particularly, Nectin-2 were down-regulated. According to Tomasec et al[285], TB40E contained two viral species: a virus carrying an intact copy of the UL141 gene (TB40/E *lisa*), and a natural mutant that contains a frameshift insertion in UL141 gene (TB40/E *bart*). The viral mutant reconstituted from the TB40/E genome cloned into a BAC (named here TB40/E-B), resembles TB40/E *bart* as no functional gpUL141 is produced and thus it can be considered as an UL141 deletion mutant[287]. Infection of moDC with TB40/E-B did not cause down-modulation of PVR and Nectin-2, indicating that UL141 glycoprotein is essential for mediating HCMV abrogation of both DNAM-1 ligands in myeloid cells (Figure 1, Chapter IV), similarly to what previously reported in HCMV infected fibroblasts[285, 286].

The levels of PVR and Nectin-2 expression in infected cells have profound effects on NK cell mediated recognition; indeed, DNAM-1 was found to participate in NK cell recognition of HCMV infected DC during the early phase of the viral cycle (48 hours post infection) but not during the late phase (72 hours post infection). Moreover, our results show that, paradoxically, at 72 hours after infection, anti DNAM-1L mAbs enhanced degranulation. This phenomenon might be explained by the involvement of TIGIT, a recently identified inhibitory receptor expressed by NK cells and T cells which binds with high affinity the DNAM-1 ligand, PVR [164,165]. The reduced surface expression of PVR and, particularly, of Nectin-2 on HCMV infected cells, during the late phase of infection, would prevent DNAM-1 recognition by NK cells, still permitting PVR binding to TIGIT. In that way, a single viral immunoevasin would modulate NK cell functions at two complementary levels, by impairing the function of an activating receptor while keeping engaged the inhibitory counterpart (Figure 8). Further studies using anti TIGIT blocking mAb are required to verify this hypothesis.

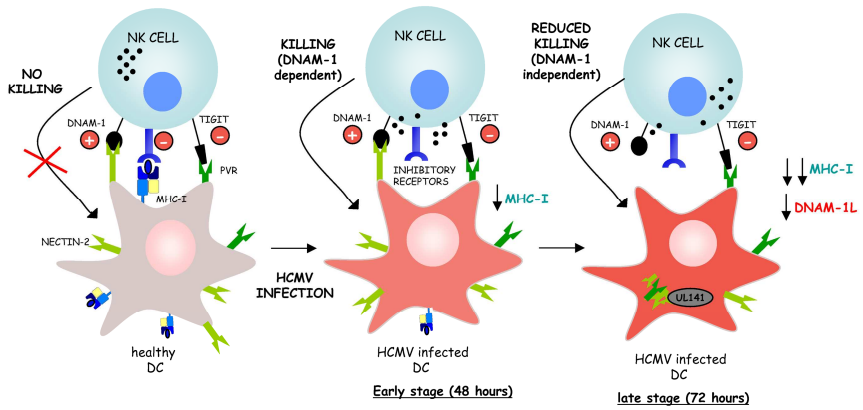


Figure 8. Model of DNAM-1-ligand interaction during HCMV infection

HCMV suppression of PVR and Nectin-2 may have consequences extending beyond the regulation of NK cell function. Nectin-2 and PVR have been described as cell adhesion molecules[155]. HCMV infection is recognized to disrupt focal adhesions and intercellular connections, by suppressing the expression of several cellular proteins involved in these processes[289]. Thus, the down-modulation of Nectin-2 and PVR on the surface of infected cells might influence cell adhesion as well.

On the other hand, DNAM-1 is reported to be expressed on cells of myeloid origin, including monocytes and moDC. In monocytes, DNAM-1 interacts directly with endothelial PVR and promotes monocyte transendothelial migration by acting specifically during the diapedesis step[156]. Infection of moDC also induced a marked down-regulation of DNAM-1 (Figure 2, Chapter IV) that might influence transendothelial migration of infected cells. Further studies are required to explore this issue.

NKp46 was the other activating receptor that clearly participated in the response of NK cells to HCMV infected DC, whereas NKp30 did not seem to play any role. The dominant role of NKp46 in the response to HCMV-infected moDC was in contrast to its lack of participation in the lysis of

immature moDC by IL-2 activated NK cells, that is mainly mediated by DNAM-1 and NKp30[143,161], suggesting that the expression of NCR ligands might be regulated during infection.

NKp46 was originally reported to directly recognize influenza hemagglutinins[135], and to contribute with NKG2D to the activation of the NK cell response against influenza infected moDC[107]. By contrast, no HCMV viral protein has been shown to interact with NKp46 and the nature of its cellular ligands remains unknown. On the other hand, NKp30, beyond its role in NK cell mediated killing of several tumor cells, has been reported to participate in the cross-talk between activated NK cells and immature moDC[143,144]. Although it has been recently shown that the B7 family member B7-H6 is a cell ligand of NKp30[147], the molecular basis of NKp30 mediated recognition of immature DC remains still uncertain, as B7-H6 is not expressed on myeloid DC[147].

Considering the lack of information regarding the specific molecular ligands of these NCR on myeloid cells, the analysis of their expression has been assessed using soluble NCR-Fc fusion proteins. Our results provide the first unequivocal evidence that the ligands for NKp30 and NKp46 are constitutively expressed on the surface of immature DC and indicate that their expression is maintained in the first 48 hours of infection, whereas, a marked decrease in their surface expression was observed at later stages, in particular for NKp46 ligand. The loss of NKp46 ligand expression at 72 hours post infection appears to have an impact on the NK cell response as it was not anymore antagonized by an anti NKp46 mAbs (Figure 3, Chapter IV). Moreover, results obtained in HCMV infected fibroblasts indicate that the decrease of surface expression of NKp46 and NKp30 ligands is not an exclusive feature of HCMV infected moDC, as a similar process occurs also in this cell type, regardless of the viral strain used (Figure 4, Chapter IV).

It is of note that the decrease of surface expression of NKR ligands homogenously affects all moDC in TB40/E treated cultures, including

infected and noninfected cells, whereas down-regulation of DNAM-1L or HLA-I was selective and limited to infected moDC. It is conceivable that soluble factors secreted in response to HCMV infection could play a role in this phenomenon. Indeed, preliminary results suggest that IFNβ1 can down-modulate the expression of NKp30 and NKp46 ligands on the surface of moDC (Figure 5, Chapter IV). This process would not represent a viral immune evasion strategy but a regulation mechanism aimed to protect bystander not infected cells from NK cell mediated killing. Previous functional studies indirectly suggested that the ligands for NCR are expressed on normal cells and their expression might be modified by cellular stress, infection or tumor transformation[142]. Thus, our results provide novel insights in the regulation of NKp30 and NKp46 ligand expression that may help to characterize these unknown molecules.

According to the analysis of NCR ligand expression, the dominant role of NKp46, as well as the lack of involvement of NKp30, could not be simply explained by a modulation of the expression of the corresponding ligands in infected cells. Indeed, our data suggest that the function of these NCR may depend on the level of HLA expression on target cells as well as on the NK cell metabolic status. In this regard, it is of note that resting NK cells do not kill immature DC that express normal levels of HLA class I molecules and IL-2 activation is necessary to trigger their function[143]. Activation of NK cells with IL-2 induces an increase of NKp30 surface density which correlates with the acquired capacity of killing immature DC. On the other hand, NKp46 is highly expressed on resting NK cells and may be the dominant killing receptor when a target cell down-modulates the expression of HLA class I molecules (i.e. HCMV infected DC). An alternative explanation would be that qualitative changes occur during infection affecting the NKR-ligand interactions, increasing the affinity of NKp46 for its ligand and/or inhibiting NKp30 activity. Interestingly, Arnon et al reported that HCMV tegument protein pp65 (UL83) binds to NKp30 and

inhibits NKp30 mediated lysis of several target cells, included immature dendritic cells, by dissociating the adaptor molecule CD3ζ from the NKR[148]. Although it is unlikely that release of pp65 occurs early in the viral cycle, this event should be also considered.

In our experimental system, activation of NK cells appeared mainly dependent on the reduced expression of HLA class I molecules on infected moDC. According to the missing self hypothesis, down-regulation of MHC class I molecules would determine a loss of inhibitory signals, contributing to the NK cell response to infected cells. On the other hand, to counteract this vulnerability, HCMV has developed different strategies that allow maintain inhibitory receptors for HLA class I molecules engaged. The UL18 glycoprotein binds with high affinity to the LILRB1 inhibitory receptors[57] thought formal evidences for its function in immune evasion are still missing[262]. In the same line, a leader signal peptide of the UL40 HCMV protein was shown to stabilize HLA-E surface expression in HLA-E transfected fibroblasts, thus repressing NK cell activation by engagement of CD94/NKG2A inhibitory receptor[83]. Artificial over-expression systems do not necessarily reflect the natural HLA-E levels and, therefore, may not allow predictions with respect to the complex regulatory processes occurring during natural infection. Compared to human fibroblasts, moDC express constitutively higher levels of HLA-E and allow to monitor the direct effect of HCMV infection on natural HLA-E expression. Unexpectedly, a down-regulation of HLA-E molecules was detected in TB40/E infected moDC compared to mock and UV-TB40/E moDC, paralleling the decreased expression of HLA-class I molecules. Gene sequence analysis revealed that TB40/E bears a point mutation in the leader peptide nonamer of UL40 reported to bind to HLA-E. Nevertheless, this mutation did not simply account for the down-regulation of HLA-E expression in HCMV infected moDC, as it was also observed upon infection with a clinical isolate (UL1271) bearing the canonical UL40 signal peptide sequence. Thus, HLA-E

expression was analysed in HCMV infected fibroblasts using the same HLA-E specific mAb, 3D12. Interestingly, while MHC class I molecules were down-regulated, the low HLA-E expression levels did not decrease in fibroblasts infected with HCMV AD169, UL1271 or TB40/E. Indeed, a slight increase in the HLA-E surface expression was observed in AD169 or UL1271, but not in TB40/E infected cells(Figure 6, Chapter IV), suggesting a participation of UL40 in this phenomenon and confirming previous reports[83].

Altogether these results highlight clear differences in HLA-E regulation between HCMV infected moDC and fibroblasts. It is conceivable that differences in HLA-E basal expression as well as in HLA-E recycling kinetics may account for this discrepancy. Variations in expression levels of viral proteins involved in the regulation of classical and non classical MHC class I molecules should be also taken in account.

In HCMV infected fibroblasts, UL-40 mediated HLA-E stabilization was described to protect infected cells from lysis by NK cells bearing the CD94/NKG2A inhibitory receptor[83,254]. It is of note that inhibitory and activating receptors specific for HLA class I molecules (i.e. KIR, CD94/NKG2, LILRB1) are not homogeneously distributed in peripheral blood NK cells and the variegated distribution of these receptors results in the generation of phenotypically and functionally distinct NK cell subsets[268]. Particularly, CD94/NKG2A and CD94/NKG2C receptors are generally displayed by different subsets of human NK cells. A comparative analysis of these two NK cell subsets indicated that $NKG2A^+$ NK cells degranulated more efficiently than $NKG2C^+$ cells upon interaction with HCMV-infected moDC, whereas both comparably reacted against the HLA-defective K562 leukemia cell line, suggesting that the decrease of HLA-E expression in infected cells released NK cells from the control by the NKG2A inhibitory receptor .

To further confirm an involvement of inhibitory receptors in our system, we infected moDC with a viral mutant (TB40/E-B) lacking 3 genes responsible of MHC class I down-regulation (i.e.) *US2*, *US3* and *US6*. Among them, *US6* has been shown to down-regulate the expression of HLA-E, by interfering with peptide loading through binding to TAP[238]. Infection of moDC with this mutant did not decrease the surface expression of HLA class I molecules, including HLA-E. Moreover moDC infected with TB40/E-B appeared less sensitive to NK cells compared to TB40/E wild type infected cells (Figure 7, Chapter IV), in line with a previous report by Falk et al. in HCMV infected fibroblasts[252].

Altogether our results support that the ability of UL40 to stabilize HLA-E expression, as originally described in fibroblasts, is inefficient to preserve the class Ib molecule surface levels in moDC and to prevent NKG2A$^+$ NK cell activation. On the other hand, a subset of CTL has been described to recognize peptides in an HLA-E restricted fashion[81] and to efficiently kill CMV infected fibroblasts by recognizing the HLA-E presented UL40 derived peptide[290,291]. Pietra and colleagues reported that autologous DC pulsed with the UL40 derived viral peptide became susceptible to lysis by this CTL subset[292]. Thus, the down-modulation of HLA-E expression observed in HCMV infected moDC might inhibit the recognition of infected DC by this CTL subset.

Additionally, the less efficient response of NKG2C$^+$ NK cells, as compared to NKG2A$^+$ cells, also observed in fibroblasts (Petersen et al[293] and data not shown) might be due to differences in their NK cell receptor expression pattern. In fact, previous observations indicate that the NKG2C$^+$ NK cell subset includes higher proportions of KIR$^+$ and LILRB1$^+$ cells, bearing lower surface levels of NKp30 and NKp46 NCR[268], the latter playing a dominant role in the response to infected moDC.

On the other hand, the lower response of CD94/NKG2C$^+$ cells is apparently contradictory with previous observations indicating a putative

anti-viral role of this cell subset. Indeed, our group has shown that the percentage of NK cells bearing the activating CD94/NKG2C receptor tends to increase in peripheral blood from HCMV$^+$ individuals[268] and furthermore NKG2C$^+$ NK cells may expand upon in vitro co-culture of PBMC with HCMV-infected fibroblasts[273]. Moreover, a CD94/NKG2C$^+$ NK lymphocytosis was detected in a patient with a selective T cell deficiency, coinciding with an acute HCMV infection and was associated to a reduction of viremia[251]. Altogether these observations suggest that NKG2C might interact with a specific ligand expressed in infected cells promoting the late expansion of cells bearing this NKR; yet, the mechanisms underlying the effect are still undefined. Several indications support that the NKG2C$^+$ subset is heterogeneous in terms of its replication and killing capacity. Experiments with CFSE labeled PBL indicated that only a fraction of CD94/NKG2C$^+$ cells proliferate in response to HCMV infected fibroblasts and the expansion was not perceived in PBL from a group of HCMV$^+$ donors that displayed lower proportions of NKG2C$^+$ cells[273]. Moreover, two populations displaying a different NKG2C staining intensity have been observed in HCMV$^+$ donors[268]. In our experimental system a reduced cytotoxic response was perceived only in the NKG2C^{bright} subset bearing lower NKp46 surface levels but not in the NKG2C^{dim} population (data not shown). We hypothesize that a small NKG2C$^+$ proliferating "progenitor" pool accounts for the expansion observed in response to HCMV infected fibroblasts and the anti viral effect. By contrast, the majority of CD94/NKG2C$^+$ NK cells, resulting of previous HCMV induced expansion *in vivo,* may correspond to terminally differentiated cells, associated to reduced effector functions against virus infected cells, as previously shown for virus-specific cytotoxic T lymphocytes[294].

Attempts to verify this hypothesis in our experimental system were unsuccessful, and no expansion of CD94/NKG2C$^+$ NK cells occurred in response to HCMV infected DC. In fact, CD3$^+$CD8$^+$ T cells expanded

presumably due to the ability of non-infected moDC of cross-presenting HCMV antigens to memory T cells in HCMV$^+$ donors. Moreover an increased expression of CD94/NKG2A in NK cells was noted. In this regard, complementary studies revealed that secretion of IL-12 by HCMV infected dendritic cells induced NKG2A expression on NKG2C$^+$ NK cell clones, providing a reversible regulatory feedback mechanism to control the activation of NKG2C$^+$ cells[66]. This process might interfere with the response of NKG2C$^+$ cells to HCMV infected DC, preventing their expansion.

Studies in experimental MCMV infection revealed that DC-derived cytokines regulate NK cell activation in response to the virus *in vivo*[247]. Moreover, type I IFN and IL-12 were shown to partially contribute to stimulate the human NK cell response to influenza-infected moDC[107]. Evidences supporting that cytokines also contribute to the NK cell response to HCMV-infected moDC have been obtained also in our experimental system. In particular, up-regulation of CD69 surface expression and secretion of IFN-γ observed upon co-culture of freshly purified NK cells with autologous HCMV infected moDC were dependent on type I interferons and IL-12 respectively, thus indicating that, as predicted, cytokines complement the signals triggered by NKR and NCR contributing to promote an optimal antiviral response.

Considering that the infection rate of moDC varied from 40% to 90% an obvious question that arises from these results is whether type I interferons and IL-12 are produced by HCMV infected DC and/or by bystander uninfected DC. Type I interferons have been described to be induced early upon MCMV infection in mice[247] and upon exposure to HCMV in human fibroblasts[295] and dendritic cells[225]; IL-12 produced by MCMV infected CD11b$^+$ DC has been described as a key cytokine in the promotion of NK cell mediated IFN-γ secretion in mice[189]. In our experimental settings, significant amount of IFN-α and IL-12 were detected in the supernatant of HCMV infected DC but not in samples recovered from moDC treated with UV inactivated virus (data not shown). Yet, studies are in progress to more

precisely define whether non-infected moDC may also contribute to cytokine production.

Altogether, our results indicate that cytokines produced upon HCMV infection activate NK cells. On the other hand, several evidences suggested that these soluble factors could also modulate the expression of NK cell receptors on NK cells, thus providing a feedback regulatory mechanism to control NK cell reactivity against normal bystander cells. IL-12 secretion by HCMV infected moDC has been shown to up-regulate the expression of the NKG2A inhibitory receptor in the $NKG2C^+$ population during the innate immune response to HCMV infection, thus likely contributing to prevent killing of activated uninfected cells[66]. Indeed, up-regulation of HLA-E expression upon virus exposure has been reported in our experimental system in healthy cells, consistent with their response to endogenous type I interferons.

Additionally, type I interferons and IL-12 inhibited transiently the expression and function of the activating NK cell receptor NKG2D during HCMV infection. When HCMV infected moDC were cultured with autologous PBMC a marked and transient down-regulation of NKG2D expression was observed in NK cells, but not in $CD8^+$ $NKG2D^+$ T cells. This down-regulation was associated to NK cell activation, as shown by increased surface expression of CD69 and CD25, as well as IFN-γ secretion, and was dependent on soluble factors released by HCMV infected DC (data not shown). In an attempt to characterize the mechanism underlying this phenomenon we found that treatment of PBMC with TB40/E infective viral particles induced a comparable down-modulation of NKG2D expression in NK cells, associated to decreased levels of NKG2D and DAP10 mRNA. This effect was dependent on the presence of HCMV infected cells, was observed in NK cells but not in most T lymphocytes, and was associated to cell activation, indicating that inhibition of NKG2D expression was dependent on sensing of virus and active HCMV infection.

Type I interferons and IL-12 secreted upon HCMV infection by both pDC and myelomonocytic cells were shown to be required for inhibiting surface NKG2D expression, by acting through different mechanisms. Particularly, IL-12 alone decreased NKG2D mRNA expression without altering cell surface expression, whereas type I interferons reduced surface NKG2D but not mRNA levels, through a still unknown mechanism. Together both cytokines exerted a synergistic effect and a significant down-regulation of NKG2D surface expression was observed. Down-modulation of NKG2D expression was associated to reduced NK cell cytotoxicity against target cells expressing NKG2D ligands, without affecting the function of other triggering receptors. In particular, NKG2D down-regulation did not impair NK cell mediated cytotoxicity triggered by NKp46 that plays a dominant role in the response to HCMV infected myelomonocytic cells.

Considering the lack of involvement of NKG2D in the killing of HCMV infected cells, the observed phenomenon might represent a physiological regulatory process to prevent the response of activated NK cells against bystander uninfected cells that may display NKG2D ligands along the course of inflammatory responses.

NKG2D has been originally described as an important activating receptor involved in the NK cell response against tumor and viral infected cells where the expression of its specific ligands is induced[86]. On the other hand, a participation of NKG2D in the cross-talk between healthy immune cells has been recently reported in several studies. It has been shown that NKG2DL can be up-regulated on immune cells upon activation making them susceptible to NKG2D mediated NK cell lysis. In macrophages, LPS has been shown to up-regulate both transcription and surface expression of ULBP1-3 and surface expression of constitutively transcribed MICA[114]. NKG2DL are also upregulated in dendritic cells by TLR stimulation[115] and Type I interferon treatment[113]. Interestingly, T cell stimulation has been shown to induce NKG2DL expression and to promote autologous NK-

cell–mediated lysis via a mechanism dependent on NKG2D[111,112]. In our experimental system, treatment of PBMC with HCMV infective viral particles was associated to increased surface expression of several NKG2D ligands, mainly MICA and MICB, in monocytes, supporting previous observations. Considering the low rate of infection, it is plausible that this phenomenon was due to cell activation rather than an intrinsic consequence of cell infection.

Sustained expression of NKG2DL, as well as shedding of the corresponding soluble forms has been described to induce internalization and degradation of NKG2D in NK cells[116]. In our experimental system the up-regulation of NKG2DL does not seem to be the main cause of the modulation of NKG2D expression as blocking mAbs against MICA, MICB and ULBP3 did not prevent the loss of NKG2D expression and no soluble MICA was found in the supernatants.

It is conceivable that, in response to HCMV infection, different danger signals simultaneously induce the expression of NKG2DL on activated immune cells and the release of cytokines inhibiting NKG2D expression in NK cells. In this scenario, the modulation of NKG2D expression induced by type I interferons and IL-12, would prevent NK cell killing of other activated cells. Indeed, the possibility that soluble factors could modulate NKG2D expression has been considered in previous studies[118-121]. Yet, this is the first original report describing that cytokines with an antiviral function regulate NK cell function through the inhibition of NKG2D expression.

Altogether our results support that human NK cells are capable of counteracting viral immune evasion strategies early upon infection, and responding to HCMV infected autologous moDC that have impaired their antigen presenting function thus favouring the development of adaptive immune responses to viral antigens cross-presented by healthy DC. Additionally, our results indicate that type I interferons and IL-12, beyond their role as antiviral cytokines, may act in concert to dampen NK cell

activation, by inhibiting expression of the activating receptor NKG2D and by inducing expression of inhibitory receptor NKG2A, thus providing reversible regulatory feedback mechanisms in the context of inflammatory responses (Figure 9). Preliminary results also suggest that prolonged exposure to type I interferons might regulate the NK cell response by decreasing NCR ligands expression, thus protecting bystander cells from NCR -mediated attack.

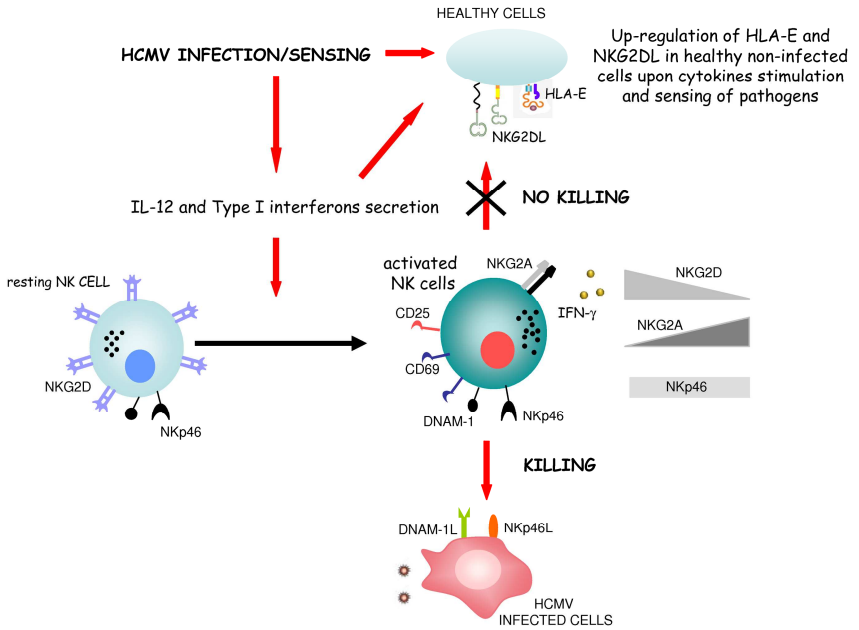


Figure 9. Model of cytokine-mediated regulation of NKR surface expression during HCMV infection

In conclusion, our data reveal a complex scenario where the stage of infection, the nature of activating and inhibitory receptors expressed by NK cells, the NK cell metabolic status as well as antiviral cytokines contribute to determine the magnitude of the NK cell response to HCMV.

Chapter VII

Conclusions

1. HCMV infected moDC downregulate HLA class I molecules and specifically activate autologous NK cell functions, triggering IFN-γ production and cytotoxicity.

2. NKp46 and DNAM-1 NK cell receptors play a dominant role in NK cell mediated recognition of HCMV-infected moDC, without a detectable participation of NKp30 and NKG2D.

3. NKG2D ligands are undetectable on the surface of HCMV infected moDC, reflecting the effectiveness of viral immune evasion mechanisms targeting this recognition system.

4. DNAM-1 participates in NK cell recognition of HCMV infected moDC at an early stage of infection whereas the effect of HCMV mediated downregulation of DNAM-1 ligands on moDC is perceived later.

5. Ligands for NKp30 and NKp46 NCR are constitutively expressed by moDC and decrease at late stages of infection in both infected and non-infected cells.

6. HLA-E expression is downregulated in HCMV-infected moDC and does not prevent $NKG2A^+$ NK cell activation.

7. Type I interferons and IL-12 secreted in response to HCMV infection contribute to activate the NK cell response against infected moDC.

8. HCMV infection of PBMC transiently inhibits the expression and function of NKG2D in NK cells, preserving other activation pathways.

9. The loss of NKG2D expression in NK cells is dependent on a synergistic effect exerted by type I interferons and IL-12.

10. The inhibition of NKG2D expression may represent a physiological regulatory process to dampen NK cell reactivity against normal bystander cells that may display NKG2D ligands along the course of inflammatory responses.

11. Our results provide novel insights on the involvement of receptor-ligand interactions and cytokines in the NK cell response to HCMV infection, stressing the importance of the dynamics of viral immune evasion mechanisms.

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ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen presenting cells
BAC	Bacterial artificial cromosome
CFSE	Carboxyfluorescein succimidyl ester
CTL	Cytotoxic Lymphocytes
DAP10	10kDa DNAX adaptor protein
DAP12	12kDa DNAX adaptor protein
DNAM-1	DNAX accessory molecule 1
DNAM-1L	DNAM-1 ligands
EC	Endothelial cell
ER	Endoplasmic reticulum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft-versus-host disease
HA	Hemagglutinin
HCMV	Human cytomegalovirus
HLA	Human leukocyte antigen
IE	Immediate early
IFN	Interferon
IFNAR	Interferon receptor chain 2
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
IL	Interleukin
ILT	Ig-like transcripts
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer Immunoglobulin-like Receptors
KLR	Killer cell lectin-like receptor
LIL	Leukocyte Immunoglobulin-Like Receptors
LRC	Leukocyte receptor complex

mAb	Monoclonal antibody
MCMV	Murine Cytomegalovirus
mDC	Myeloid DC
MHC	Major histocompatibility complex
MIC	MHC class I chain related
miRNA	micro RNA
moDC	Monocyte derived dendritic cells
NCR	Natural cytotoxicity receptors
NK	Natural killer
NKG2	Natural killer group 2
NKG2DL	NKG2D ligands
NKR	Natural killer cell receptors
ORF	Open reading frames
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid DC
PI3K	Phosphotidylinositol 3-kinase
PRR	Pattern recognition receptors
PVR	Poliovirus receptor
RAET1	Retinoic acid early transcript 1
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
UL	Unique long
ULBP	UL-16 binding protein
US	Unique short

PUBLICATIONS

1. **Magri G**, Muntasell A, Romo N, Sáez-Borderías A, Pende D, Geraghty DE, Hengel H, Angulo A, Moretta A, López-Botet M. NKp46 and DNAM-1 NK cell receptors drive the response to Human cytomegalovirus infected myeloid dendritic cells overcoming viral immune evasion strategies. Blood. 2011 Jan 20;117(3):848-56.

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