Interplay of human adaptive NKG2C^{+} NK cells with specific antibodies and T cells in the response to cytomegalovirus infection

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ABSTRACT-RESUMEN

ABSTRACT

Human cytomegalovirus (HCMV) infection induces persistent changes in the host immune system, namely a sustained expansion of effector memory and cytotoxic specific T lymphocytes, the production of specific antibodies (Ab) and, in some individuals, associates with the differentiation of a functionally competent Natural Killer (NK) cell subset with adaptive features, identified by the elevated expression of the activating receptor CD94/NKG2C. NK cells are considered important players in the defence against HCMV, yet the exact mechanisms by which these cells contribute to primary infection and viral reactivation control remain partially understood. In the present study, we have characterized three distinct mechanisms by which NK cells could participate in the defence to HCMV infection. On one hand, we have shown that NK cell direct sensing of HCMV particles leads to the priming of their anti-viral effector functions by mechanisms involving TLR2 and type I IFN. We have also shown that NK cells cooperate with specific antibodies in the recognition of HCMV-infected cells, overriding viral immunoevasion strategies from early to late stages of the lytic cycle. Of note, adaptive NKG2C+ NK cells were highly responsive to Ab-driven activation, being particularly efficient in the production of antiviral cytokines. Finally, we have also shown that the increased expression of HLA class II molecules in circulating adaptive NKG2C+ NK cells endows them with the capacity to present HCMV-derived antigens to specific CD4+ T

cells. Remarkably, CD4+ T cells activated in response to HLA-DR+ NK cells displayed an effector memory phenotype, lacked the co-stimulatory molecule CD28 and showed a cytotoxic and Th1 functional profile. Altogether, these results support that the interplay of human NK cells, particularly the adaptive NKG2C+ subset, with specific antibodies and T cells contributes to the defence against HCMV infection.

RESUMEN

La infección por el citomegalovirus humano (HCMV) induce cambios persistentes en el sistema inmunitario del huésped, consistentes en la expansión de linfocitos T citotóxicos específicos, la producción de anticuerpos específicos y, en algunos individuos, la diferenciación y expansión adaptativa de una subpoblación de células Natural Killer (NK), identificada por la elevada expresión del receptor activador CD94/NKG2C. Se considera que las células NK juegan un papel importante en la defensa contra el HCMV, aunque el conocimiento de los mecanismos con los que contribuyen al control de la infección primaria y de la reactivación viral es parcial. En este estudio, hemos caracterizado tres mecanismos mediados por células NK que pueden contribuir a la defensa frente la infección por HCMV. Por un lado, mostramos que el reconocimiento directo de partículas de HCMV incrementa sus funciones anti-virales mediante mecanismos dependientes de TLR2 e IFN de tipo I. Por otra parte, demostramos in vitro la importancia de la citotoxicidad dependiente de anticuerpos mediada por las células NK como mecanismo de respuesta frente a las células infectadas, superando los mecanismos virales de inmunoevasión. En comparación con la subpoblación NK NKG2A+, las células adaptativas NKG2Cbright mostraron una producción de citocinas anti-virales superior en respuesta a la activación mediada por anticuerpos. Finalmente, describimos como la expresión constitutiva de HLA-DR en células NK NKG2C+ adaptativas

circulantes les permite la presentación de antígenos del HCMV a linfocitos T CD4+ específicos con un fenotipo efector-memoria, carentes de la molécula co-estimuladora CD28 y con un perfil funcional Th1 y citotóxico. En conjunto, estos resultados apoyan que la interacción de las células NK, en especial la subpoblación NKG2C+ adaptativa, con anticuerpos y linfocitos T específicos contribuye a la defensa frente al HCMV.

PREFACE

PREFACE

Human Cytomegalovirus (HCMV) is a β herpesvirus that infects with high prevalence all human populations. Although HCMV infection generally follows a subclinical course in healthy hosts and the virus remains latent with occasional reactivations, it may have severe consequences in congenitally infected infants and immunocompromised individuals. An effective defense against HCMV requires the participation of T lymphocytes, specific antibodies and NK cells. HCMV infection promotes, to a variable extent in different individuals, a persistent expansion of an NK cell subset with adaptive traits characterized by high levels of the CD94/NKG2C receptor, together with other phenotypic and functional features. Among a number of open questions, little information is available about their direct interaction with HCMV particles and their interrelation with the anti-viral adaptive immunity. These questions have been addressed in the present work, providing novel insights on the immune mechanisms contributing to the defense against this viral infection.

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INTRODUCTION AND AIMS

CHAPTER 1: INTRODUCTION

1 Immunobiology of Natural Killer cells

1.1 NK cell distribution and characterization

Natural Killer cells (NK) were initially described by Rolf Kiessling, Hans Wigzell (Karolinska Institute, Sweden) and Ronald Herberman (NCI-NIH, USA) in 1975 as lymphocytes with large granular morphology with the ability to kill tumor cells *in vitro* without previous sensitization (1,2). NK cells are cytotoxic lymphocytes of the innate immune system that contribute to the control of microbial pathogens and tumors (3). In addition to their cytotoxic capacity, NK cells also secrete pro-inflammatory and anti-viral cytokines which regulate the adaptive immune response (3).

NK cells were the first innate lymphocytes described, followed by lymphoid tissue inducer (LTi) cells, which induce the development of lymph nodes and Peyer's patches. More recently, the discovery of innate lymphocytes with different functional capacities revealed a more complex family (4). Innate lymphoid cells (ILCs) are divided into cytotoxic ILCs (paralleling $CD8^+$ cytotoxic T cells), represented by NK cells, and non-cytotoxic ILCs (resembling to $CD4^+$ helper T cells) (5). ILCs are further classified into ILC1 (which include NK cells), ILC2 and ILC3 according to the cytokines they produce and the transcription factors that regulate their development (6).

T, B, NK cells and the rest of ILCs derive from a Common Lymphoid Progenitor (CLP), which differentiates to NK cell

Progenitor (NKP). Studies in mice have identified Common helper ILC precursors (ChILP) that give rise to group 1, 2 and 3 non-cytotoxic ILCs but not to NK cells. Eomesodermin (Eomes) and T-box transcription factor T-bet (shared with ILC1) (6) appear essential for NK cell differentiation.

NK cells develop in the bone marrow from $CD34^{+}$ progenitors. Mature NK cells localize in peripheral blood, spleen, liver, lung, gut as well as in uterus, particularly during pregnancy (7). NK cells comprise 5-25% of human peripheral blood lymphocytes but a minor fraction of lymph node lymphocytes. In humans, NK cells are phenotypically defined based on the lack of CD3 and expression of CD56, an isoform of the neural cell adhesion molecule 1 (NCAM-1). $CD56^{bright}$ NK cells are characterized by higher expression of CD56 and lack of the CD16A (FcγRIIIA) receptor. They represent a minor population in blood but are predominant in secondary lymphoid tissues. They have a low cytotoxic potential but produce pro-inflammatory cytokines when activated. In contrast, $CD56^{dim}$ NK cells express lower levels of CD56 but display CD16A, which allow them to respond against antibody-coated targets. This NK cell subset has a developed cytotoxic machinery and also produces cytokines when activated by target cell recognition.

$CD56^{bright}$ and $CD56^{dim}$ NK cell subsets also differ in their chemokine receptor profile, which determine their location in homeostasis and under pathological conditions. Resting $CD56^{bright}$ $CD16A^{-}$ NK cells express high levels of CCR5, CCR7 and CXCR3

but low or no levels of CXCR1, CXCR2 and CX3CR1, while $CD56^{dim}$ $CD16A^{+}$ express high levels of CXCR1, CX3CR1, lower levels of CXCR2 but very low or no levels of CC receptors. Both NK cell subsets express high levels of CXCR4 (8). Upon activation, NK cells up-regulate some chemokine receptors (9,10). The $CD56^{bright}$ subset is also characterized by the expression of CD62L, which mediates the interaction with high endothelial venules allowing migration to lymph nodes (8).

1.2 NK cell functions

Upon activation, NK cells kill their targets mainly through perforin and granzyme release, as well as through the expression of death receptor ligands (11). The main cytotoxic pathway consists of releasing by exocytosis the content of specialized secretory granules (granulosomes), including granzymes and perforin, a process called degranulation (12). Perforin subunits polymerize forming pores across the target cell membrane, disrupting the oncotic/osmotic balance and enabling the entry of granzymes into the cytoplasm (12). Granzymes belong to a family of serine proteases that are able to induce caspase-dependent and -independent apoptosis, leading to chromatin condensation and nuclear fragmentation (13). NK cells also express the death receptors Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) which are able to mediate caspase-dependent apoptosis in target cells (14).

NK cell activation also leads to the production of several soluble factors (15). NK cells are characterized for producing tumor necrosis factor α (TNFα) and interferon γ (IFNγ), which have pro-inflammatory and anti-viral functions, and different chemokines, namely macrophage inflammatory protein 1α (MIP-1α), MIP-1β, interleukin 8 (IL-8) and CCL5 (also known as regulated on activation, normal T cell expressed and secreted or RANTES) (16,17). Moreover, $CD56^{bright}$ NK cells have been reported to secrete granulocyte/macrophage colony-stimulating factor (GM-CSF).

In addition to direct effector functions, NK cells can regulate T cell responses through direct and indirect mechanisms. NK cells produce IFNγ, which has a direct effect on primary and memory $CD4^+$ priming towards Th1 cell differentiation (18). Moreover, a mice model of influenza infection showed that NK cell-dependent IFNγ production was essential for $CD8^+$ T cell recruitment to lymph nodes (19).

NK cells have also been described to lyse activated T cells (20–23), which up-regulate ligands for activating receptors on NK cells that may contribute to their susceptibility to NK cell-mediated lysis. Upon activation, $CD4^+$ and $CD8^+$ T cells up-regulate NKG2D ligands MHC class I polypeptide-related sequence A (MICA) and UL16 binding protein 1-3 (ULBP1-3) surface expression, becoming susceptible to killing by autologous IL-2 activated NK cells in a NKG2D-dependent fashion (20). The involvement of NKG2D in NK cell-mediated lysis of activated T cells is also supported in mice

by *in vitro* NKG2D-dependent NK cell lysis of activated T cells (21) and by *in vivo* data showing an increase of memory $CD8^+$ T cells following immunization in NK cell-depleted mice (24). NKG2D also mediate NK cell control of antigen-specific $CD4^+$ T cells in mice undergoing chronic graft-versus-host disease, involving T cell surface expression of Fas rather than on perforin-mediated lytic activity of NK cells (25). In addition to NKG2D, $CD56^{bright}$ NK cells expressing TRAIL were reported to induce apoptosis of activated T cells. $CD8^+$ T cells from patients chronically infected with hepatitis B virus (HBV) express higher levels of the TRAIL-R2 receptor, which renders T cells susceptible to NK cell-mediated elimination (22). While NK cells are able to negatively regulate T cell responses, they may also promote them by killing activated regulatory T cells (Treg), as it has been shown *in vitro* through NKG2D and NKp46 (23). In addition to activating receptors, inhibitory receptors also regulate NK cell-mediated T cell lysis. CD94/NKG2A in NK cells binds to human leukocyte antigen E (HLA-E), up-regulated on activated $CD4^+$ T cells, delivering inhibitory signaling and dampening NK cell cytotoxicity (26).

NK cell can also regulate adaptive immunity by interacting with dendritic cells (DCs), affecting their capacity to prime T cell responses. Cell-cell contact as well as NK cell-mediated secretion of IFNγ and TNFα promotes DC maturation, resulting in up-regulation of HLA class II, CD80, CD83 and CD86 (27); on the other hand, activated NK cells can eliminate immature DCs (28),

enhancing the T cell response by the consequent selection of activated, immunogenic DCs (29).

1.3 Regulation of NK cell functions

The “missing-self hypothesis” was postulated by Klas Kärre in 1986 (30), showing that NK lymphocytes were able to kill cells lacking self MHC class I surface molecules, whose expression conferred resistance. Subsequently, molecular basis for the effect was explained confirming the existence of inhibitory NK cell receptors specific for MHC-I which counteracted signals triggered by an array of different activating receptors (31). According to the current paradigm, NK cell functions are tightly controlled by a balance between inhibitory and activating signals, triggered either upon direct recognition of target cells or indirectly through CD16A interaction with antibody-bound targets (covered in more detail in the following section).

Aside from surface receptors, cytokines secreted by accessory cells can regulate NK cell cytotoxicity, IFNγ secretion, survival and proliferation (15,32). IFN-I, IL-12 and IL-18 stimulate NK cell effector functions. IL-2 and IL-15 are two growth factors and particularly IL-15 is essential for NK cell maturation, proliferation and survival (33).

1.3.1 Receptors involved in target cell recognition

Main inhibitory receptors on NK cells recognize MHC class I molecules and are classified in three families: Killer Immunoglobulin-like Receptors (KIRs), Leukocyte Immunoglobulin-Like Receptors (LILRs) and CD94/NKG2 heterodimers (34). All inhibitory receptors included in these families share in their cytoplasmic region immunoreceptor tyrosine-based inhibitory motifs (ITIM). This motif is phosphorylated upon receptor engagement, recruiting Src homology region 2 domain-containing tyrosine phosphatases (SHP-1 and SHP-2), which interfere with NK cell activation by dephosphorylating key substrates in activating pathways; inhibition is transient and spatially localized at the NK cell-target synapse (35).

Although inhibitory receptors specific for MHC class I are the best characterized, there are other inhibitory receptors bearing ITIMs that regulate NK cell activation. These receptors include carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), sialic acid-binding immunoglobulin-like lectin 7 (SIGLEC7), leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) and killer cell lectin-like receptor subfamily G member 1 (KLRG1) (35).

Activating receptors lack intrinsic signaling domains and couple to adaptor proteins through charged residues in their transmembrane domain. The majority of activating receptors couples to FcεRIγ, CD3ζ or DNAX adapter protein (DAP)-12 which contain

immunoreceptor tyrosine-based activating motifs (ITAM). ITAM are phosphorylated by SRC-family kinases upon receptor engagement, recruiting spleen tyrosine kinase (SYK) or ζ-associated protein (ZAP)-70 (36).

A selection of NK cell receptor-ligand pairs with relevance in this work are described in more detail in the following section (**Figure 1**).

1.3.1.1 Inhibitory receptors specific for HLA-I

CD94 and NKG2 are type II integral membrane glycoproteins bearing an extracellular C-type carbohydrate recognition domain (CRD) that form a gene cluster on chromosome 12p13 (37). The NKG2 family includes NKG2A (and its splice variant NKG2B), NKG2C, NKG2E (and its splice variant NKG2H) and NKG2F. The formation of stable heterodimers with the invariant common CD94 subunit is required in order to stabilize them on the cell surface (38,39). CD94 homodimers can reach the cell surface, although they lack signaling capacity (40).

NKG2A (and also NKG2B) cytoplasmic tail contain ITIM motifs that confer the heterodimer the capacity to inhibit NK cell activation (41). CD94/NKG2A is the first inhibitory receptor specific for HLA class I acquired by mature NK cells and is lost by some subsets at the final stages of NK cell development (42). NKG2A transcription in NK cells is regulated by GATA-binding factor 3 (GATA3) (43) and is up-regulated by IL-2 and IFN-α (44).

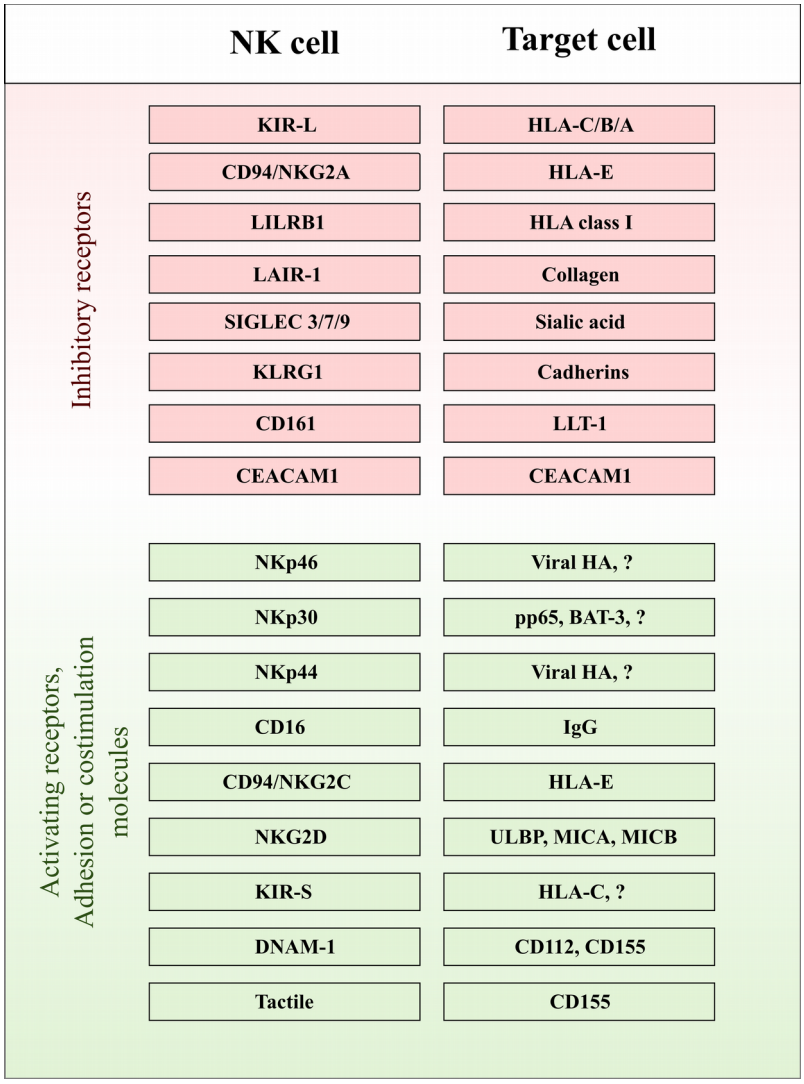

Figure 1. Activating and inhibitory NK cell Receptors and their ligands. Based on Vivier et al. Nat Immunol 2008.

The natural ligand for CD94/NKG2A is the non-classical MHC class I HLA-E (45–47). HLA-E is ubiquitously expressed, although at lower levels than classic MHC class I (48). HLA-E is composed

of a heavy chain, β_2 microglobulin and a nonamer peptide derived from residues 3-11 of the leader sequence of some MHC class I (49). According to the crystal structure of CD94/NKG2A bound to HLA-E (50,51), the interaction is dominated by CD94, suggesting that the main role of NKG2A is to confer signaling capacity to the heterodimer.

Killer Immunoglobulin-like Receptors constitute a family of polymorphic molecules expressed by NK cells and by a minor subpopulation of αβ and γδ T cells. KIR can display two (KIR2DL) or three (KIR3DL) Ig-like extracellular domains and are classified accordingly (52). KIRs are encoded in the leukocyte receptor complex (LRC) region in chromosome 19q13.4 (53), including both inhibitory and activating receptors, whose ligands are incompletely characterized. Inhibitory KIR display a long cytoplasmic tail (KIRXD**L**) bearing an inhibitory ITIM motif while activating KIR have a short tail (KIRXD**S**) lacking signaling motifs, but include a charged transmembrane residue (Lys/Arg) which facilitates their interaction with ITAM-bearing DAP12 adaptor. KIR2DL4 contains both an ITIM and a charged transmembrane residue (52).

At the population level, two different haplotype sets termed A and B have been defined which vary in KIR gene content, copy number and alleles. Group A haplotypes include seven genes and two pseudogenes. KIR2DS4, the only activating gene encoded in A haplotype, is non-functional in 80% of the Caucasian population (54). Group B comprises a variety of haplotypes which contain

different combinations of inhibitory and activating KIR genes. The frequencies of A and B haplotypes vary in ethnically different populations (53).

Inhibitory KIR2DL1 recognizes group 2 HLA-C alleles (containing Lys in position 80) while KIR2DL2 and KIR2DL3, which are alleles from the same locus, interact preferentially with group 1 HLA-C (containing Asn in position 80). KIR3DL1 recognizes HLA-B alleles and few HLA-A alleles bearing the Bw4 epitope and KIR3DL2 interacts with HLA-A3/11 (55). KIR variability between individuals combined by HLA polymorphism generates a low probability to find two individuals with same KIR-HLA profile, placing inhibitory KIR as key players for NK cell-mediated recognition of allogenic cells.

The Leukocyte Ig-Like Receptors (LILR) superfamily, also known as Ig-like Transcripts (ILT), LIR and CD85, is also encoded in the leukocyte receptor complex (LRC). The LILR family, which consists of 11 genes and 2 pseudogenes, includes both activating and inhibitory receptors and are predominantly expressed in the myeloid lineage (56), with the exception of LILRB1 (ILT2, LILR1 and CD85j), an inhibitory receptor specific for HLA class I molecules that is also expressed on NK cells at late differentiation stages (57), B cells and some T cell subsets. LILRB1 recognizes a wide spectrum of HLA class I molecules, yet with different affinities and preferentially interacts with HLA class I dimers, particularly HLA-G and certain class Ia alleles (58).

1.3.1.2 Activating receptors

Natural Cytotoxicity Receptors (NCR) were discovered in the late 1990s and defined for their preferential expression on human NK cells and their ability to induce NK cell activation and killing of tumor cells *in vitro (59)*. NCR are type I proteins with one or two Ig-like domains and a charged amino acid in their transmembrane region that allows the interaction with signaling adaptors (59).

NKp46 was the first NCR to be identified and is expressed on NK cells, some T cells subsets and a fraction of ILC3 (4,60,61). NKp46 is a 46 kDa protein encoded in the LRC that couples to CD3ζ homodimers or CD3ζ-FcεRIγ heterodimers.

NKp30 is a 30 kDa protein encoded in the class III region of the human MHC on chromosome 6p21.1. It is mainly expressed on NK cells but can also be found on a fraction of T cells after IL-2 and IL-15 exposure (62,63). NKp30 can bind to CD3ζ homodimers or CD3ζ-FcεRIγ heterodimers through a charged residue in its transmembrane domain (59). There are six splice variants of NKp30 expressed on the cell surface with different intracellular domains that can transmit qualitatively different signals (64).

NKp44 is a 44 kDa protein encoded in the class III region of the human MHC, induced upon activation. It couples to DAP12 through a lysine in the transmembrane domain triggering NK cell activation (65). Intriguingly, NKp44 has an ITIM motif in the cytoplasmic tail recently shown to inhibit NK cell function upon interaction with proliferating cell nuclear antigen (PCNA), a protein

overexpressed in cancer cells, inhibiting NK cell cytotoxicity and IFNγ secretion (66).

NCRs ligands include viral, bacterial, parasite and cellular proteins (59); however, the identification remains incomplete. NKp46 and NKp44 can interact with a broad range of hemagglutinins (HA) and hemmagglutinin neuramidases (HN) (59). NKp30 binds to HA of the vaccinia virus, although this interaction mediates NK cell inhibition and infected cell resistance to NK cell-mediated lysis (67). The human cytomegalovirus (HCMV) tegument protein pp65 has been reported to bind NKp30, resulting in NK cell inhibition by disrupting its interaction with adaptor proteins (68). *Mycobacterium tuberculosis* components interact with NKp44 (69), although no NK cell activation is observed. In contrast, NKp46 triggers NK cell activation upon binding to vimentin, up-regulated on the surface of *M. tuberculosis*-infected cells (70). NCRs also interact with poorly defined cellular molecules that are up-regulated on tumor cells. It has been reported that the three NCRs could bind to different heparan sulphates (HS) (71). In addition, NKp44 recognizes an isoform of the *mixed-lineage leukemia-5* gene (*MLL5*) *(72)*, while NKp30 binds to HLA-B-associated transcript 3 (BAT3) (73) and B7-H6 (74).

The NKG2 family includes the activating receptor NKG2C, expressed on NK cells and on a fraction of αβ and γδ T cells (75,76). The activating heterodimer CD94/NKG2C interacts with the ITAM-bearing adaptor molecule DAP12 through a positively

charged residue (lysine) in the NKG2C transmembrane domain, forming the trimer CD94/NKG2C/DAP12 (40). A deletion in the *NKG2C* gene (officially annotated as *KLRC2*) has been described (77). Individuals carrying the homozygous deletion has been reported to represent ~4% in different populations (78–80), ranging from 0.7% in Mexican to 10% in Gambian populations (81,82). CD94/NKG2C recognizes HLA-E, although its binding affinity is lower than that of the inhibitory CD94/NKG2A receptor (83). The interaction affinity is modulated by the peptide bound to HLA-E (46,48,83). A peptide derived from HLA-G is the one that provides the most efficient recognition by both CD94/NKG2A and CD94/NKG2C receptors (46).

CD94/NKG2C and CD94/NKG2A heterodimers can be co-expressed in a small fraction of NK cells. IL-12 is able to transiently up-regulate CD94/NKG2A expression in CD94/NKG2C$^+$ NK cells dampening their capacity to be activated, which might represent a negative regulatory feedback mechanism (84).

NKG2D is constitutively expressed on NK cells, CD8$^+$ T cells and a subset of differentiated CD4$^+$ T cells (85). Unlike members of the C type lectin-like NKG2 family, NKG2D is expressed on the surface as a homodimer and couples to the DAP10 adaptor. DAP10 recruits phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K), growth factor receptor-bound protein 2 (GRB2) and Vav-1 (86), regulating NK-cell cytotoxicity and cytokine production (87). NKG2D-

dependent activation may override inhibitory signals delivered by NK receptors for HLA class I (88,89) and co-stimulates T cell receptor (TCR)-dependent activation of $CD8^+$ T cells (88,90).

NKG2D recognizes MICA, MICB and ULBP1-6, which in healthy tissues are undetectable or expressed at low levels but are induced in cells subjected to various types of cellular stress. Viral infections or oncogenic transformation promotes expression of these "induced self" ligands (86).

1.3.2 Pattern recognition receptors (PRRs) in NK cells

As other innate immune cells, NK cells express germline-encoded PRRs recognizing pathogen associated molecular patterns (PAMPs) that are shared by various microorganisms (91). In addition, some PRRs can sense non-pathogenic epitopes known as danger-associated molecular patterns (DAMPs) released after tissue damage or from stressed cells (92). PRRs up-regulate the transcription of genes involved in inflammatory responses, "priming" NK cells and enhancing their effector functions (91).

Among PRRs, Toll-like receptors (TLRs) play an essential role in the innate responses against microbial pathogens. To date, 10 human TLRs (TLR1-10) and 12 murine (TLR1-9, TLR11-13) have been identified, with a broad specificity for different PAMPs. The expression of the TLRs varies between cell types such as NK cells, monocytes/macrophages, granulocytes, DC, mast cells and some non-hematopoietic cells (91,93). Initial studies based on human NK

cell mRNA detection described expression of all TLRs, being TLR1, TLR2 and TLR3 the highest expressed, followed by moderate levels of TLR5 and TLR6 and low levels of TLR4, TLR7, TLR8, TLR9 and TLR10 (94–97). Based on flow cytometry analysis, TLR2, TLR3, TLR4, TLR7, TLR8, and TLR9 have been described to be intracellular in circulating NK cells (96–101), though surface expression for TLR2 and TLR3 has also been described (96,102). In uterine NK cells, surface TLR2, TLR3 and TLR4 were found (99,103).

In general, exposure of primary NK cells to individual synthetic TLR agonists does not enhance NK cell effector functions, requiring co-stimulation with cytokines such as IL-12 or type I IFNs. However, some reports suggest that TLR2, TLR3, TLR4 and TLR5 stimulation alone or with low concentrations of IL-2 are sufficient to trigger NK cell effector functions. These are further stimulated in combination with IL-12 or type I IFNs (95,97,102,104), promoting the production of IFNγ and TNFα and their cytotoxic function. TLR2, TLR4 and TLR9 activation enhance both the production of cytokines and cytotoxicity (97,102,105) while TLR3, TLR7/8 activation enhance the secretion of IFNγ but have little effect in NK cell cytotoxicity (96,100).

In addition to TLR, NK cells express other PRR involved in sensing viral infections, including the RIG-I-like receptor (RLR) family members retinoic acid-inducible gene I (RIG-I) itself and melanoma differentiation-associated gene 5 (MDA5) (106,107). RLRs are

localized in the cytoplasm and recognize viral RNA and DNA PAMPs. Some studies support the role of RLRs in the control of herpes virus infections (108). Herpes simplex virus 1 (HSV-1)-induced IFN response by primary human monocyte-derived macrophages depend on MDA5 (109), while RIG-I was involved in type I IFN induction during Epstein-Barr virus (EBV) infections (110). Another cytosolic sensor for viral DNA is the DNA-dependent activator of IRF (DAI), which promotes type I IFN expression. DAI was shown to be essential for IFNβ production by HCMV-infected fibroblasts (111).

1.3.3 Antibody-dependent Cellular Cytotoxicity (ADCC)

Fcγ Receptors (FcγR) family is composed by proteins that are able to bind the constant fragment (Fc) of immunoglobulin G (IgG) (112). FcγR are classified in three groups based on their affinity for IgG and to their signaling characteristics. FcγRI (CD64) is a high-affinity receptor capable of binding monomeric IgG. FcγRIIA/B/C (CD32) are single-chain low affinity-receptors that contain ITAM (CD32A/C) or ITIM (CD32B) motifs capable of respectively triggering activating and inhibitory signals. FcγRIIIA/B (CD16A/B) are low affinity-receptors. CD16A is coupled to either CD3ζ chain or FcεRIγ chain respectively containing three or one ITAMs. Human IgG subclasses have different affinities for each receptor and CD16A binds more efficiently to IgG1 and IgG3 than to IgG2 and IgG4 (113).

The majority of $CD56^{dim}$ NK cells express high surface levels of CD16A mediating ADCC whereas $CD56^{bright}$ NK cells show low or undetectable CD16A levels. $CD56^{dim}$ NK cells express activating FcγR in the absence of the inhibitory counterpart. A low expression of the activating FcγRIIC, determined by an allelic polymorphism of the *FcγRII* gene (114), has been described in NK cells from some donors, triggering degranulation and cytokine production. Expression of the inhibitory FcγRIIB was reported in a small subset of NK cells showing reduced ADCC against IgG-coated target cells (115). Confirmation of these results is warranted.

CD16A was the first activating NK cell receptor identified (116) and one of the most extensively studied. Signaling via CD16A activates nuclear factor of activated cells (NFAT), inducing the production of cytokines including IFNγ and TNFα and several chemokines, as well as NK cell degranulation (37,112). CD16A is capable of triggering cytokine secretion and degranulation by resting NK cells without requiring additional signals upon engagement with specific antibodies (117).

A polymorphism on position 158 of the CD16A receptor resulting in a phenylalanine (F) to valine (V) substitution has been described (118,119). Product of *FCGR3A-158V* allele binds with higher affinity to human IgG1-4 and NK cells from V/V homozygous individuals displayed greater ADCC compared to F/F homozygous individuals (118,119). Due to the impact on ADCC function, it has been postulated that the *FCGR3A* polymorphism could modulate

the efficacy of monoclonal antibody-based therapies in different tumors such as breast cancer or colon cancer, although inconsistent results have been obtained by different studies (120).

1.3.3.1 ADCC in the context of viral infections

There exist a growing interest for the role of ADCC in the context of viral infections and its potential for the design of successful vaccines. The use of therapeutic antibodies has been proposed as a complement therapy for currently used anti-viral drugs, presumably allowing to limit long-term exposure to drug toxicity (121).

This has been best explored in the context of human immunodeficiency virus (HIV) infection. Animal models support a protective effect of specific antibodies against HIV infection dependent on Fc-mediated effector functions (122,123). In human, the RV144 vaccine trial showed a modest efficacy in preventing HIV infection (124). This study showed a negative correlation of ADCC with infection in a fraction of the subjects (125). A number of studies following natural infection also support a protective role of ADCC-inducing antibodies against the progression to AIDS (126).

ADCC has also been associated to control of HSV (127,128), influenza (129), dengue virus (130), respiratory syncytial virus (RSV) (131) and EBV (132,133). CMV-specific antibodies' capacity to activate NK cells has been poorly studied and was initially described by two studies that found a synergistic effect between NK

cells and specific antibodies in controlling the viral titer *in vitro (134)* and in the lysis of HCMV-infected fibroblasts (135). Recently, the possible role of NK cells as Ab-dependent effectors in the context of HCMV infection was again outlined. The newly defined FcεRIγ$^-$ NK cell subpopulation was found to be highly responsive against HCMV or HSV-1 infected cells in the presence of specific antibodies (136). In addition, HCMV-expanded NKG2C$^+$ NK cells showed enhanced responses against antibody-coated HCMV-infected cells (137).

ADCC involvement in viral infections control is further supported by epidemiological data. The *FCGR3A-158V* allele has been associated to the risk of developing Kaposi's sarcoma in HIV-infected subjects (138). In the same line, homozygozis for *FCGR3A-158V* allele was found a risk factor for HIV infection and progression (139). On the contrary, *FCGR3A-158V/V* genotype was associated with an asymptomatic course of HSV-1 infection, but only in homozygotes for G1m3 IgG1H chain allele (140). Following kidney transplant, *FCGR3A-158V/V* was associated with a lower incidence of urinary tract infections (141). In the context of HCMV infection, an NK cell line transfected with the high affinity *FCGR3A-158V* allele showed increased efficacy to inhibit cell-to-cell HCMV transmission *in vitro* compared to cells transfected with the *FCGR3A-158F* allele (142).

2 Human Cytomegalovirus

Human Cytomegalovirus (HCMV) or Human Herpesvirus 5 (HHV-5) belongs to the *herpesviridae* family, being the prototypic member of the *betaherpesvirinae* subfamily, while HSV-1/2 and varicella zoster virus (VZV) belong to the *alphaherpesvirinae* subfamily and EBV and Kaposi's sarcoma-associated herpesvirus (KSHV) to the *gammaherpesvirinae*. Herpesviruses are highly species-specific and closely related members of the herpesvirus family have evolved to infect other mammalian species such as MCMV or Rhesus CMV (RhCMV), which have provided animal models to study cytomegalovirus infection *in vivo (143)*.

HCMV is widespread reaching a seroprevalence between 40 and 90% in different populations, depending on socioeconomic factors and age. Acute HCMV infection is usually asymptomatic and the virus establishes a life-long persistent infection characterized by latency with occasional reactivations that allow transmission to new hosts. The mechanisms of latency are not fully understood, although the hematopoietic cells of the myeloid lineage have been proposed to be an important reservoir (144). HCMV can be transmitted through secretions (eg. saliva, sexual contact, breastfeeding), placental transfer, blood transfusion, solid-organ transplantation (SOT) or hematopoietic stem cell transplantation (HSCT) (145).

HCMV acute infection is usually asymptomatic or mild in healthy individuals. In contrast, the virus may become severely pathogenic in congenital infection and in preterm infants infected through

breast milk as well as in immunocompromised patients (i.e. primary immunodeficiencies, AIDS and immunosuppressed patients). HIV-infected individuals that have low $CD4^+$ T cell counts are in high risk for HCMV disease. In HSCT and SOT recipients, HCMV infection is a frequent event that may lead to severe complications, being associated with diminished graft survival (146).

2.1 Biology of HCMV

HCMV is the largest human herpesvirus with a double stranded linear DNA genome of ~240 Kb. Conventionally, it has been considered that HCMV genome encodes for >170 ORF (143). However, a recent study using ribosome profiling technology has raised to >700 the number of potential ORFs encoded by HCMV genome (147). In addition, 24 non-coding HCMV miRNAs regulate different functions such as cell cycle and immunoevasion (148). The genome of HCMV is divided into two domains termed long and short genome segments (L and S) and each domain is composed of a central unique region (UL and US) flanked by repeated segments with terminal (TRL and TRS) or internal (IRL and IRS) location (149). In common with other herpesviruses, DNA is contained inside an icosahedral nucleocapsid, surrounded by the tegument or matrix which is enclosed by a lipid bilayer (envelope) displaying several viral and host molecules (150) (**Figure 2**).

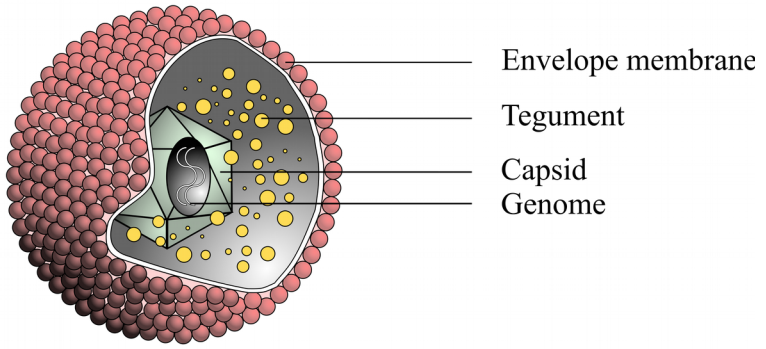

Figure 2. HCMV virion structure.

HCMV is able to infect a number of different human cell types including epithelial, endothelial, smooth muscle and myelomonocytic cells, as well as fibroblasts, neurons, hepatocytes and trophoblasts (151). The mechanism of virus entry is cell-type dependent and incompletely understood. While entry into fibroblasts involves direct fusion of the virus envelope with the plasma membrane, internalization by epithelial/endothelial cells involves macropinocytosis and virus fusion in endosomes (152). Complexes formed by the envelope glycoproteins gH and gL determine the tropism for fibroblasts, epithelial and endothelial cells. The trimer gH/gL/gO is required for cell-free virions entry to all cell types (153) and is sufficient to infect fibroblasts, but the pentamer gH/gL/UL128/UL130/UL131 is required to infect epithelial, endothelial and myeloid cells (151). Some laboratory strains have lost the Ulb' genome region, comprising UL133-151 non-essential genes (154) and accumulate mutations in UL128,

UL130 and UL131 (155). These genomic changes respectively increase the production of cell-free virions and decrease the capacity of infecting epithelial, endothelial and myeloid cells (156).

HCMV infection leads to the coordinated synthesis of viral proteins, originally classified in three overlapping phases based on their time of synthesis after infection (157). Immediate Early (IE) proteins are expressed from the first hours of infection and do not require *de novo* transcription because preformed mRNA is incorporated into virions (158). Immediate Early protein 1 and 2 are major transcription factors required for the subsequent expression of Early genes (E), including a number involved in immunoevasion (149). Finally, Late genes (L) comprise those encoding for most of the envelope glycoproteins, tegument proteins and capsid components among others (157). Expression of L genes requires an active viral DNA polymerase and can be further classified, according to their partial or full-dependence on this enzyme, respectively as Early-Late (E-L) or True Late genes (L). Recently, an exhaustive study analyzing the proteome of infected fibroblasts using mass-spectrometry further characterized the kinetics of viral protein expression and classified them into 5 temporal profiles (Tp1-5) (159). Approximately 50 genes are essential for HCMV replication, virion assembly and propagation in tissue culture (160).

2.2 Immune response to HCMV

The innate immune system, critical to control HCMV infection and to develop the adaptive immune response, is activated by HCMV through TLR-dependent and -independent pathways. Several TLRs including TLR2, TLR3, TLR7 and TLR9 have been reported to play a role in innate response against herpesvirus infections (108). TLR2 binds to glycoptrotein B (gB) and gH on the surface of HCMV viral particles, and trigger cytokine secretion in human fibroblasts (161). The use of specific knock-out mice has revealed an involvement of TLR2 in the recognition of MCMV envelope glycoproteins and the sensing of MCMV DNA by TLR3, TLR7 and TLR9 (162–165). Epidemiological data also supports the involvement of TLR2 and TLR9 in HCMV sensing. Specific single nucleotide polymorphisms (SNPs) in TLR2 and TLR9 genes have been associated to susceptibility to HCMV infection and reactivation in recipients of liver and allogeneic stem cell transplants, respectively (166,167). Polymorphisms in TLR3 and TLR4 that might constitute a genetic risk factor for the development of HCMV congenital infection have recently been identified (168,169).

2.2.1 T cell response to HCMV

T cells are key players in the control of HCMV infection. Strong evidence that HCMV-specific $CD8^+$ T cells play a crucial role in controlling this viral infection comes from both MCMV models and from patients undergoing HSCT. In mice, adoptive transfer of IE1-

specific $CD8^+$ T cells to immunodepleted hosts infected with MCMV prevented lethal infection in the absence of $CD4^+$ T cells (170). In addition, murine models of BMT show that reconstituted $CD8^+$ T cells are crucial for preventing MCMV disease (171). In human HSCT, a strong correlation between recovery of the $CD8^+$ T cell population and protection from HCMV disease (172) has been observed, and adoptive transfer studies reveal that *ex vivo* expanded HCMV-specific $CD8^+$ T cells are able to protect from primary infection and control reactivation (172).

HCMV-specific $CD8^+$ T cells are found at an exceptionally high frequency in peripheral blood of healthy donors, becoming up to 10% of the circulating $CD8^+$ T cells (143); the frequency may further increase in the elder. This phenomenon termed "memory inflation" (173) was initially described in mice, being characterized by an oligoclonal expansion over time of HCMV-specific $CD8^+$ T cells specific for a reduced number of epitopes. The magnitude and specificity of the expansion is variable between donors, and common immunodominant antigens are IE1, IE2 and pp65 (143). The underlying mechanisms are not fully understood, although a persistent/recurrent re-stimulation by latently infected cells is thought to drive the inflation. During acute HCMV infection, effector $CD8^+$ T cells show mainly a $CD45RA^-$ $CD27^+$ $CD28^{+/-}$ $CCR7^-$ phenotype, while in persistent infection/latency they display a $CD45RA^{+/-}$ $CD27^-$ $CD28^-$ $CCR7^-$ phenotype, associated to terminally differentiated T cells (174) (**Figure 3**).

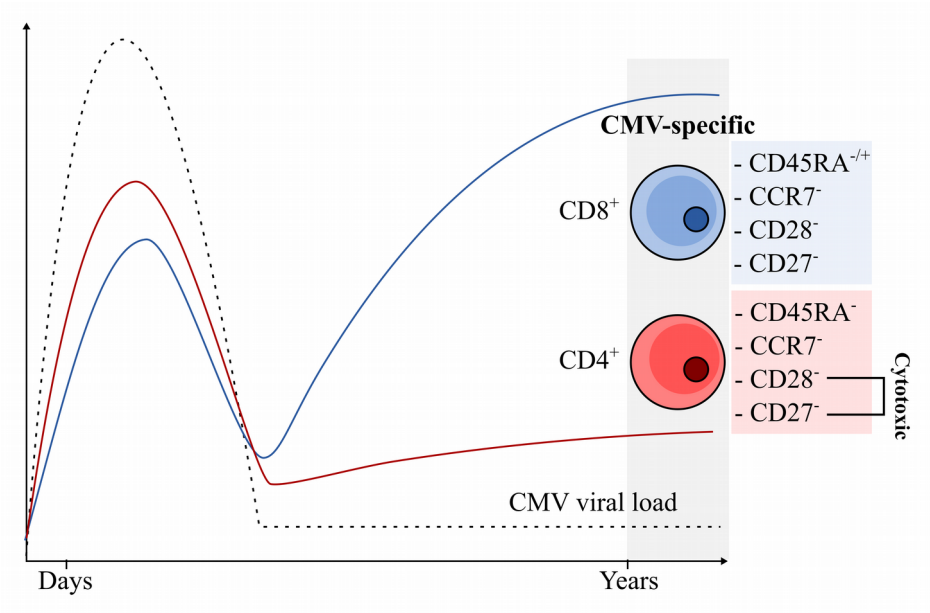

Figure 3. T cell compartment adaptation in response to HCMV.

While the role of the $CD8^+$ T cell response against HCMV infection is clear, there is increasing evidence that $CD4^+$ T cells are also important for the control of the pathogen. In mice, selective depletion of $CD4^+$ T cells impaired their ability to clear the virus from salivary glands (175), which is a site of rapid virus shedding (171), and resulted in an increased incidence of recurrent MCMV infection (171). In humans, symptomatic primary infection correlates with a delayed detection of HCMV-specific functional $CD4^+$ T cell response (176). In addition, healthy young children following HCMV acquisition persistently shed virus in urine and saliva, concomitant with low levels of virus-specific $CD4^+$ T cells (177). Studies in humans undergoing lung (178), renal (176,179) and bone marrow transplantation (180) have shown the association

between low $CD4^+$ T cell levels with susceptibility to clinically symptomatic HCMV reactivation episodes. $CD4^+$ T cells critically contributed to maintain $CD8^+$ CTL activity upon adoptive transfusion of HCMV-specific T cell lines (180,181).

Like HCMV-specific $CD8^+$ T cells, high proportions of specific $CD4^+$ T cells are also found in peripheral blood of healthy donors (a median of 2% (182)). HCMV-specific $CD4^+$ T cell responses do not undergo "inflation" to the levels of $CD8^+$ T cells, although some degree of expansion has been reported (183). $CD4^+$ T cells specific for pp65, IE and gB have been detected in a high proportion of individuals (143,172). An analysis of the $CD4^+$ T cell response comprising the whole HCMV proteome revealed a broader response and suggested that individuals have $CD4^+$ T cells specific for a median of 12 different HCMV ORFs (184).

HCMV-specific $CD4^+$ T cells are characterized by the production of Th1 cytokines, i.e. IFNγ and TNFα, while very few produce IL-4 and other cytokines including IL-10 and IL-17 (185–188). These cells show a particular phenotype characterized by $CD45RA^-$ $CCR7^-$ $CD28^-$ $CD27^-$, which correspond to an effector memory phenotype (186,187,189) (**Figure 3**). The loss of CD28 has been associated with the expression of perforin and granzyme B and the ability to mediate MHC class II restricted cytotoxicity in addition to produce IFNγ (190); these cells, rare in HCMV seronegative individuals, are enriched in $HCMV^+$ individuals (190,191). In mice, the presence of $CD4^+$ cytotoxic T cells was associated with reduced

MCMV replication, supporting the role of these cells in protection against the viral infection (192).

The major role of T cells in the control of HCMV infection is also highlighted by the fact that HCMV encodes for several molecules that interfere with antigen presentation by the infected cell. US2, US3, US6, and US11 are HCMV proteins with different expression kinetics along the lytic cycle that impair surface HLA-I expression through different mechanisms. US2 and US11 are endoplasmic reticulum (ER)-associated proteins that interact with HLA-I molecules, translocate them to the cytoplasm and lead to their degradation by ubiquitination in a TRC8-dependent and Delphin-1-dependent way respectively (193,194). US3 interacts with HLA-I in the ER and interferes with its maturation (195). US6 blocks peptide transport into ER by transporter associated with antigen processing (TAP), precluding peptide loading onto HLA-I (196). US2, US3 and UL83 (pp65) also interfere with HLA-II expression. US2 and UL83 accumulate HLA-DR molecules in lysosomes and induce their degradation (197,198). US3 inhibits HLA-DR assembly, trafficking and antigen loading (199). HCMV also interferes with HLA-II expression in infected cells by preventing the expression of the class II major histocompatibility complex transactivator CIITA (200,201).

2.2.2 Antibody response to HCMV

Specific antibodies against viral proteins have an important role in limiting the disease severity, preventing congenital infection

(202,203) and controlling the virus spread in blood (204). Most studies characterizing the antibody response against HCMV have focused on antibodies with virus neutralizing activity (**Figure 4**). There is a broad spectra of antigens recognized by HCMV-specific antibodies and the best characterized are the glycoprotein complexes on the virion envelope. They are probably the major targets for neutralizing antibodies and comprise glycoprotein (g)B, gM/gN and gH/gL.

The reactivity of sera from seropositive donors against gB is of 100% and specific antibodies represent 40% to 70% of their neutralizing activity on fibroblasts (205,206). However, it was recently discovered that antibodies against the gH/gL/UL128/UL130/UL132 pentamer are produced very early upon HCMV infection and have >100-fold higher neutralizing titer when tested on endothelial/epithelial cells as compared to antibodies tested on fibroblasts, which appear with a delay of 2-3 months (207,208). Thus, antibodies against the pentamer may have a higher contribution than initially thought, especially in the early steps of the infection.

Although viral glycoproteins are the dominant targets for antibodies with neutralizing activity, there is a large proportion of HCMV-specific antibodies without neutralizing capacity targeting these proteins (209) and other viral antigens including proteins contained in the virion tegument such as ppUL25, pp28, pp50, pp52, pp65, pp150, UL94, US22 (210–214), structural proteins including UL48,

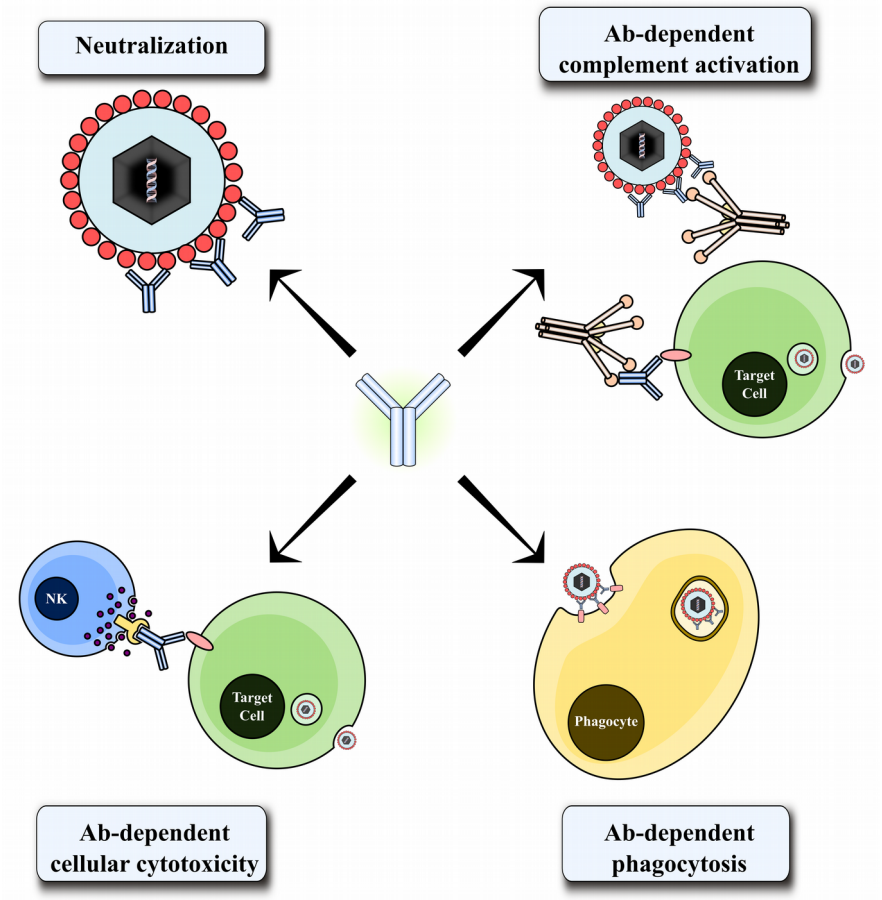

Figure 4. Antibody(Ab)-mediated effector functions involved in the control of viral infections.

UL80A (215,216) and also non-structural proteins such as IE1, UL44, UL56, UL70 and UL71 (217,218). Recently, Pérez-Bercoff and colleagues analyzed the HCMV-specific antibody-epitope recognition repertoire after HSCT transplantation using the unbiased technology peptide-microarray, revealing many more epitopes (219). The role of these antibodies in protection against HCMV-infection and reactivation has not been studied.

The involvement of IgG antibodies in the defense against HCMV infection is supported by the development of specific immunoevasion molecules targeting them. HCMV codifies for several proteins capable of binding to IgG through the Fc portion called viral Fcγ receptors (vFcγRs). Four molecules have been identified with IgG-binding capacity: gp34 (RL11) and gp68 (UL119-118) (220) and more recently gpRL13 (RL13) and gp95 (RL12) (221). Experiments using target cells infected with viral strains lacking gp34 and gp68 demonstrate their capacity to interfere with NK cell activation mediated by polyclonal HCMV-immune IgG (222). In contrast, they don't seem to interfere with neutralization and complement-mediated effector functions (223).

2.2.3 NK cell response to HCMV

NK cells play an important role in the control of HCMV, as it is indirectly suggested by the fact that patients with NK cell deficiencies develop severe susceptibility to herpesviruses, including HCMV (224). In addition, studies performed in mice show that NK cells limit the severity, extent and duration of MCMV infection (225) and are able to provide natural resistance to the viral infection when adoptively transferred (226). However, the mechanism conferring this protection is not fully understood.

The development of multiple immunoevasion strategies by HCMV to counteract NK cell response indirectly supports the involvement of this lymphocyte lineage in the infection control. Viral protein

UL18 is an HLA class I homologue that binds with high affinity to the inhibitory receptor LILRB1 (227) and is expressed on the surface of infected cells at low levels, increasing at late time-points of infection (228,229). The role of UL18 in modulating NK cell activity has been controversial, with studies showing enhanced NK cell killing or an inhibitory effect using UL18-transfected target cells (228,230,231), and may be influenced by UL18 polymorphisms between different viral strains (232). HCMV is also able to maintain HLA-E on the infected cell surface, despite reducing surface expression of other HLA class I molecules. HLA-E expression is resistant to US2 and US11 proteins and is displayed at the surface of infected cells presenting a nonamer from the leader peptide of UL40 polymorphic viral glycoprotein; UL40 peptide-loading to HLA-E is TAP-independent and thus resistant to US6 (233). HLA-E loaded with the canonical UL40 leader peptide has been shown *in vitro* to prevent the activation of CD94/NKG2A^{+} NK cells in experimental systems based on the use of UL40-derived synthetic peptides (233,234). Binding of distinct HLA-E/UL40 complexes with the activating receptor CD94/NKG2C has also been shown by surface plasmon resonance (235). Nonetheless, the involvement of these interactions in the regulation of NK cell recognition of HCMV infected targets remain elusive (236). On the other hand, HCMV hampers surface expression of ligands for NK cell activating receptors. UL16, US18, US20, UL142 and miR-UL112 limit surface expression of NKG2D ligands (NKG2D-L) (237–240). Moreover, UL141 sequesters CD155 and CD112,

ligands for DNAM-1, T cell activation increased late expression (TACTILE or CD96) and T cell immunoreceptor with Ig and ITIM domains (TIGIT) co-receptors with activating and inhibitory function, respectively (241,242). In addition, ligands for NKp46 and NKp30 have also been shown to be reduced in HCMV infected cells by as yet unknown mechanisms (243). The tegument protein pp65 (UL83) was reported to interact with NKp30, inducing its dissociation from the CD3ζ adaptor (68) and inhibiting NK cell activation. Recently, downregulation of B7-H6, a NKp30 ligand, on the infected cell by US18 and US20 was also described, indirectly supporting the involvement of this NKR in the control of HCMV (244). The *in vivo* relevance of these immune evasion mechanisms is unknown.

2.2.3.1 HCMV-induced reconfiguration of the NK cell receptor repertoire

Some observations support the development of a memory-like pattern of NK cell-mediated response to HCMV infection. MCMV-encoded glycoprotein m157 on infected cells is recognized by the activating receptor Ly49H in NK cells from C57BL/6 mice. Activation of Ly49H drives the expansion of virus-specific NK cells during the acute phase of MCMV infection (245–247), followed by a contraction phase and the survival of a stable pool of "memory-like" Ly49H$^+$ NK cells showing enhanced cytotoxicity and cytokine production upon re-challenge with MCMV (248). These "memory-

like" NK cells have a particular transcriptional signature as compared to conventional NK cells (249).

In humans, HCMV infection has been shown to induce a persistent reconfiguration of the NK cell compartment characterized by the expansion of an NK cell subset displaying high surface levels of the CD94/NKG2C activating receptor (NKG2C^{bright} cells, **Figure 5**) (75). This pattern of response to the viral infection was reported to be detected in some healthy individuals independently of KIR haplotypes, HLA-E dimorphism and past infections by other herpesvirus (i.e. EBV and HSV-1) (75). NKG2C^{bright} NK cell expansions are associated to HCMV in newborns with congenital infection as well as in healthy children, indicating that the reconfiguration of the NK cell compartment may occur early in life and following primary infection (250,251). Although some reports describe NKG2C^{bright} NK cell expansions in patients with other viral infections such as HCV, HBV, HIV, Chikungunya Virus, Hantavirus or EBV, HCMV co-infection is systematically observed (252–257).

NKG2C^{bright} NK cells, which lack CD94/NKG2A, may coexist with another subset of NK cells expressing lower levels of CD94/NKG2C (NKG2C^{dim}) that can co-express CD94/NKG2A and is detectable in both HCMV seronegative and some HCMV^{+} individuals (258). Whether NKG2C^{bright} NK cells may differentiate from NKG2C^{dim} precursors remains unknown. The magnitude of NKG2C^{bright} expansions is quite variable in different individuals, from a small proportion up to 80% of the NK cell compartment in

some subjects, and remains stable over time (75,259). The *NKG2C* genotype influences this reconfiguration process, as the *NKG2C*$^{+/+}$ genotype is associated to higher surface receptor levels and to greater numbers of NKG2C^{+} NK cells in HCMV seropositive subjects compared to the *NKG2C*$^{+/-}$ genotype (80,251).

Besides NKG2C^{bright} NK cells, oligoclonal expansions of differentiated NK cells lacking NKG2C and expressing self-specific activating KIR have also been described in some HCMV^{+} healthy adults (260) as well as in HCMV-infected HSCT recipients transplanted with NKG2C$^{del/del}$ grafts (261). In addition, the expansion of an NK cell subset that has down-modulated the adaptor molecule FcεRIγ-chain has been recently associated to HCMV infection (262). FcεRIγ loss is generally predominant in NKG2C^{bright} NK cells, but is also detectable in NK cells lacking NKG2C expression.

Adaptive HCMV-induced NK cells are characterized by a particular phenotypic and epigenetic profile. They express lower surface levels of NKp30, NKp46 and CD161, higher levels of LILRB1 while maintaining comparable levels of CD16 and NKG2D (263). Adaptive NK cells show variable degrees of epigenetic silencing of adaptor molecules (FcεRIγ, Syk and EWS/FLI1 activated transcript 2 (Eat2)) and transcription factors (promyelocytic Leukemia zinc finger (PLZF), Helios and Ikaros family zinc finger 2 (IKZF2)) (264,265). NKG2C^{bright} NK cells predominantly display inhibitory KIR specific for self HLA-C1 or/and -C2 allotypes, suggesting that

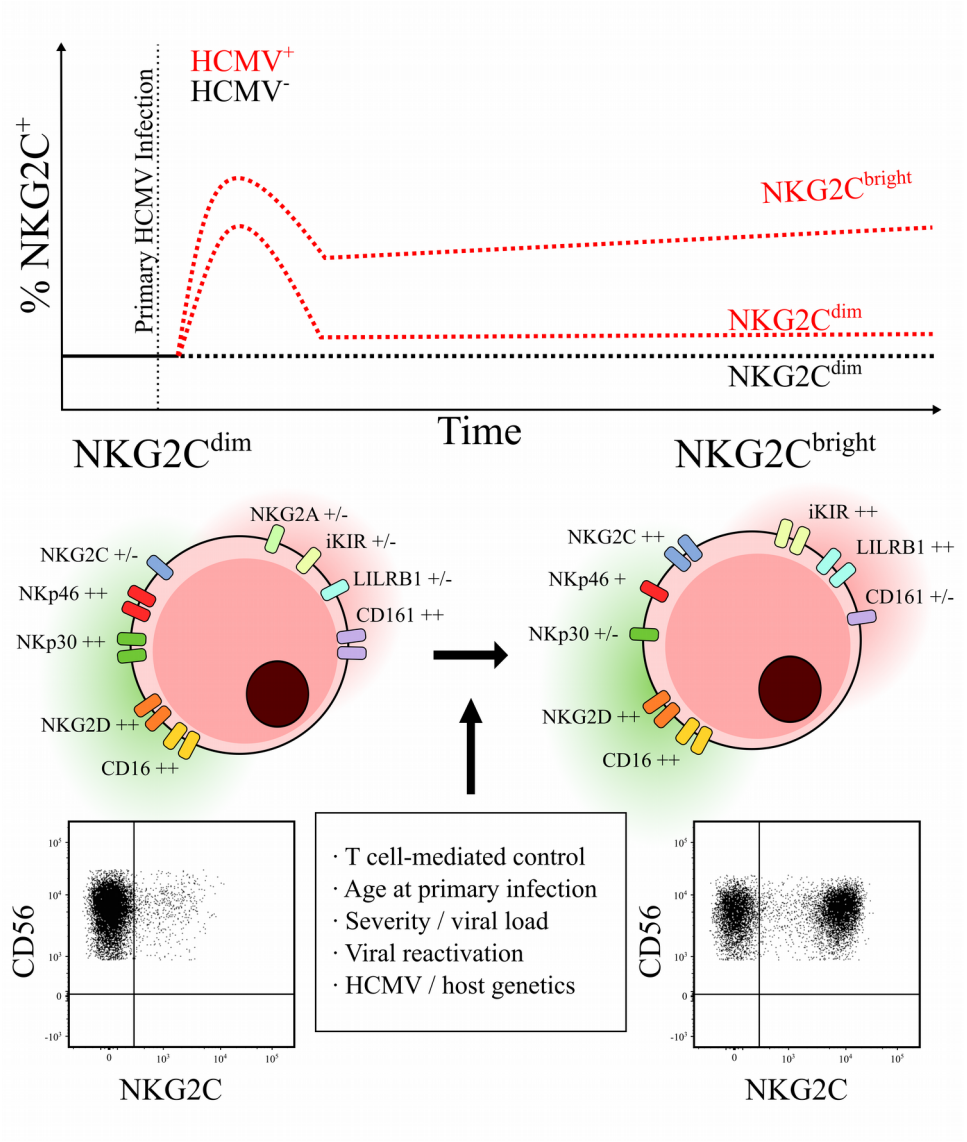

Figure 5. NK-cell compartment adaptation in response to HCMV.

these cognate interactions modulate their differentiation and/or expansion driven by HCMV (259).

Adaptive NKG2C$^+$ NK cells show a limited direct *in vitro* response against HCMV-infected cells (137,243,266). Nevertheless, their

protective role in HCMV infections is suggested by recent published observations were we found that high pretransplant levels of $NKG2C^+$ NK cells in kidney transplant recipients (KTR) were associated with a reduced incidence of posttransplant HCMV viremia (267).

CHAPTER 2: AIMS

Human cytomegalovirus (HCMV) infection induces persistent changes in the host immune system, namely the adaptive stimulation and expansion of antigen-specific T lymphocytes, the production of specific antibodies and, in some individuals, the generation of a differentiated NK cell subset with adaptive features. The interplay between NK cells and adaptive immune responses to the viral infection remains poorly characterized. To explore this issue the following aims have been addressed:

1. To assess the functional impact and mechanism(s) involved in NK cell-mediated sensing of HCMV.
2. To characterize the antibody-dependent NK cell response to HCMV-infected cells and, particularly, the involvement of adaptive NKG2C^{+} NK cells.
3. To explore the ability of HLA-DR^{+} NK cell subsets to present HCMV-antigens to specific CD4^{+} T cells.

PART II
RESULTS

CHAPTER 3

Priming of NK cell anti-viral effector mechanisms by direct recognition of human cytomegalovirus.

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CHAPTER 4

Antibody-Mediated Response of NKG2C^{bright} NK cells against Human Cytomegalovirus.

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CHAPTER 5

Exploring the ability of HLA-DR$^+$ NK cell subsets to present HCMV-antigens to specific CD4$^+$ T cells.

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

MATERIAL AND METHODS

Ethics statement

PBMC and serum samples used in this study were obtained from volunteer healthy adults. Written informed consent was obtained from every donor, and the study protocol was approved by the local ethics committee (Clinical Research Ethics Committee, Parc de Salut Mar n˚2013/5470/I).

Abs and flow-cytometry analysis

FACS analysis was performed using mAbs specific for the following molecules: HLA-DR-fluorescein isothiocyanate (FITC), CD86-FITC, CD45RA-FITC, Perforin-FITC, CD69-Phycoerythrin (PE), CD80-PE, IFNγ-PE, CD4-allophycocyanin (APC), CD3-peridinin-chlorophyll protein (PerCP), CCR7-PE-Cy7, CD16-PE-Cy7 (BD Biosciences Pharmingen, San Diego, CA), CD8-V500, CD28-PE-CF594 (BD Biosciences Horizon, San Diego, CA), CD56-APC, CD25-PE, (eBioscience, San Diego, CA), NKG2C-PE (clone 134591), NKG2C-Alexa Fluor 700 (clone 134591) and unlabeled-NKG2C (clone MAB1381; R&D Systems, Minneapolis, MN), anti-FcεRI Ab, γ subunit-FITC (Merck, Millipore), CD4-FITC, CD4-PE-Cy7, Granzyme B-Pacific Blue (PB; Biolegend, San Diego, CA) and NKG2D-APC (Miltenyi Biotec, Bergisch Gladbach, Germany). Anti–TNF-α (infliximab; REMICADE) was

directly labeled with CF-Blue by Immunostep (Salamanca, Spain). mAb anti-NKp46 (clone Bab281) and anti-NKp30 (AZ20) were kindly provided by Dr. A. Moretta (University of Genova, Genova, Italy); anti-CD57 (clone HNK-1), anti-LILRB1 (clone HP-F1) and anti-CD161 (clone HP-3G10) were produced in our laboratory and employed as hybridoma culture supernatants. Cells were pretreated with human aggregated IgG (10 μg/ml) to block Fc receptors and were subsequently labeled with specific Abs. For indirect immunostaining, samples were incubated with unlabeled Abs followed by PE-Cy7-conjugated or APC-Cy7-conjugated $F(ab')_2$ polyclonal goat antimouse IgG (Biolegend). Samples were acquired in LSRII or LSRFortessa flow cytometers (BD Biosciences), and data analyzed with FlowJo software (Tree Star). For blocking experiments, the anti-HLA-DR D1.12, kindly provided by Dr. R. Accolla (Università of Insubria, Varese) was used at saturating concentration. Depending on the assay, the anti-HLA class I W6/32 or an isotype control were used.

HCMV stock preparation

The MRC5 fetal human lung fibroblast cell line was obtained from the American Type Culture Collection (Manassas, VA) and grown in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Purified stocks of HCMV AD169 strain were prepared by infecting MRC5 cells at 0.25 multiplicity of infection (MOI) and harvesting supernatants when maximum

cytopathic effect was reached. Cells and debris were removed from virus containing supernatant by centrifugation 10 min at 5,000 x g and stored at -80˚C. Viral stocks were titrated on MRC5 cells analyzed by detection of IE-1/IE-2 viral antigens with specific mAb (clone mab810; Millipore) by immunofluorescence as previously described (268).

Functional assays with primary cells

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood samples by separation on Ficoll-Hypaque gradient (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway). Serum samples were collected, heat-inactivated and aliquoted before storage at -20˚C. Standard clinical diagnostic tests were used to determine HCMV specific IgG titer (Roche Diagnostics, Basel, Switzerland). PBMC were kept overnight with complete RPMI 1640 medium supplemented with 200 U/ml of interleukin-2 (hrIL-2; Proleukin, Chiron, Emeryville, CA (200 U/ml) prior to functional assays.

NK cells were purified by negative selection using NK Cell Isolation kit (Miltenyi) according to the manufacturer instructions. MoDCs were generated as previously described (243); briefly, monocytes were obtained by positive selection from PBMCs using anti-CD14 microbeads (StemCell Technologies, Grenoble, France), and cultured for 6 days in RPMI 1650 medium supplemented with 10% FBS, interleukin-4 (IL-4; 25 ng/ml, R&D Systems), and

granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/ml, PeproTech).

NK cells or moDCs were cultured overnight with HCMV virions (2.5 infective particles/cell) in the presence or absence of 10% sera from HCMV seropositive donors. Subsequently, these APCs were incubated with autologous $CD4^+$ T cells (purified using $CD4^+$ T cell Isolation Kit, Miltenyi) or PBMC (5:2 E:T ratio) for 18h at 37ºC in the presence of Brefeldin A (10 μg/ml; Sigma-Aldrich). Next, cells were stained with antibodies recognizing surface markers, fixed, permeabilized (fixation/permeabilization kit; eBioscience), stained with anti-TNFα and anti-IFNγ, and analyzed by flow cytometry. In some experiments, $CD4^+$ T cell degranulation was monitored by measuring CD107a mobilization with the additional presence of monensin (5 μg/ml; Sigma-Aldrich) and CD107a-FITC (BD Biosciences Pharmingen, San Diego, CA). Boolean gating function was used to identify all possible combinations of markers stained for on $CD3^+$ $CD4^+$ T cell populations (Flowjo software).

$CD4^+$ T cell and NK cell expansions

Expanded $CD4^+$ T cells were obtained by incubating PBMC in 24-well plates ($3x10^6$ cells/well) in complete RPMI 1640 medium in the presence of HCMV virions ($2x10^5$ PFU/well). At day 3, cell cultures were supplemented with 25 U/mL hrIL-2. Cell cultures were maintained at 37º C in a 5% CO2 humid atmosphere for 10–12 days; every 3 days 50% of the supernatant was replaced with fresh

medium supplemented with IL-2; when high cell density was attained, cell cultures were split.

NK cells were expanded by incubating PBMC in 24-well plates ($3x10^6$ cells/well) in complete RPMI 1640 medium together with the HLA-E^+ 721.221-AEH lymphoblastoid cell line irradiated at 40 Gy ($1x10^6$ cells/well). Cell cultures were maintained at 37º C in a 5% CO2 humid atmosphere for 10–12 days; every 3 days 50% of the supernatant was replaced with fresh medium; when high cell density was attained, cell cultures were split. Expanded NK cells were further purified using the Miltenyi NK cell Enrichment kit. NK cell loading with HCMV immunocomplexes and functional assays were performed in the same conditions as described for primary cells.

Gene expression analysis

Publicly available gene expression data was obtained from NIH GEO Accession GSE66564 (265) and GSE66124 (264). Already normalized data was log2 transformed, linear model was designed using R *limma* package (269) and a moderated t-test between the two population groups groups was applied for paired-wise comparison using *eBayes* function. A volcano plot of the modeled data was generated using the *ggplot2* package.

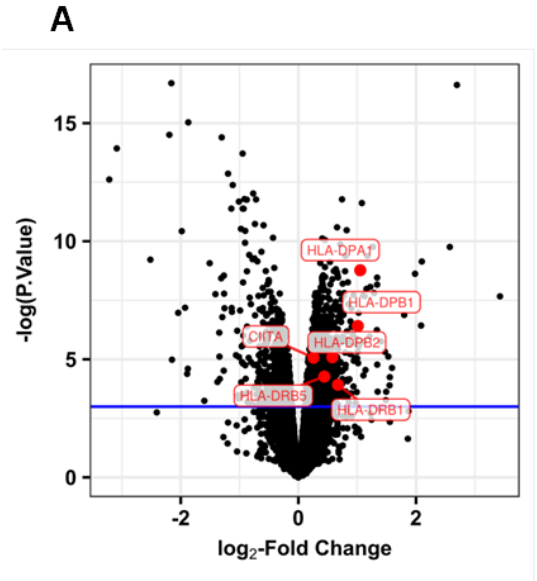
Statistical analysis

Statistical analysis was performed by the Mann Whitney U test using GraphPad Prism 5 software. Results were considered significant at the two-sided P level of 0.05.

RESULTS

1. Circulating HCMV adaptive NKG2C^{bright} NK cells express HLA-DR molecules.

The analysis of stable changes in published transcriptional programs of adaptive NK cells (defined as CD56^{dim} NKG2C^{+} (CD57^{+}/FcεRIγ^{-})) identified antigen processing and presentation of peptides via MHC class II as an overrepresented functional pathway (**Figure 1**).



Gene symbol	Fold Change
HLA-DRB5	13.7
HLA-DQB1	12.2
HLA-DQA2	11.4
HLA-DQA1	10.6
CIITA	9.8
HLA-DQA1	8.2
HLA-DRB4	7.1
HLA-DRA	6.8
HLA-DRB1	3.8
HLA-DPB1	3.3
HLA-DPB2	3.1
HLA-DPA1	2.7
HLA-DRB3	2.6
HLA-DMA	2.3
CD74	2.3

Figure 1: Differential expression of genes involved in antigen presentation by MHC class II in adaptive NKG2C^{+} NK cells. A) Re-analysis of the available microarray data published in Schlums et al. Immunity. 2015. Volcano plot showing fold-change of gene expression in NKG2C^{+} CD57^{bright} compared to NKG2A^{+} CD57^{-} CD56dim NK cells. Blue horizontal line indicates a p-value of 0.05 and selected genes are labeled. **B)** Table extracted from the gene expression

microarray analysis data published by Lee et al. Immunity. 2015. comparing sorted NKG2C^{+} versus NKG2C^{-} NK cells from a donor displaying expansions of adaptive NK cells.

Transcripts for CIITA, HLA-DQ, HLA-DP, HLA-DMA and HLA-DRA were reported to be enriched in adaptive NK cells (264,265) and these findings were confirmed by microarray analysis in our laboratory (data not shown). In order to ascertain the predicted expression of MHC class II molecules, we analysed the expression of HLA-DR in circulating NK cells from healthy individuals displaying or not expansions of the HCMV adaptive NKG2C^{bright} NK cell subset by flow cytometry. As shown in **Figure 2**, HLA-DR was expressed in approximately ~50% of circulating CD56^{bright} NK cells in all donors, regardless of their HCMV serology. Surface HLA-DR expression was variable among circulating CD56^{dim} NK cells and generally higher in HCMV^{+} individuals with expansions of NKG2C^{bright} NK cells as compared to HCMV^{-} and HCMV^{+} individuals without detectable NKG2C^{bright} NK cell expansions (**Figure 2A-B**). Remarkably, the proportions of HLA-DR^{+} NKG2C^{bright} NK cells remained stable along time (**Figure 2C**) and were not associated with the expression of activation markers (i.e. CD69 and CD25) (**Figure 2D)**. NKG2C^{bright} NK cells have been proposed to undergo sequential differentiation associated to the down-regulation of FcεRIγ adaptor, NKp30, NKp46 and CD161 as well as the acquisition of CD57 and LILRB1 (270,271).

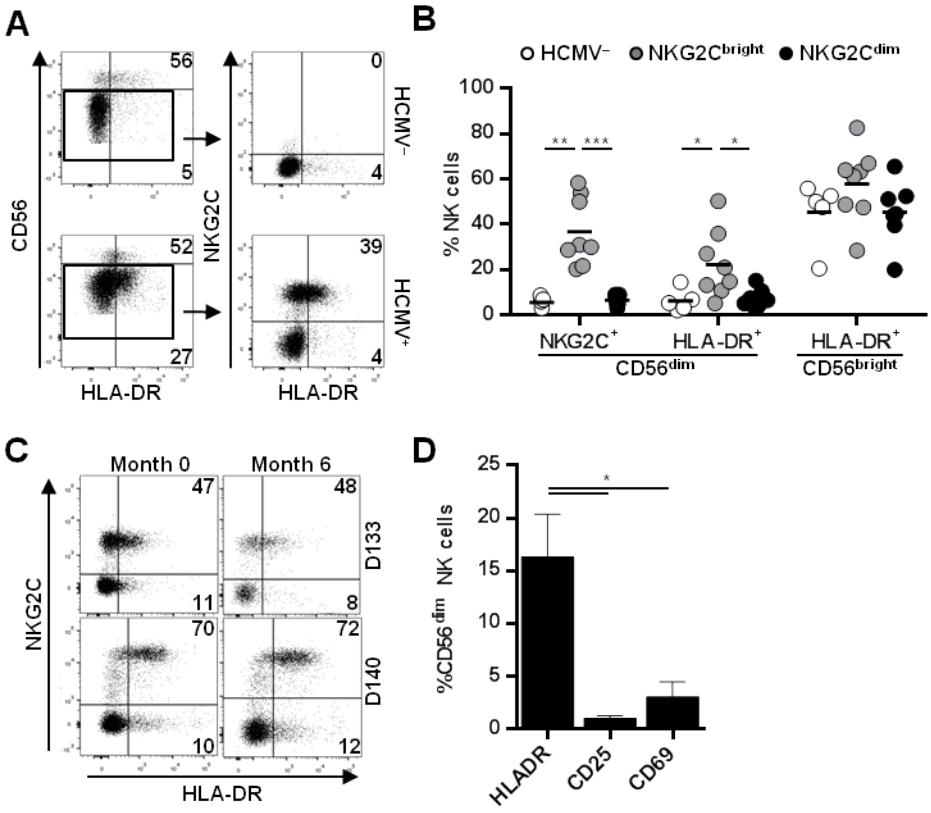


Figure 2. Circulating NKG2C^{bright} NK cells express variable levels of HLA-DR in the absence of other activation markers. NKG2C and HLA-DR expression was analysed by flow cytometry in circulating NK cells from seronegative (HCMV^{-}) and seropositive (HCMV^{+}) individuals with (NKG2C^{bright}) or without (NKG2C^{dim}) adaptive NKG2C^{+} NK cell expansions. **A)** Representative dot plots of NKG2C and HLA-DR expression. **B)** Frequency of NKG2C^{+} and HLA-DR^{+} cells in CD56^{dim} and CD56^{bright} NK cell subsets in individuals categorized according to their HCMV serology and NKG2C$^{bright/dim}$ NK cell profile. **C**) Dot plots showing the stability of the NKG2C and HLA-DR phenotype along time in two representative HCMV^{+} individuals analyzed out of 5. **D**) Frequency of HLA-DR^{+}, CD25^{+} and CD69^{+} CD56^{dim} cells in circulating

NK cells from HCMV$^+$ individuals with NKG2C^{bright} NK cell profile (mean ± SEM, n=6) (**p < 0.01, ***p < 0.001).

Since surface HLA-DR was variable among NKG2C^{bright} NK cells in different individuals, we analyzed its co-expression with KIR, CD57, LILRB1, NKp30, NKp46, CD161 and FcεRIγ in NK cells from five HCMV$^+$ individuals displaying an average expansion of ~30% NKG2C$^+$ NK cells. Of note, expression of all assessed markers was comparable in HLA-DR$^+$ and HLA-DR$^-$ NKG2C^{bright} NK cells (**Figure 3A**). Detailed analysis of HLA-DR expression in two individuals concomitantly displaying NKG2C$^+$ and NKG2C$^-$ FcεRIγ$^-$ NK cell subpopulations confirmed the preferential expression of HLA-DR in adaptive NKG2C^{bright} NK cells regardless of FcεRIγ levels (**Figure 3B**). Altogether, these results indicate that HLA-DR expression may reflect a differentiation event in NKG2C^{bright} NK cells uncoupled from FcεRIγ down-regulation.

2. NKG2C^{bright} NK cells may uptake HCMV-antibody immunocomplexes and present antigens to CD4$^+$ T cells.

We have previously shown that NK cells can directly sense the presence of HCMV virions and are activated in response to HCMV-antibody immunocomplexes (IC) (272,273). We addressed whether HLA-DR$^+$ NK might present antigens to specific CD4$^+$ T cells in the context of HCMV infection and whether this function could be facilitated by the uptake of HCMV-antibody immunocomplexes. To address this question purified NK cells were cultured overnight with

HCMV particles at MOI 2.5, including or not serum from seropositive donors. Monocyte-derived dendritic cells (moDC) were cultured in parallel in the same conditions for comparison.

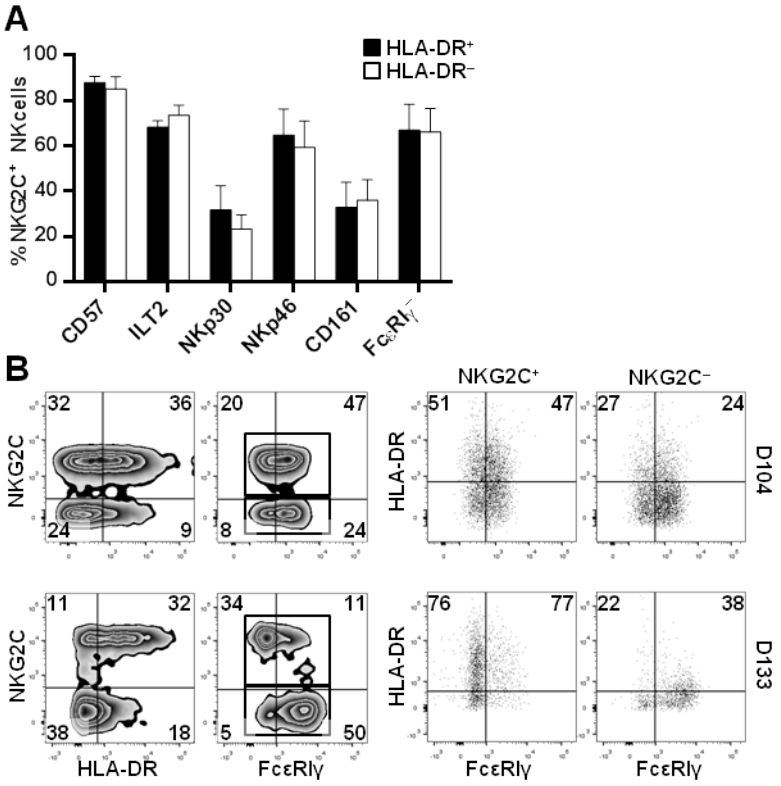


Figure 3. HLA-DR expression in NKG2C^{bright} NK cells is uncoupled from other NKR or differentiation markers. The expression of FcεRIγ, NKp30 and NKp46 NCR, CD161, CD57 and ILT2/LILRB1 was analysed in NKG2C^{+} HLA-DR^{+} and NKG2C^{+} HLA-DR^{-} circulating NK cells from seropositive individuals with adaptive NKG2C^{bright} NK cell profile. **A)** Frequencies of CD57^{+}, ILT2^{+}, NKp30^{+}, NKp46^{+}, CD161^{+} and FcεRIγ^{low} cells in CD56^{dim} NKG2C^{+} NK cells according to HLA-DR co-expression (mean ± SEM, n=5). **B)** Co-expression of HLA-DR and FcεRIγ in NKG2C^{bright} and NKG2C^{-} CD56^{dim} NK cells from two representative donors out of 5.

Overnight co-culture with HCMV virions promoted the up-regulation of surface HLA-DR and CD86 in moDC, yet no significant changes were noticed in NK cells. In contrast, co-culture with HCMV in the presence of HCMV^{+} serum promoted an up-regulation of surface HLA-DR in both NK and moDC, enhancing CD86 expression only in the latter **(Figure 4A-B** and not shown). Enhancement of surface HLA-DR expression was more evident in NKG2C^{bright} NK cells (**Figure 4C**).

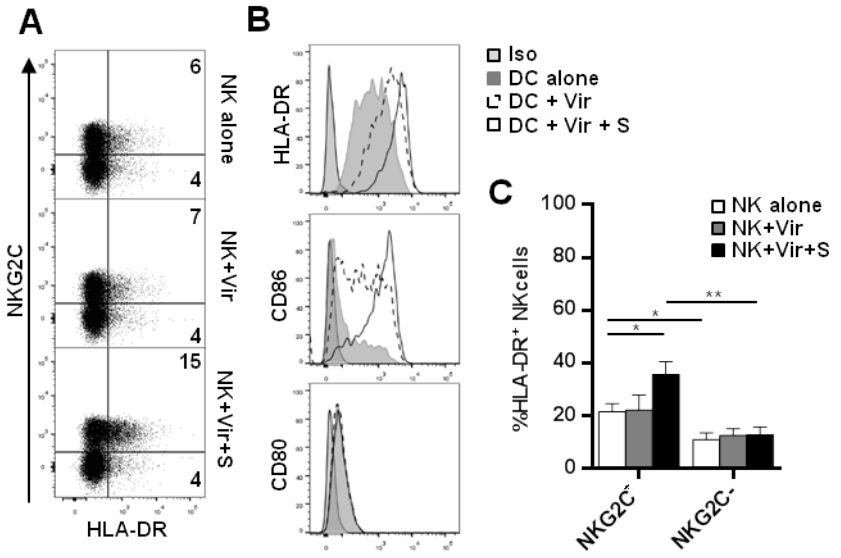


Figure 4. NKG2C^{bright} NK cells up-regulate HLA-DR in response to HCMV in the presence of specific antibodies. Purified primary NK cells and monocyte-derived DC were cultured overnight with HCMV viral particles in the presence or absence of serum from HCMV^{+} donors. Expression of HLA-DR, CD86 and CD80 was analysed by flow cytometry. **(A)** Dot plots of HLA-DR and NKG2C expression in one representative donor out of 4 tested. **B)** Histograms displaying HLA-DR, CD86 and CD80 expression in moDC under the different conditions tested. **(C)** Bar graph showing the average expression of HLA-DR in NKG2C^{+}

and NKG2C$^-$ NK cells under the different conditions tested (mean ± SEM, n=4) (*$p < 0.05$, **$p < 0.01$).

In order to analyze antigen presentation, NK cells and moDCs were pre-incubated overnight with HCMV in the presence or absence of HCMV$^+$ sera and subsequently used as antigen presenting cells in co-cultures with autologous CD4$^+$ T lymphocytes. TNFα and IFNγ production was monitored at 20 h by intracellular staining analysed by flow cytometry (**Figure 5**). NK cells pre-incubated with HCMV viral particles triggered the activation of a small percentage of CD4$^+$ T lymphocytes as detected by the simultaneous production of TNFα and IFNγ; a proportion that increased when NK cells were pre-loaded with HCMV in the presence of serum, though this trend did not reach statistical significance. Co-culture with autologous CD4$^+$ T lymphocytes did not promote cytokine production by NK cells (not shown). The average proportion of CD4$^+$ T cells activated by virus-loaded moDC was ten-fold higher in comparison to the activation achieved by antigen-presenting NK cells, and was not enhanced by HCMV$^+$ sera (**Figure 5**). The lack of CD8$^+$ T cell activation in response to HCMV-loaded NK cells, when using autologous PBMC instead of purified CD4$^+$ T cells as effectors, pointed to the involvement of HLA class II molecules in the observed NK cell-dependent CD4$^+$ T cell activation (**Figure 6A**). In fact, NK cell-induced CD4$^+$ T cell activation was partially blocked by an α-HLA-DR antibody (**Figure 6B**). Finally, CD4$^+$ T cell activation could not be detected in experiments with cells from HCMV-seronegative donors, supporting the requirement of an

expanded pool of antigen-experienced $CD4^+$ T cells capable of responding to HCMV pre-loaded NK cells (**Figure 6C**).

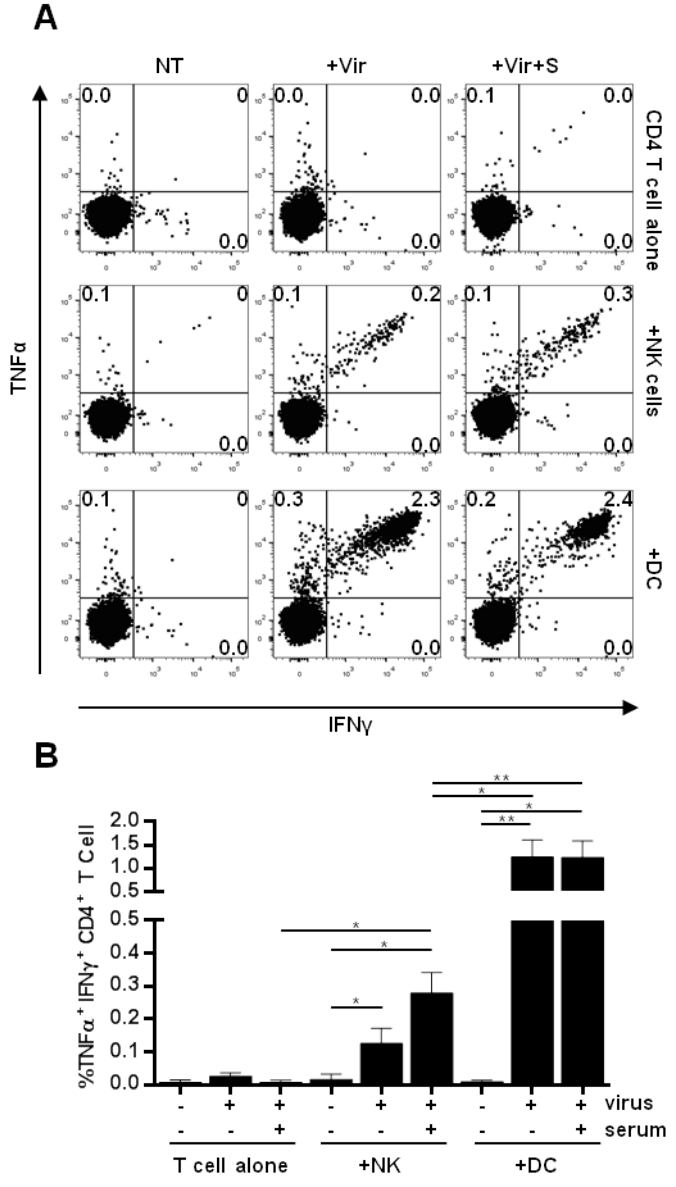


Figure 5. Comparison of $CD4^+$ T cell activation in response to NK cells or DCs. Purified NK cells previously loaded with HCMV-Ab immunocomplexes were cultured O/N with isolated autologous $CD4^+$ T cells in the presence of

Brefeldin A (2.5:1 T /NK cell). TNFα and IFNγ production by $CD4^+$ T cells was measured by intracellular staining analysed by flow cytometry. **A)** TNFα and IFNγ production by $CD4^+$ T cells in the different conditions. Data from one representative donor out of 5 tested. **B)** Mean frequency of $IFN\gamma^+$ and $TNF\alpha^+$ $CD4^+$ T cells upon activation with different APCs (mean ± SEM, n=5) (*$p < 0.05$, **$p < 0.01$).

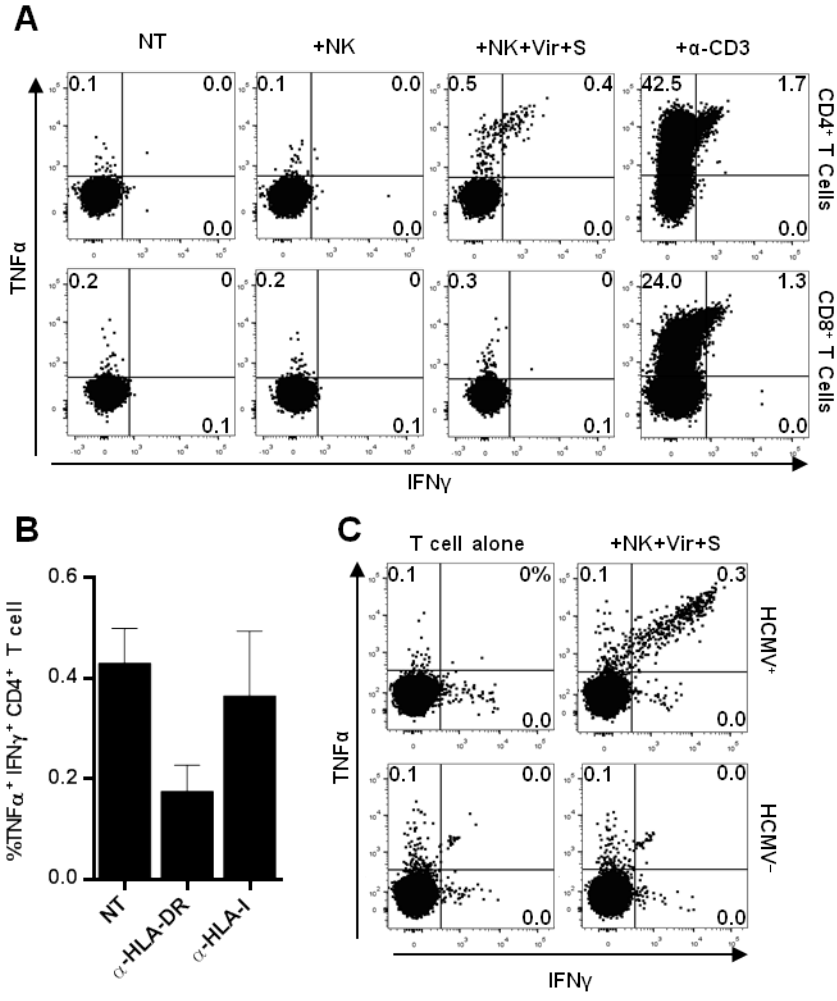


Figure 6. NK cell-mediated activation of $CD4^+$ T cells is HCMV specific and involves HLA-II-peptide complexes. NK cells previously loaded with HCMV-

Ab immunocomplexes were cultured O/N with PBMC (2.5:1 PBMC/NK cell), and TNFα and IFNγ production in $CD4^+$ or $CD8^+$ T cells was analysed by flow cytometry. α-CD3 antibody (clone SPV-T3b) was used as a positive control. **A)** Dot plots correspond to data from one representative seropositive ($HCMV^+$) donor out of 2 tested. **B)** Frequency of $IFN\gamma^+$ and $TNF\alpha^+$ $CD4^+$ T cells detected upon co-culture with NK cells previously loaded with HCMV-Ab immunocomplexes in the presence or absence of α-HLA-DR (clone D1.12) or α-HLA-I (clone W6/32) antibodies. **C)** Dot plots showing TNFα and IFNγ production by $CD4^+$ T cells from a pair of seropositive and seronegative ($HCMV^-$) donors out of 3 tested.

Overall, these data indicate that circulating NK cells can process HCMV virions and present HCMV-derived peptides to antigen-primed $CD4^+$ T cells in a process that may be enhanced by the presence of HCMV-antibody immunocomplexes.

3. HCMV-specific $CD4^+$ T cells activated by NK cells display an effector memory phenotype, lack the co-stimulatory CD28 receptor and have cytotoxic potential.

We analyzed the differentiation profile of $CD4^+$ T cells activated in response to antigen presenting NK cells by monitoring the expression of CCR7, CD45RA and of CD28 as previously defined (190,274). CCR7 and CD45RA expression define four T cell populations: naïve ($CD45RA^+$ $CCR7^+$), central memory ($CD45RA^-$ $CCR7^+$), effector memory ($CD45RA^-$ $CCR7^-$) and terminally differentiated effector memory (TEMRA) ($CD45RA^+$, $CCR7^-$) T cells, whereas CD28 negative circulating $CD4^+$ T cells have been

identified in individuals with chronic/persistent viral infections, particularly associated to HCMV (190). The majority of $CD4^+$ T cells activated in response to HCMV-loaded NK cells displayed an effector memory (EM) phenotype, and lacked CD28. On the other hand, both $CD28^+$ and $CD28^-$ effector memory $CD4^+$ T cells were activated upon co-culture with HCMV-loaded moDC (**Figure 7 A-B**). Thus, the activation of $CD28^-$ memory $CD4^+$ T cells by antigen presenting NK cells likely reflects their less stringent activation requirements and their capacity for responding to lower HLA class II-peptide levels. In agreement with the described association between CD28 down-regulation and acquisition of cytotoxic potential (190,275), $CD28^-$ $CD4^+$ T cells in HCMV seropositive donors were $perforin^+$ and granzyme B^+ with variable co-expression of the activating NK cell receptor NKG2D (**Figure 7C**) (85). Hence, NK cells can present HCMV-derived antigens in an HLA-DR restricted manner, specifically activating $CD4^+$ $CD28^-$ effector memory T cells with cytotoxic potential.

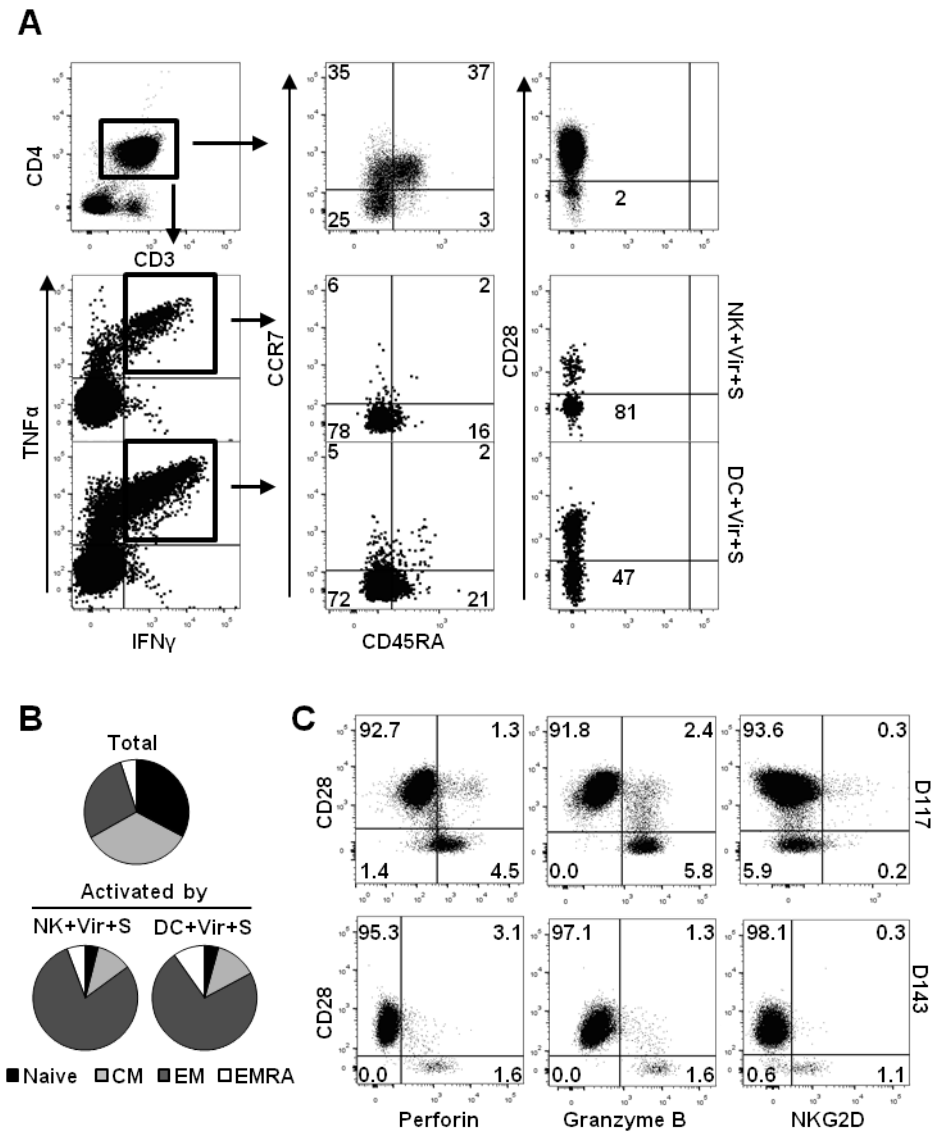


Figure 7. Differentiation and functional profile of HCMV-specific CD4$^+$ T cells activated by antigen-presenting NK cells. NK cells or moDC previously loaded with HCMV-Ab immunocomplexes were cultured with purified autologous CD4$^+$ T cells and the production of TNFα and IFNγ in combination with CD45RA, CCR7 and CD28 differentiation markers was analysed using specific mAbs by multiparametric flow cytometry. **A)** Dot plots showing

CD45RA, CCR7 and CD28 expression in total and activated $CD4^+$ T cells from a representative donor in the different conditions tested. **B)** Pie chart showing the distribution of total and NK cell-activated $CD4^+$ T cell subpopulations based on CCR7 and CD45RA (n=2). **C)** Perforin, granzyme B and NKG2D expression in $CD28^+$ and $CD28^-$ cells $CD4^+$ T cells from two representative $HCMV^+$ individuals.

4. Setting up an experimental system including expanded $CD4^+$ T cells and NK cells for the analysis of HLA class II-dependent antigen presentation.

An experimental system including *in vitro* expanded $CD4^+$ T cells and HLA-DR^+ NK lymphocytes was set up to further characterize the consequences of their cognate interaction during antigen presentation. HCMV-specific $CD4^+$ T cells were enriched by culturing PBMC from HCMV seropositive individuals with HCMV viral particles in the presence of low amounts of IL-2 as previously described (85). Under these conditions, $CD4^+$ T cells with an effector-memory phenotype containing high levels of perforin and granzyme B, accompanied by the variable expression of NKG2D and CD28, were obtained after 9-11 days (**Figure 8**). $NKG2C^+$ NK cells were expanded in parallel by co-culture with the .221-AEH cell line. After 9 days, the majority of expanded NK cells displayed $CD16^+$, HLA-DR^+, $CD86^+$ and were notably enriched in the $NKG2C^+$ NK cell subset (**Figure 9**). To reproduce the experimental conditions previously tested with primary lymphocytes, expanded NK cells pre-loaded with HCMV in the presence or absence of

HCMV$^+$ serum were co-cultured with CD4$^+$ T cells purified post-expansion. As shown in **Figure 10**, the proportion of CD4$^+$ T cells activated by NK cells was 12 to 52 fold higher on average in the absence or presence of HCMV$^+$ serum, respectively, as compared to co-cultures with primary NK and T cells, reaching up to 10% of total CD4$^+$ T lymphocytes. Also in this experimental setting, NK cell-dependent CD4$^+$ T cell activation could be blocked by an anti-HLA-DR antibody (**Figure 10B**) and no cytokine production was detected in NK cells (not shown).

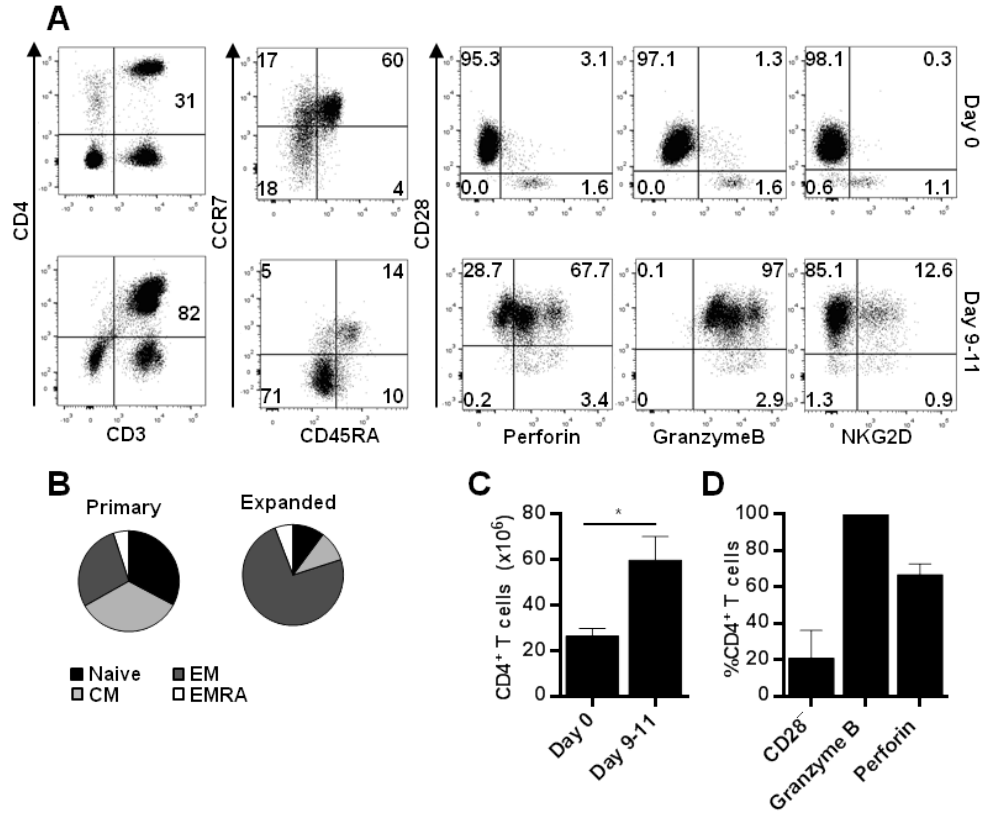


Figure 8. Cytotoxic CD4$^+$ T cells expand upon PBMC co-culture with HCMV. CD4$^+$ T cells were expanded by co-culturing PBMC from seropositive donors

with HCMV particles (MOI = 0.3) for 9-11 days with 25 U/ml IL-2. A) Dot plots showing CD45RA, CCR7 and CD28 expression versus perforin, granzyme B and NKG2D in $CD4^+$ T cells prior and after stimulation with HCMV. Data correspond to a representative donor out of 4 expanded. B) Pie charts showing the distribution of T cell subpopulations based on CCR7 and CD45RA expression prior and after expansion (mean, n=4). C) Average $CD4^+$ T cell numbers prior and after expansion (mean+SEM, n=6). D) Frequency of $CD28^-$, $perforin^+$ and granzyme B^+ $CD4^+$ T cells after expansion (mean+SEM, n=4).

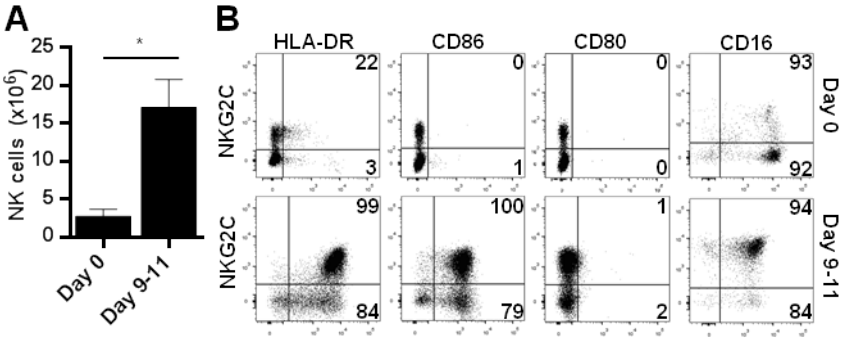


Figure 9. **$NKG2C^+$ $HLA\text{-}DR^+$ NK cell expansion with the $HLA\text{-}E^+$ 721.221-AEH cell line.** PBMC were cultured with irradiated 721.221-AEH lymphoblastoid cells for 9-11 days. **A)** Average NK cell numbers obtained upon expansion (mean+SEM, n=4). **B)** Dot plots displaying HLA-DR, CD86, CD80, CD16 and NKG2C in NK cells at days 0 and 9-11 (*$p < 0.05$).

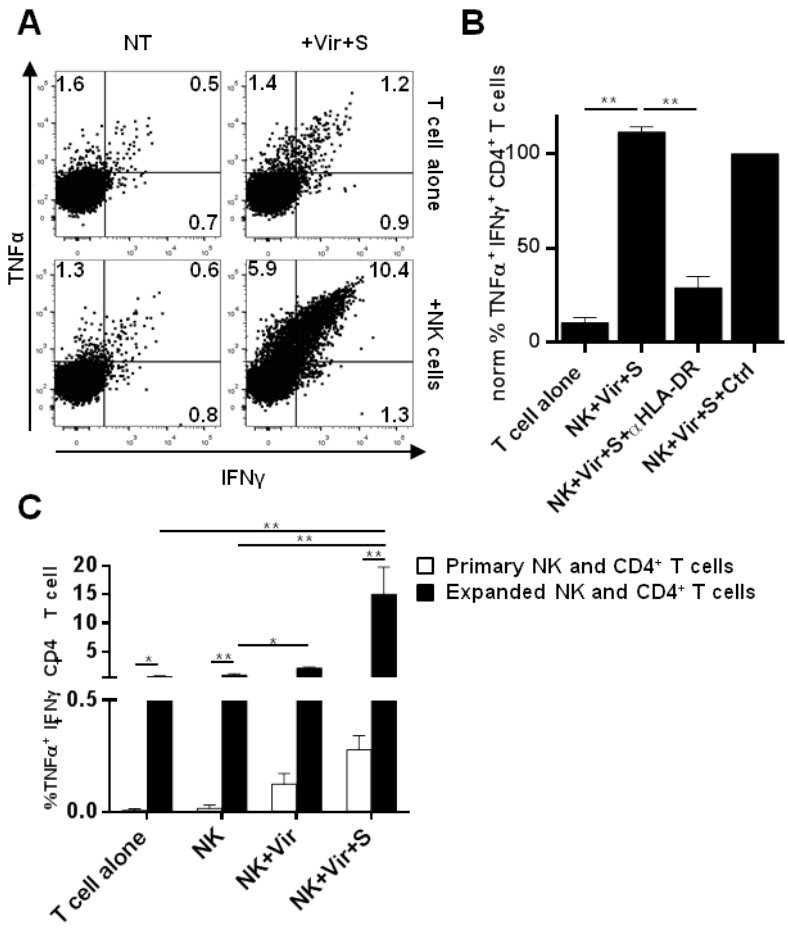


Figure 10. Antigen presentation by NK cells to autologous CD4$^+$ T cells using expanded cells. Expanded NK cells were purified and cultured O/N with HCMV in the presence of immune serum washed twice and co-cultured O/N with enriched HCMV-specific CD4$^+$ T cells. TNFα and IFNγ production was measured by intracellular staining analysed by flow cytometry. **A)** TNFα and IFNγ production in CD4$^+$ T cells from a representative donor. **B)** Frequency of TNFα$^+$ IFNγ$^+$ CD4$^+$ T cells in the different conditions tested. An α-HLA-DR (clone D1.12) and an isotype control antibody were included in some conditions (mean ± SEM, n=5). **C)** Comparison of the frequency of TNFα$^+$ IFNγ$^+$ CD4$^+$ T cells in

co-culture experiments using primary or expanded antigen presenting and effector cells (mean ± SEM, n=5) (*p < 0.05, **p < 0.01).

5. HCMV-antigen presenting NK cells trigger $CD4^+$ T cell polyfunctional activation including degranulation.

Based on the observation that $CD4^+$ $CD28^-$ T cells are endowed with cytotoxic potential, we next assessed whether HCMV-loaded NK cells could trigger $CD4^+$ T cell activation. For that purpose $CD4^+$ T cell degranulation as well as IFNγ and TNFα production were analysed by flow cytometry upon co-culture experiments including NK cells previously incubated with HCMV-antibody immunocomplexes and autologous $CD4^+$ T cells, both in the primary as well as in the expanded setting. In experiments with primary cells, moDC were also included as professional APC. As shown in **Figure 11A**, degranulation was detected in ~25% and 40% of HCMV-specific primary $CD4^+$ T cells respectively activated by NK and moDC antigen presenting cells. In most activated $CD4^+$ T cells degranulation was concomitant to cytokine production (**Figure 11E**). In co-cultures using expanded NK and $CD4^+$ T cells, the proportion of $CD4^+$ T cells degranulating in response to NK cell-mediated antigen presentation increased to up to ~80%. Also in this case, degranulation was accompanied by TNFα and IFNγ production (**Figure 11B/C/E**).

Collectively, this set of experiments explores a previously unrecognized facet of adaptive $NKG2C^{bright}$ NK cells in the context

of HCMV infection defense. Our results indicate that NK cells can directly process and present HCMV-derived antigens in an HLA-DR restricted manner, specifically activating $CD4^+$ $CD28^-$ effector memory T cells endowed with cytotoxic potential. Interestingly, this function appears preferentially mediated by adaptive $NKG2C^{bright}$ NK cells displaying low although persistent expression of surface HLA class II in homeostatic conditions and enhanced by the presence of HCMV specific antibodies. Our observations are in line with the recently appreciated role of other ILC subsets as HLA class II-dependent regulatory cells (276–279). Further experiments addressing the bidirectional consequences of NK cell-mediated antigen presentation to cytotoxic $CD4^+$ T cells are warranted to understand the putative role of this unconventional HLA class II–dependent antigen presentation pathway in the anti-viral defense along HCMV reactivation.

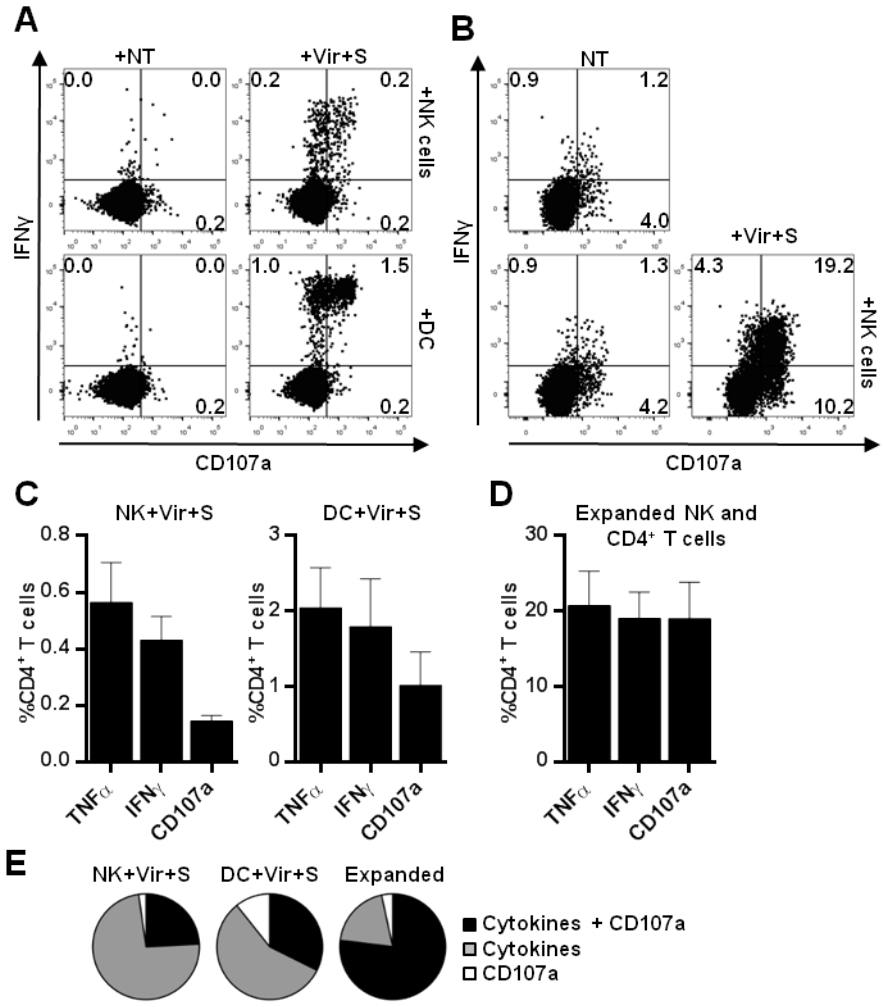


Figure 11. Primary and expanded HCMV-specific CD4$^+$ T cells degranulate in response to antigen loaded NK and moDC. TNFα and IFNγ production concomitant to CD107a mobilization was monitored by flow cytometry in co-culture experiments using HCMV-loaded primary NK cells or moDC and primary autologous CD4$^+$ T cells (**A, C and E**) or, alternatively, antigen loaded expanded NK cells and HCMV-expanded CD4$^+$ T cells (**B, D and E**). **A-B)** Dot plots displaying the proportions of IFNγ$^+$ CD107a$^+$ CD4$^+$ T cells in the different experimental systems used. Data from a representative experiment. **C-D)** Overall

frequency of TNFα^{+}, IFNγ^{+} and CD107^{+} CD4^{+} T cells in the different experimental systems assayed (mean+SEM; n=2(C), n=4(D)). **E)** Pie charts showing the distribution of CD4^{+} T cells that produce cytokines and degranulate against HCMV loaded antigen presenting cells in the different experimental systems used (mean; n primary system=2, n expanded system=4).

PART III

DISCUSSION AND CONCLUSIONS

CHAPTER 6: DISCUSSION

NK cells are lymphocytes that, together with T cells and B cells, play an essential role in the control of infection by HCMV. Supporting their contribution, patients with NK cell deficiencies show increased susceptibility to herpesvirus infections including HCMV (224), which establishes an asymptomatic persistent infection in immunocompetent individuals. During acute infection and latency, HCMV activates innate and adaptive immune mechanisms and impacts on lymphoid compartments, particularly T and NK cells. The mechanisms by which NK cells interact with viral particles, infected cells and other immune response players against viral infections are not fully understood. Results included in this thesis contribute to characterize this interaction at different levels: 1) NK cells are capable to directly recognize HCMV particles through different sensors; 2) HCMV specific antibodies target infected cells and trigger CD16-mediated NK cell activation; and 3) NK cells are activated by HCMV-antibody immunocomplexes (IC) and present antigens to specific $CD4^+$ T cells.

NK cells are conventionally considered effectors of innate immunity because they lack antigen-specific rearranged receptors and rely on germline encoded molecules recognizing PAMPs as well as alterations in the expression of self-proteins occurring along pathogen challenge, cellular stress or inflammation (91,280). In this regard, the study presented in the first part of this thesis reveals the involvement of TLR2 and autocrine type I IFN as mechanisms underlying the direct recognition of HCMV virions by NK cells,

which result in their priming evidenced by the acquisition of surface CD69 expression and the enhancement of anti-viral functions (i.e. increased IFNγ production and degranulation against HCMV-infected moDCs). Our results did not address whether HCMV sensing could vary among different NK cell subsets. Taking into account the enhanced capacity of adaptive NK cells to produce cytokines (265,273,281), their specific contribution to direct recognition of HCMV viral particles deserves attention.

CD69 up-regulation was dependent on autocrine type I IFN (IFN-I), while the enhancement of IL-12-dependent IFNγ production relied on both endosomal TLR2 activation and autocrine IFN-I production. Inactivation of the viral particles abolished CD69 up-regulation but only partially reduced IFNγ production, suggesting that infective viral particles were required for NK priming through autocrine type I IFN but not for TLR2-mediated HCMV recognition. TLR2 is directly activated by HCMV gB and gH (161), and its role in HCMV-infection control is indirectly supported by previously published observations. First, a single-nucleotide polymorphism (SNP) described in TLR2 gene has been associated with a significantly higher degree of CMV replication in liver transplant patients, and subjects homozygous for this polymorphism showed increased risk of CMV disease (166,282). Assessing the impact of the polymorphism in TLR2-mediated HCMV particle recognition by NK cells deserves further attention. Second, a recent report shows that HCMV miR-UL112-3p, which targets NKG2D-ligand MICB, also targets TLR2 and down-regulates its protein

expression levels in infected cells (283). While NK cells do not support a productive infection, the authors detected the presence of miR-UL112-3p in viral particles and also in sera from healthy individuals, opening the possibility to be delivered into NK cells, dampening its activation. In contrast, identity of the sensor triggering IFN-I production was not disclosed by our results. Polymorphisms in TLR3 and TLR9 genes have also been associated to HCMV infection susceptibility (167,168), and mouse models support their involvement in the recognition of MCMV (162,163,165). As NK cells express both TLRs, they may contribute to their capacity to sense HCMV. Taking into account the lack of response to UV-inactivated viral particles, cytosolic PRRs also appeared likely candidates. NK cells express RIG-I and MDA5 (106,107), which are known to recognize cytosolic dsRNA and dsDNA (through the RNA polymerase III as intermediate) (110,284). HCMV has also been reported to stimulate DNA sensors such as DNA-dependent activator of IRF (DAI) in fibroblasts (111). DAI activation by MCMV infection requires IE3-dependent transcription (HCMV IE2 homologue) (285). While dependency on viral transcription would fit with our observations showing that UV-inactivated virions did not trigger type I IFN response in NK cells, IE1/2 was not detected in NK cells cultured with HCMV virions.

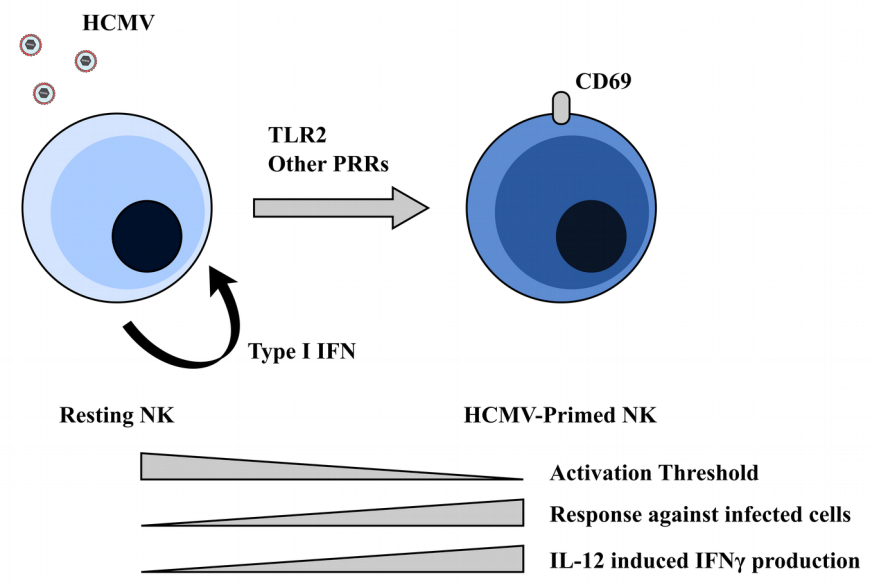


Figure 6. Direct HCMV sensing by NK cells.

In our experiments, NK cells failed to recognize HCMV-infected MRC5. The study presented in the second part of the thesis demonstrates that HCMV-specific antibodies significantly enhance the capacity of NK cell to recognize and respond against HCMV-infected cells. Despite the evident down-regulation of surface HLA-I in HCMV-infected cells, their direct recognition by NK cells was rather modest likely owing to a number of immunoevasion mechanisms developed by the virus. The synergism between NK cells and IgGs enhances the immune response to HCMV infection.

Redirected degranulation assays showed that NK stimulation with HCMV viral particles enhanced the activity of several receptors, including CD16, suggesting that priming with HCMV particles could contribute to antibody-dependent NK cell responses to

infected cells. In our experimental system by co-culturing NK cells with HCMV-infected MRC5 at 48-72 hours (h) post infection (p.i.), NK cells may interact with both infected cells and HCMV particles. While assays measuring NK degranulation and TNFα production may be too short to allow ADCC modulation by viral particles, these may have an effect in delayed IFNγ and TNFα production. Priming of antibody-dependent NK cell functions by viral particles deserve further attention.

Our results highlight the sensitivity of NK cell-mediated ADCC-mechanisms. We detected NK cell ADCC against infected cells as early as 12 h p.i. and the response increased along the viral cycle (until 48-72 h). The temporal characterization of antibody-dependent NK cell response against HCMV-infected cells allowed us to address different aspects of this interaction. While blockade of HLA-I by a mAb capable of interfering with specific inhibitory receptors (i.e. KIR2DL and/or ILT2) indicated their ability to negatively regulate IgG-mediated activation of NKG2C^{bright} NK cells at early times p.i. (12 h), experiments using the ΔUS2-11 HCMV deletion mutant, which preserves surface HLA-I levels on infected cells, showed no influence at later times p.i. (48-72 h). In parallel, the density of HCMV antigens on the surface of infected cells recognized by polyclonal IgG from seropositive donors as detected by flow cytometry increased progressively, reaching the highest levels at 72 h p.i., correlating with the magnitude of the NK cell response. Coinciding, structural HCMV proteins including

those targeted by neutralizing antibodies are generally expressed at late stages of the viral cycle (157), suggesting their contribution to NK cell-mediated ADCC.

The set of HCMV antigens recognized by IgG in seropositive individuals has not been fully characterized. Generally, studies have focused on some immunodominant antigens (i.e. pp150 or IE1) and in viral proteins targeted by neutralizing antibodies, including gB, gH and gM (286–288). The only study that systematically screened the entire HCMV proteome to visualize the epitope profile targeted by antibodies took advantage of peptide microarray technology to analyze the breadth and dynamics of the HCMV-specific humoral response in HSCT transplant recipients (219). The identification and characterization of viral antigens on the surface of infected cells targeted by the humoral response with ADCC capacity remains largely unexplored. Putative candidates would include several HCMV viral proteins described to reach/traffic through the infected cell surface along productive infection (i.e. gB, gH, gL, gO, gM, gN, RL11, UL11, UL78, UL119, UL132, US28 (205,222,287,289–294)) or shown to be displayed on the surface of transfected cells (i.e. with RL12, UL16, UL33, UL37, UL133, UL135, UL142, UL144 and US27 (221,295–301)). In addition to components of the glycoprotein complexes on the viral envelope, a number of these molecules have been related to immunoevasion mechanisms that require their location on the plasma membrane. Some examples are viral chemokine receptor homologues (e.g. UL78, UL33, US27, US28) and UL11, which has been reported to interact with CD45 in

T cells reducing activation and promoting IL-10 secretion (302,303). Some of these viral proteins are rapidly internalized remaining in endosomal vesicles close to the plasma membrane (e.g. UL16, UL37, UL33, US27 and UL78) (292,295,297,301). Viral protein internalization may constitute a novel immunoevasion mechanism for reducing ADCC responses along HCMV infection, as previously suggested for HIV infection (304).

Neutralizing antibodies prevent HCMV propagation through cell-free particles, but show limited efficacy to reduce cell-cell transmission (305). In contrast, ADCC targets infected cells, regardless of the viral propagation mechanism. The capacity of NK cell-mediated ADCC to target cells in the initial steps of the infection process, before the virion-producing machinery is set up, might be important to prevent pathogen dissemination. Although the majority of proteins reported to be detectable on the plasma membrane of infected cells are expressed late in the viral cycle, some are expressed earlier, including UL78, UL16 and UL37 which might be responsible for the activation detected at 12 h p.i (157,159). Other molecules (e.g. UL133 and UL135) are also expressed early but are encoded within the Ulb' region (157), absent in AD169 HCMV strain (154), and despite not contributing to the observed response in our experimental conditions, might be relevant for HCMV infection control. Studies are required to precisely define the breadth and nature of the antigens targeted by antibodies capable of eliciting an efficient ADCC response against HCMV-infected cells, valuable in the context of vaccine development.

Recent studies highlighted the intrinsic increased responsiveness of adaptive NK cells to signaling through CD16, showing enhanced antibody-mediated responses against HCMV-infected cells (136,137). Monitorization of NKG2C^{bright} contribution to antibody-mediated responses confirmed their capacity to efficiently degranulate against antibody-coated target cells and their particular proficiency to produce cytokines upon activation as compared to NKG2A^{+} NK cell subset. Remarkably, NKG2C^{bright} NK cells not only showed greater Ab-driven cytokine secretion, but also produced higher levels of TNFα and IFNγ upon CD16-independent activation.

Previous studies comparing direct cytotoxicity against HCMV-infected cells showed a lower activation of NKG2C^{bright} compared to NKG2A^{+} NK cells (243,266). Accordingly, we also observed lower degranulation in NKG2C^{bright} NK cells against HCMV-infected fibroblasts compared to NKG2A^{+} NK cells. It has been discussed (243) that NKG2C^{bright} NK cells may be more repressed compared to NKG2A^{+} NK cells due to their expression of HLA-I-specific receptors (i.e. KIR, LILRB1) and lower surface expression of the activating NCR (75), involved in the recognition of HCMV-infected cells (243). In this regard, HLA-I blockade concomitant to ADCC against HCMV-infected fibroblasts at 12 hours p.i. increased only NKG2C^{+} NK cell activation, supporting the involvement of inhibitory KIRs and/or LILRB1 in the control of this NK cell subset. In contrast, infection with a ΔUS2-US11 HCMV mutant revealed that during late infection, when antibody recognition of

infected cells was considerably increased, antibody-mediated activation overcame HLA-I-dependent inhibitory mechanisms controlling NKG2C^{bright} NK cells.

Two different mechanisms may contribute to the increased cytokine production capacity of NKG2C^{bright} NK cells: 1) differentiation-driven epigenetic modifications on cytokine loci as regulators of their effector program; and 2) preferential coupling of CD16 to CD3ζ as a consequence of FcεRIγ loss. The rationale behind the latter is that CD3ζ contains three ITAM motifs while FcεRIγ contains only one (262). However, several observations support a major involvement of the first mechanism. First, it has recently been described that *IFNG* and *TNF* loci are hypomethylated in HCMV-associated adaptive NK cells, reducing the signaling threshold for induction of cytokine expression (265,281). Second, we showed that NKG2C^{bright} NK cells produced more TNFα in response to Ab-dependent activation regardless of their FcεRIγ levels (271). Moreover, increased IFNγ and TNFα production by NKG2C^{bright} NK cells were detected upon CD16-independent stimulation with phorbol-12-myristate-13-acetate (PMA) and Ionomycin (data not shown).

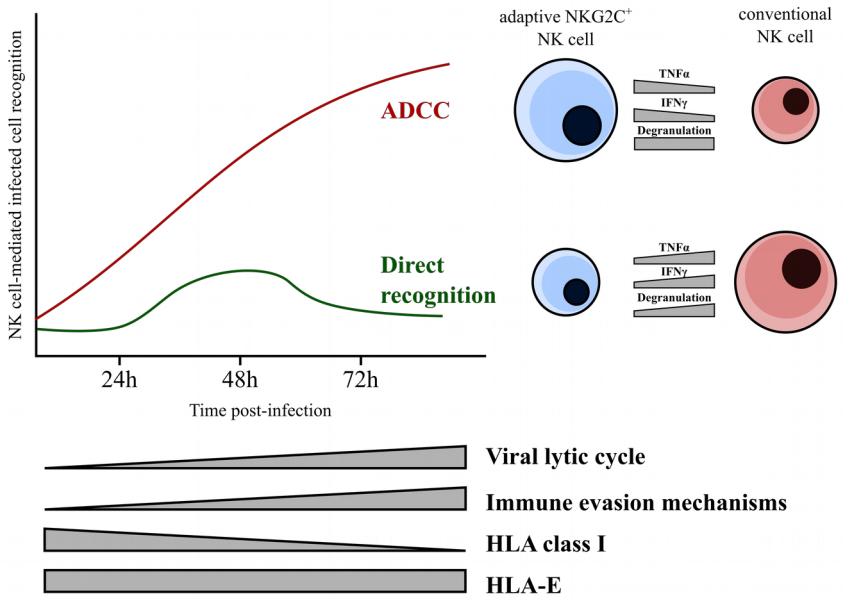


Figure 7. HCMV-infected cell recognition by NK cells along lytic viral cycle, contribution of conventional and adaptive NKG2C^{+} NK cell subsets.

In addition to the detection of infected cells, antibodies also enhanced NK cell activation by cell-free viral particles. It has been previously reported that γδ^{+} T cells expressing CD16 were activated by IgG-opsonized HCMV viral particles and produced IFNγ (306). Similarly, NK cells were activated by low concentrations of HCMV immune complexes (<3 infective viral particles/NK cell) as indicated by the production of TNFα over degranulation and IFNγ, which was dominated by the NKG2C^{bright} NK cell subset.

NK cells belong to the newly defined Innate lymphoid cells (ILCs) family based on their functional characteristics and development relationship (4). Recent studies have reported the expression of MHC class II molecules in ILC2 and ILC3 subsets demonstrating

their capacity to process and present antigens to $CD4^+$ T cells as an important regulatory mechanism along intestinal homeostasis (307) and immunity against helminths (278). The results presented in the third part explore the capacity of NK cells to present HCMV peptides to $CD4^+$ T cells. HLA class II expression on a fraction of resting NK cells in peripheral blood was described long time ago (308–310). The proportion of HLA class II^+ NK cells varied between reported studies, ranging from less than 1% (309) to 21% (308). More recently, the analysis of transcriptional programs in adaptive NK cells identified antigen presentation of peptides via MHC class II as a developed functional pathway in this NK cell subset (e.g. enriched transcripts: CIITA, HLA-DQ, HLA-DP, HLA-DMA and HLA-DRA) (264,265). Our results showed that $NKG2C^{bright}$ NK cells concentrate baseline HLA-DR expression, which remained stable on fresh NK cells at least for a six-month follow up and was not associated to CD69, coinciding with a previous report (310), or CD25 activation markers. These observations suggest that HLA-DR is associated to a differentiated phenotype rather than reflecting a transient activation. It was recently proposed that $NKG2C^{bright}$ NK cell subset undergoes sequential differentiation associated to down-regulation of transcription factors (e.g. PLZF1), signaling molecules (e.g. FcεRIγ), surface NKR (e.g. NKp30, NKp46 and CD161), as well as acquisition of LILRB1 and CD57 (259,264,265,271). HLA-DR expression in $NKG2C^{bright}$ NK cells was not related to FcεRIγ levels or any differentiation marker analyzed (i.e. FcεRIγ, NKp30,

NKp46, CD161, CD57 and LILRB1), supporting the acquisition of HLA-DR expression by NKG2C^{bright} NK cells as another consequence of the HCMV-induced epigenetic diversification previously described in adaptive NK cells (265). A recent study showed that enhanced NKG2C^{bright} NK cell capacity to produce TNFα was independent of FcεRIγ loss (271). Taken together, these results suggest that functional features are acquired throughout the NKG2C^{bright} expansion, prior to down-regulation of the adapter molecule.

The role of HLA class II in NK cells remains largely unexplored, and the majority of studies addressing antigen presentation on NK cells rely on the use of bacterial-derived soluble peptides and superantigens such as *Staphylococcus aureus* protein A (SpA) and *Staphylococcal enterotoxin* B (SEB) (308,311–313). Few studies have addressed the role of HLA class II on NK cells in the context of viral infections. Kim and colleagues (314) showed that NK cells up-regulated HLA-DR and HLA-DQ upon activation by UV-inactivated HSV1/2 or upon incubation with immunodominant HSV2 glycoprotein D conjugated to a TLR2 agonist, inducing IFNγ production in autologous CD4^{+} T cells. Although the authors did not demonstrate the antigen specificity of the interaction, they showed a physical contact and establishment of an immunological synapse between antigen-stimulated NK cells and CD4^{+} T lymphocytes.

We developed an experimental system where NK cells were incubated with viral particles in the presence of HCMV-specific

antibodies and cultured with autologous $CD4^+$ T cells. Our results indicate that NK cells interact with the ICs, resulting in TNFα production and HLA-DR up-regulation. HCMV-loaded NK cells were able to activate specific $CD4^+$ T cells inducing the production of IFNγ and TNFα. IFNγ, characteristic of a Th1 profile, is the main cytokine used to identify HCMV-specific $CD4^+$ T cells (172) and, accordingly, IFNγ production was detected in a majority of activated $CD4^+$ T cells. Production of other cytokines such as IL-4, IL-17 or IL-10 by a minority of HCMV-specific $CD4^+$ T cells has also been described (186,187,315). Because we only monitored IFNγ and TNFα production, the main anti-viral cytokines produced by HCMV-specific $CD4^+$ T cells, we cannot exclude that NK cells may also activate a fraction of $CD4^+$ T cells producing a different cytokine profile.

We compared the NK cell capacity to activate $CD4^+$ T cells with that of autologous moDC as professional APC. $CD4^+$ T cells activated by NK cells and moDC showed similar functional profile, although the magnitude of the response induced by professional APCs was significantly higher, similar to previous reports (189,316,317). NK cells incubated with specific IgG in addition to HCMV viral particles up-regulated surface HLA-DR and induced a higher $CD4^+$ T cell response compared to NK cells incubated with the viral particles alone, suggesting that interaction of IC with CD16 facilitated viral antigen uptake/processing/loading onto HLA class II. The capacity of FcγR to enhance antigen uptake has been extensively described in APCs such as DCs and macrophages

(318,319). In our experiments, DCs incubated with viral particles forming ICs up-regulated HLA-DR and CD86 as compared to DCs incubated with viral particles alone, but induced similar $CD4^+$ T cell activation. It is conceivable that in the experimental conditions used, DC activated the majority of HCMV-specific $CD4^+$ T cells regardless of the antigen uptake mechanism while NK cells required antibodies to optimize antigen presentation.

$CD4^+$ T cells activated by both NK cells and DCs showed mainly a differentiated effector memory phenotype characterized by absence of CD45RA and CCR7 expression, in line with previous reports describing HCMV-specific $CD4^+$ T cells (186,187,189). In addition, CD28 loss marks terminally differentiated T cells, a phenotype that is markedly enriched in HCMV-specific $CD8^+$ and $CD4^+$ T cells (320); accordingly, $CD4^+$ T cells activated by NK cells were mainly $CD28^-$. Memory T cells are responsive to TCR stimulation in the absence of co-stimulatory signals (321); yet, several molecules have been proposed to enhance the effector potential in the absence of CD28. Natural Killer Receptors (NKRs) and particularly NKG2D are expressed by a fraction of $CD4^+$ T cells with cytotoxic potential that expands *in vitro* when PBMC from healthy seropositive donors are stimulated with HCMV (85). In this system, NKG2D synergized with TCR-dependent activation of $CD4^+$ T cells, triggering proliferation and pro-inflammatory cytokine production, supporting the role of NKG2D as a co-stimulatory receptor in a subset of HCMV-specific $CD4^+$ T cells. Nonetheless, NKG2D expression on HCMV-specific $CD4^+$ T cells was rather variable, and was not

strictly associated to cell activation by HCMV-loaded NK cells (data not shown).

As previously reported (320), perforin and granzyme B expression correlated with CD28 down-regulation in $CD4^+$ T cells, pointing out the cytotoxic potential of HCMV-specific $CD4^+$ T cells, which degranulated upon NK-cell mediated activation. Cytokine production predominated over degranulation independently of the APC used in the assay, similar to previous reports using HCMV-infected cells (187) or PBMCs stimulated with viral lysate (322) as APCs.

Interaction of APCs and T cells through TCR triggers a cross-talk involving contact-dependent and soluble factors, activating both cells (323) with different outcomes depending on the cell types involved. DC-$CD4^+$ T cell interaction induces up-regulation of antigen presentation-related molecules and pro-inflammatory cytokine production by the APC (324), while ILC2-$CD4^+$ T cell interaction promotes APC proliferation and IL-13 secretion (278). In contrast, activation of cytotoxic $CD4^+$ T cells can induce apoptosis in the APC, as it has been shown for B cells (325), lymphoblastoid cell lines (186,326), glioblastoma cell line (191,327) and moDC (328). While in the context of viral reinfection/reactivation elimination of APCs supporting a productive infection may prevent further spread of the virus, the resistance of the APC to cytotoxic effector mechanisms may extend their antigen presenting function and, hence, the production of Th1

anti-viral cytokines by $CD4^+$ cells. Cytotoxic lymphocytes possess some degree of resistance to the cytolytic mediators that prevents self-destruction upon activation (329–331). The mechanisms behind this resistance are not well-understood, although different molecules have been identified in cytotoxic lymphocytes that interfere with the function of perforin and granzyme B (332–337). LAMP1 (also known as CD107a), protein disulfide isomerases and cathepsin B are molecules that translocate to the lymphocyte cell surface upon degranulation and protect the cell from degranulation-associated damage by interfering with perforin function (332–334). Complementary, serpinB9 (also known as Protease Inhibitor 9) is the only described inhibitor of human granzyme B (335–337). SerpinB9 is an intracellular protein present in several immune cell types: particularly expressed by CTLs, NK cells and some DC subsets. Evaluation of NK cell susceptibility to cytotoxic $CD4^+$ T cells is ongoing and should help to understand the consequences of their interaction as well as their role as APC.

In summary, our results reveal the complexity of NK cell interaction with HCMV that involves direct sensing of viral particles and antibody-mediated recognition of infected cells, resulting in NK cell activation, enhanced cytotoxicity and pro-inflammatory cytokine production. In addition, we describe for the first time the capacity of NK cells incubated with HCMV IC to present viral antigens to HCMV-specific $CD4^+$ T cells, triggering their effector functions. Our work also contributes to characterize the specialization of the HCMV-induced $NKG2C^{bright}$ NK cell subset to mediate antibody-

dependent responses, especially the production of pro-inflammatory cytokines. Additionally, our data indicate that NKG2C^{bright} NK cells are competent to present HCMV antigens to CD4^{+} T cells, culminating in the release of Th1 cytokines. Altogether, our results point out that the NK cells, and particularly the adaptive NKG2C^{bright} subset, may play a role in the control of viral infections by secreting key anti-viral cytokines and promoting Th1 responses.

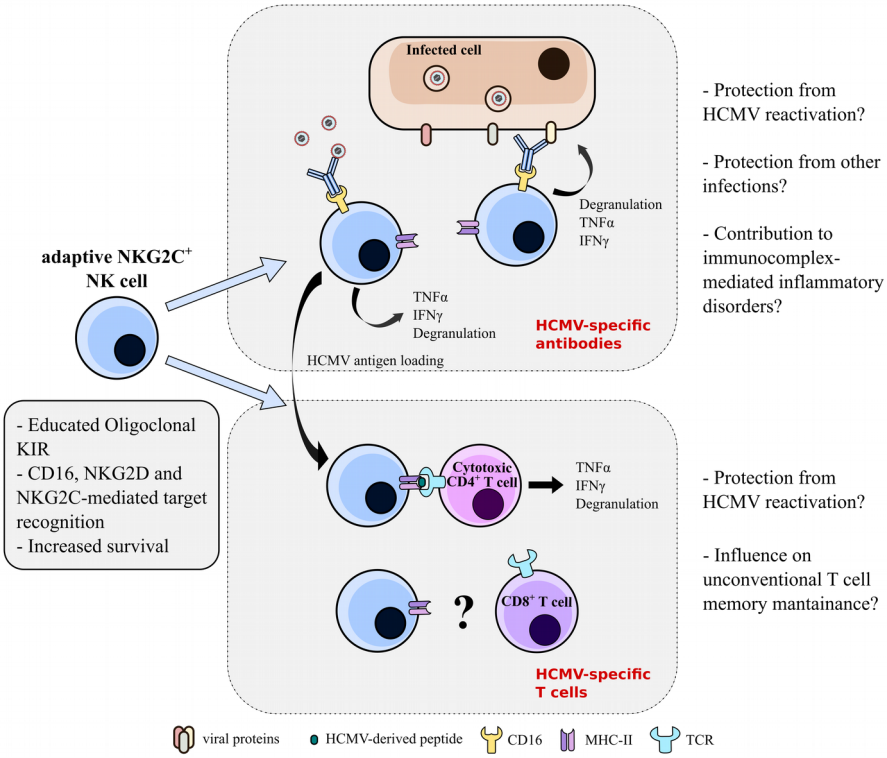


Figure 8. Crosstalk between adaptive NKG2C^{+} NK cells and the adaptive immune response to HCMV.

CHAPTER 7: CONCLUSIONS

1. Interaction with HCMV particles primes NK cells for the development of effector functions by a mechanism involving TLR2 and type I IFNs.
2. HCMV-specific antibodies enhance NK cell degranulation and cytokine production (i.e. TNFα and IFNγ) against infected fibroblasts, overcoming viral immune evasion strategies.
3. The antibody-dependent NK cell response to HCMV-infected cells can be detected early after infection (12h), progressively increasing along the lytic cycle development.
4. In response to antibody-coated target cells, adaptive NKG2C^{+} NK cells display a more proficient cytokine production than other NK cell subsets.
5. HCMV particles in the presence of specific Abs activate NK cells, particularly the adaptive NKG2C^{+} subset, promoting TNFα production and, to a lesser extent, degranulation and IFNγ secretion.
6. HLA-DR expression in circulating adaptive NKG2C^{+} NK cells is variable in healthy donors, and appears uncoupled from other activation (e.g. CD69, CD25) or differentiation markers (e.g. FcεRI γ-chain down-regulation).
7. NK cells can present HCMV antigens to specific CD4^{+} T cells, a process that is enhanced by specific antibodies.
8. CD4^{+} T cells activated by antigen-presenting NK cells are polyfunctional CD28^{-} effector-memory cells, with cytotoxic/Th1 functional profiles.

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ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen-presenting cell
CD	Cluster of differentiation
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
E	Early
FcR	Fc-receptor
HCMV	Human cytomegalovirus
HCV	Hepatitits C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
IC	Immunocomplex
IE	Immediate-early
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
ILC	Innate lymphoid cells
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer Immunoglobulin-like receptors

L	Late
LILR	Leukocyte Immunoglobulin-like Receptor
mAb	Monoclonal antibody
MCMV	Murin cytomegalovirus
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptors
NK	Natura Killer cells
p.i.	Post-infection
PBMC	Peripheral blood mononuclear cells
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UL	Unique long
US	Unique short

APPENDIX

LIST OF PUBLICATIONS

Publications included in the thesis

1. Muntasell A, **Costa-Garcia M**, Vera A, Marina-Garcia N, Kirschning CJ, López-Botet M. Priming of NK cell anti-viral effector mechanisms by direct recognition of human cytomegalovirus. Front Immunol. 2013 Feb 21;4:40.
2. **Costa-Garcia M**, Vera A, Moraru M, Vilches C, López-Botet M, Muntasell A. Antibody-mediated response of NKG2C^{bright} NK cells against human cytomegalovirus. J Immunol. 2015 Mar 15;194(6):2715-24.

Publications not included in the thesis

3. López-Botet M, Muntasell A, Martínez-Rodríguez JE, López-Montañés M, **Costa-García M**, Pupuleku A. Development of the adaptive NK cell response to human cytomegalovirus in the context of aging. Mech Ageing Dev. 2016 Sep;158:23-6.
4. Pupuleku A, **Costa-García M**, Farré D, Hengel H, Angulo A, Muntasell A, López-Botet M. Elusive Role of the CD94/NKG2C NK Cell Receptor in the Response to Cytomegalovirus: Novel Experimental Observations in a Reporter Cell System. Front Immunol. 2017 Oct 24;8:1317.