

Influence of the *NKG2C* genotype in the  
development of adaptive NK cells and  
characterization of the antibody-mediated  
response to Epstein-Barr virus.

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A mi familia



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## THESIS ABSTRACT

Human cytomegalovirus (HCMV) promotes to a variable degree a redistribution of the Natural Killer (NK) cell compartment hallmarked by increased proportions of mature NK cells displaying high expression levels of the CD94/NKG2C activating receptor. The mechanisms underlying the differentiation/expansion of NKG2C<sup>+</sup> NK cells and their implication in the response to other pathogens are open issues. We analyzed the putative influence of *NKG2C* gene (*KLRG2*) deletion on the HCMV-induced NK cell repertoire reconfiguration and CD94/NKG2C receptor function. NKG2C<sup>+/+</sup> HCMV<sup>+</sup> individuals displayed increased proportions and absolute numbers of adaptive NKG2C<sup>+</sup> NK cells as compared to hemizygous subjects, independently of HLA-E dimorphism. Moreover, *NKG2C* gene dose influenced early signaling events, degranulation and IL-15-dependent proliferation in response to CD94/NKG2C receptor engagement. These results further supported an active role of the CD94/NKG2C receptor in the adaptive NK cell response.

NKG2C<sup>bright</sup> NK cells display efficient antibody-dependent functions against HCMV-infected cells. We characterized the NK cell response triggered by serum antibodies specific for Epstein-Barr virus (EBV), evaluating the contribution of adaptive NKG2C<sup>+</sup> NK cells. EBV<sup>+</sup> sera triggered vigorous NK-cell degranulation and cytokine secretion (i.e. TNF- $\alpha$  and IFN- $\gamma$ ) in response to EBV-infected B cells in lytic viral cycle, as compared to direct NK cell activation. The EBV-specific antibody-driven cytokine response was dominated by adaptive NKG2C<sup>+</sup> NK cells. Binding of gp350/220 viral antigen-containing vesicles, released by EBV-infected cells, to B lymphocytes triggered antibody-dependent degranulation and TNF- $\alpha$  production, but induced low levels of IFN- $\gamma$  secretion and target cell damage. These results evidenced the potential of antibody-driven NK cell activation in the control of EBV infection suggesting that gp350<sup>+</sup> vesicles may divert the cytotoxic machinery, potentially favoring viral immune evasion.

## RESUMEN DE LA TESIS

El citomegalovirus humano (HCMV) promueve una redistribución del compartimento de células Natural Killer (NK) marcada por un incremento de las células NK maduras que expresan niveles elevados del receptor activador CD94/NKG2C. Los mecanismos subyacentes a la diferenciación/expansión de las células NK NKG2C<sup>+</sup> y su implicación en la defensa anti-viral son cuestiones no resueltas. Analizamos la influencia de la delección del gen *NKG2C* (*KLRG2*) en la reconfiguración del compartimento NK y en la función del receptor CD94/NKG2C. Los individuos *NKG2C*<sup>+/+</sup> HCMV<sup>+</sup> presentaron mayor porcentaje y número de células NKG2C<sup>+</sup> comparados con los individuos hemicigotos, independientemente del dimorfismo de HLA-E. Además, la dosis génica de *NKG2C* influyó en la movilización de Ca<sup>2+</sup>, degranulación y en la proliferación dependiente de IL-15 tras la activación vía NKG2C. Estos resultados apoyan un papel activo del receptor CD94/NKG2C en el desarrollo de la respuesta NK adaptativa.

Las células NKG2C<sup>+</sup> presentan una respuesta eficaz contra células infectadas por HCMV en presencia de anticuerpos específicos. Caracterizamos la respuesta de las células NK mediada por anticuerpos específicos para el virus Epstein-Barr (EBV), evaluando la contribución de las células NKG2C<sup>+</sup> adaptativas. Los sueros EBV<sup>+</sup> promovieron una potente degranulación y secreción de citocinas (TNF- $\alpha$  e IFN- $\gamma$ ) por parte de las células NK contra células B infectadas en la fase de ciclo lítico, siendo la secreción de citocinas mediada por anticuerpos dominada por las células NKG2C<sup>+</sup> adaptativas. La unión de vesículas liberadas por células infectadas por EBV, que contenían el antígeno viral gp350/220, a linfocitos B promovió la degranulación y secreción de TNF- $\alpha$  dependiente de anticuerpos, induciendo bajos niveles de IFN- $\gamma$  y citotoxicidad. Estos resultados muestran el potencial de la respuesta mediada por anticuerpo de las células NK en el control de la infección por EBV, y sugieren que las vesículas gp350<sup>+</sup> pueden desviar la maquinaria citotóxica, interfiriendo con la defensa anti-viral.

## **PREFACE**

Human Cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) cause highly prevalent and lifelong infections. In immunocompetent individuals, primary infection by both herpesviruses is generally asymptomatic, followed by the establishment of viral latency and occasional reactivation episodes. EBV and HCMV infection/reactivation become a threat in immunocompromised patients. HCMV has been related with the development of atherosclerosis and immunosenescence. EBV contributes to the development of hematopoietic and epithelial neoplasms, being associated with some autoimmune disorders. T, B and NK cells contribute to control both infections and the pathogens have reciprocally evolved a variety of immune evasion strategies, favoring their successful persistence and transmission. NK cell effector functions are triggered upon direct interaction with target cells, regulated by activating and inhibitory receptors. Moreover, NK cells mediate specific IgG-dependent cytotoxicity and cytokine production triggered through FcγR-IIIa (CD16). HCMV promotes to a variable extent in different individuals a persistent adaptive redistribution of the NK cell compartment, characterized by the differentiation and expansion of an NK cell subset which displays high levels of the CD94/NKG2C receptor, together with other differential phenotypic and functional features. Unraveling which factors underlie the development of adaptive NKG2C<sup>+</sup> NK cells and their putative influence on the immune response to other pathogens and tumors is warranted to understand their role in health and disease.



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**PART I**  
**INTRODUCTION AND AIMS**



## Chapter 1

### Introduction

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## 1. IMMUNOBIOLOGY OF NATURAL KILLER CELLS

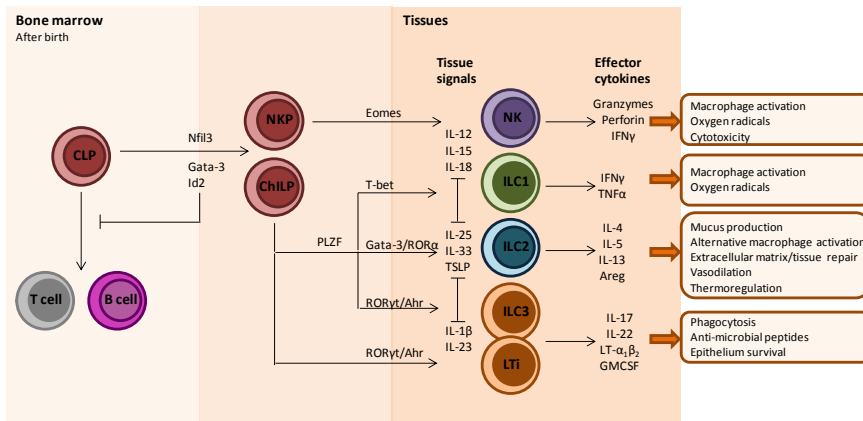
### 1.1 NK cell characterization, distribution and development

In 1975, Rolf Kiessling, Hans Wigzell (Karolinska Institute, Sweden) and Ronald Herberman (NCI-NIH, USA) described Natural Killer (NK) cells as large granular lymphocytes with the capacity to kill transformed cells without previous stimulation *in vivo* or *in vitro*, displaying spontaneous (natural) cytotoxicity against tumors<sup>1,2</sup>. NK cells were later identified as a separate lymphocyte lineage of the innate immune system which contributes to the control of a variety of pathogens<sup>3</sup>.

NK cells display cytotoxicity and cytokine-producing effector functions. NK cell activation is regulated by a repertoire of germ-line encoded surface receptors that recognize their ligands on the target cell surface and by cytokines present in the microenvironment<sup>4</sup>. NK cells are primarily found in peripheral blood, spleen, liver, lung and bone marrow whereas a limited number are localized in lymph nodes and mucosal associated lymphoid tissues (e.g. tonsils)<sup>5</sup>.

More recently, NK cells have been included within an expanded family of lymphocytes known as innate lymphoid cells (ILCs). ILCs are distributed in lymphoid and non-lymphoid tissues across multiple species, coupled with their functional heterogeneity. All members of the ILC family are characterized by lymphoid cell morphology, and lack the expression of antigen-specific receptors. The ILC family comprises canonical/conventional “cytotoxic” NK cells and “non-cytotoxic” ILC, subdivided in three subsets (ILC1, ILC2 and ILC3) based on their differential requirements for transcription factors during development and their secretion pattern of effector cytokines. Perforin and Granzyme B expression as well as the capacity to kill target cells are cardinal features to distinguish NK cells from other ILC subsets<sup>6,7</sup>.

NK cells are generated from CD34<sup>+</sup> bone-marrow resident hematopoietic stem cells (HSC) through common lymphoid progenitors (CLP). CLP give rise to B, T, NK cells, and common helper ILC precursors (ChILPs). The development of ILCs is driven by the expression of Nfil3, Gata3 as well as Id-2 transcription factors. The expression of Eomes determines NK cell development whilst the expression of the PLZF regulates the differentiation of non-cytotoxic ILCs, except for lymphoid tissue inducer (LTi) cells which are PLZF-independent<sup>6,7</sup> (**Figure 1**).

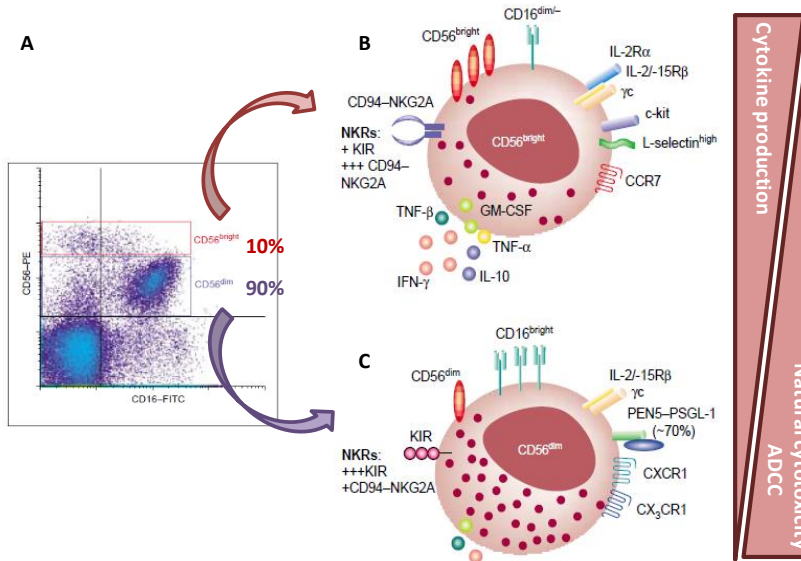


**Figure 1:** Development, activation and function of ILCs. Based on Eberl et al. Science 2015<sup>6</sup>.

Human NK cells comprise around 15% of total blood lymphocytes. They are phenotypically defined by the expression of CD56 (the 140-kDa isoform of the neural cell adhesion-molecule) and the lack of CD3 expression. Two major NK cell subsets can be identified according to cell-surface CD56 density and the co-expression of CD16 (Fcγ receptor IIIa). Around 10% of peripheral blood NK cells express high levels of CD56 (CD56<sup>bright</sup>), CD94/NKG2A and CD62L, involved in lymphocyte homing to secondary lymphoid organs, lacking or displaying low levels of CD16 and killer cell Ig-like receptors (KIR). CD56<sup>bright</sup> NK cells secrete cytokines but have a limited cytolytic activity. By contrast, the majority of peripheral blood NK cells express low levels of CD56 (CD56<sup>dim</sup>) and high levels of CD16. This subset

has preformed cytotoxic granules and is primarily responsible for mediating cytotoxic activity and cytokine production<sup>8</sup> (**Figure 2**).

Both NK cell subsets also differ in their basal expression of chemokine receptors. CD56<sup>bright</sup> NK cells express high levels of CCR7, the molecule responsible of driving these cells towards the lymph node. Moreover, they express high CCR4, CCR6 and CXCR6. By contrast, CD56<sup>dim</sup> NK cells express low levels of CXCR2 and CXCR3 and high levels of CXCR1, CX3CR1 and ChemR23, promoting NK cell activation and extravasation upon inflammation<sup>9</sup>.



**Figure 2:** Peripheral blood NK cells. **(A)** Flow cytometry analysis of CD56<sup>bright</sup> (red box) and CD56<sup>dim</sup> (purple box) NK cells. Numbers indicate the average percentage of each NK-cell subset among total NK cells in peripheral blood. **(B and C)** Scheme of major NKR, cytokine/chemokine receptors and effector functions of both human NK-cells subsets. **(B)** CD56<sup>bright</sup> NK cells **(C)** CD56<sup>dim</sup> NK cells. Cooper et. al. Trends Immunol. 2001<sup>8</sup>.

A lineage relationship between CD56<sup>bright</sup> and CD56<sup>dim</sup> subsets has been postulated. According to this view of NK cell development, precursor CD56<sup>bright</sup> NK cells would sequentially develop into CD56<sup>dim</sup> subset. Indeed, CD56<sup>bright</sup> display longer telomeres compared with CD56<sup>dim</sup> NK cells, suggesting that they have undergone fewer proliferation cycles. Moreover, CD56<sup>bright</sup> cells can express CD117 (c-KIT), a receptor expressed in progenitor cells and usually absent in CD56<sup>dim</sup> cells. By contrast, CD56<sup>dim</sup> cells express CD57, a maturation marker. Experiments of NK cell differentiation from hematopoietic stem cells indicate the early appearance of CD56<sup>bright</sup>CD16<sup>-</sup>KIR<sup>-</sup> NK cells. *In vivo*, CD56<sup>bright</sup> NK cells are the first lymphocytes that reconstitute/engraft following hematopoietic stem cell transplantation, being conventionally considered immature precursors of the CD56<sup>dim</sup> population<sup>10</sup>. Yet, this linear differentiation pathway has not been directly validated, and thus the possibility that some CD56<sup>dim</sup> NK cell subsets, particularly NKG2A<sup>-</sup> KIR<sup>+</sup> cells, may originate independently remains open. Of note, a CD3<sup>-</sup> CD56<sup>-</sup> CD16<sup>+</sup> NK cell subset is also found in healthy individuals, expanding in some chronic viral diseases (e.g. immunodeficiency virus (HIV) and hepatitis C virus (HCV))<sup>11</sup>.

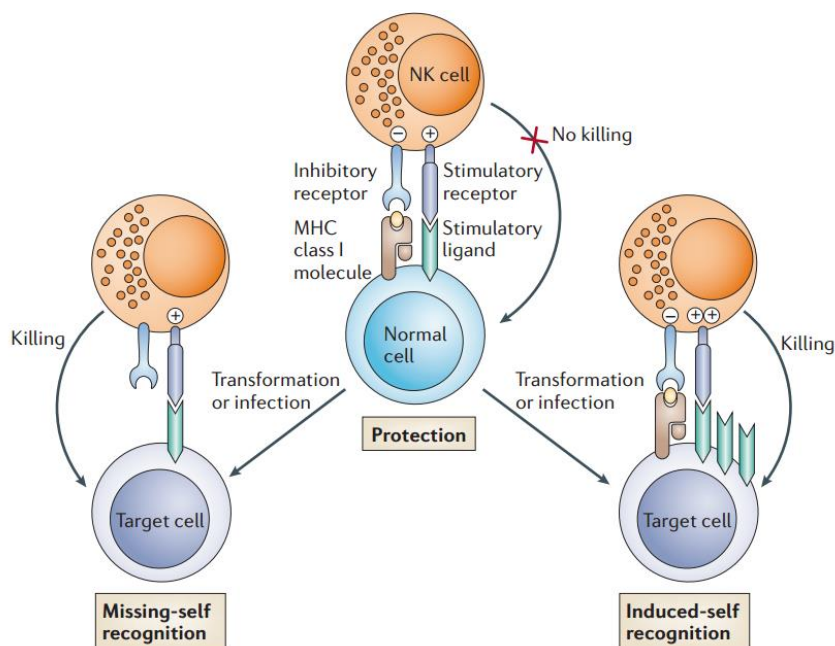
### 1.2 Target cell recognition and effector functions

In 1986 Klas Kärre postulated the “missing-self hypothesis” based on observations showing that NK cells can recognize and kill autologous transformed and healthy hematopoietic cells lacking surface major histocompatibility complex (MHC) class-I molecules<sup>12</sup>. This hypothesis was further validated by demonstrating NK cell-dependent rejection of MHC class-I-deficient bone marrow grafts (i.e.  $\beta$ 2-microglobulin<sup>-/-</sup> and/or transporter associated with antigen processing-1 (TAP-1)<sup>-/-</sup> knockout grafts) otherwise genetically identical<sup>13</sup>, leading to postulate the existence of inhibitory receptors for MHC class-I. As normal cells displaying low MHC class-I expression levels are resistant to NK cells, the existence of other inhibitory and activating receptor required to trigger NK cell effector

functions upon engagement by specific ligands present on target cells, was considered<sup>14</sup>.

The current paradigm is that NK cells express an array of germline encoded surface receptors devoted to identify molecular alterations in the target cell surface. The balance between activating and inhibitory signals received by the NK cell determines the outcome of the interaction with the target cell. Healthy cells are protected from NK cell killing by the engagement of inhibitory receptors by self MHC class-I molecules, preventing autoreactivity. In some pathological conditions (e.g. viral infection, neoplastic transformation), down-regulation of MHC class-I molecules renders target cells susceptible to NK cell recognition. On the other hand, up-regulated expression of stimulatory ligands in target cells (e.g. stress-induced self-proteins, viral moieties) can overcome MHC class-I-dependent inhibition, triggering NK-cell activation<sup>15</sup> **(Figure 3)**.

Apart from direct target cell recognition, NK cell effector functions can be indirectly triggered upon recognition of antibody-coated cells through CD16 (FcγRIII-A) mediating antibody dependent cellular cytotoxicity (ADCC) and cytokine production.



**Figure 3:** MHC class-I and stimulatory signals regulate NK cell activation upon target cell recognition. Raulet and Vance. Nat. Rev. 2006<sup>15</sup>

Upon activation, NK cells release cytotoxic granules at the synapse with the target cell. Among other molecules, these granules contain perforin, and members of a family of serine proteases called granzymes. Perforin disrupts the target cell membrane by forming a pore through which granzymes enter into the cytoplasm and initiate the apoptotic process in target cells, leading to chromatin condensation, membrane blebbing and ultimately nuclear DNA fragmentation<sup>16</sup>. In addition, NK cells express death receptor ligands including TNF-related apoptosis-inducing ligand (TRAIL), also known as APO2, FAS ligand and others that are engaged by death receptors (i.e. Fas/CD95) on the target cells resulting in extrinsic caspase-dependent apoptosis<sup>17</sup>.

The human NK-cell compartment is heterogeneous, including cells at distinct maturation stages together with a variety of subsets which display different NKR combinations with a clonal distribution pattern. The natural

killer receptor (NKR) repertoire is shaped in part by the specific interaction of inhibitory receptors with MHC class-I, which promotes the functional maturation of NK cells<sup>18</sup>. Data from different laboratories show the plasticity of NK cell education depending on MHC class-I environment<sup>19</sup>.

### **1.3 Crosstalk between NK cells and other immune cells**

The NK-cell activation threshold and specific effector functions can be further modulated by cytokines secreted by immune and stromal cells. NK cell function is enhanced by pro-inflammatory cytokines (i.e. interleukin (IL)-12, IL-1 $\alpha$ ,  $\beta$ , IL-15 and IL-18) produced by activated macrophages and dendritic cells (DC). Type I interferons (IFNs), are potent activators of NK cell function. Cytokines such as transforming growth factor- $\beta$  (TGF- $\beta$ ) produced by some tumors and regulatory T cells dampen NK cell activity. Reciprocally, IFN- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$  produced by NK cells promote DC and macrophage activation and contribute to the skewing of T cell differentiation towards a pro-inflammatory phenotype. Furthermore, NK cells secrete chemokines such as macrophage inflammatory protein-1 $\alpha$ / $\beta$  (MIP-1 $\alpha$ , MIP-1 $\beta$ ), IL-8, and RANTES, which may recruit effector cells during the immune response<sup>20</sup>.

### **1.4 NK cell receptor repertoire and signaling**

NK cells sense and properly respond to alterations caused by infections, cellular stress and transformation by integrating signals from a combination of surface receptors.

Activating receptors lack intrinsic signaling domains and couple to adaptor proteins through charged residues in their transmembrane domain. Fc $\epsilon$ RI $\gamma$ , CD3 $\zeta$  and DNAX adapter protein (DAP)-12 are the major signaling adaptors used by activating NKR. All of them contain at least one immunoreceptor tyrosine-based activating motif (ITAM). When the receptor is engaged, SRC-family kinases phosphorylate the ITAM, which recruits spleen tyrosine kinase (SYK) and/or  $\zeta$ -associated protein (ZAP)-70<sup>21</sup>.

Inhibitory receptors signal through cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) which become phosphorylated upon receptor engagement and recruit the tyrosine phosphatases SHP-1 and SHP-2. These phosphatases dampen or prevent NK cell activation, by dephosphorylating different substrates at a membrane proximal location<sup>21</sup>.

Additional cell surface receptors that are not directly coupled to ITAMs also participate in NK cell activation. These include NKG2D, associated to the DAP10 transmembrane signaling adaptor that contains an YxxM motif, which recruits p85 subunit of phosphatidylinositol 3-kinase (PI3K), as well as adhesion molecules (e.g. integrins, CD2) and cytokine receptors<sup>21</sup>.

A selection of NK cell receptors-ligand pairs with relevance in this work are described in more detail in the following sections.

#### **1.4.1 C-type lectin like receptors**

- **CD94/NKG2**

CD94 and NKG2 are type II integral membrane glycoproteins that contain an extracellular C-type carbohydrate recognition domain and display distant homology with the murine NK gene complex families (NKR-P1 and Ly-49). They are composed of the invariant common subunit CD94 that is linked to distinct glycoproteins encoded by genes of the NKG2 family. The NKG2 family includes: NKG2A (and its splice variant NKG2B), NKG2C, NKG2E (and its splice variant NKG2H), and NKG2F. They are all closely linked in the human NK gene complex at chromosome 12. CD94 binds to NKG2 receptors through disulfide bridges and is required to stabilize their surface expression, forming surface heterodimers<sup>22,23</sup>.

All CD94/NKG2 receptors show a high degree of sequence homology in their ligand-binding domain and different cytoplasmic domains. The best characterized are the inhibitory receptor CD94/NKG2A and the activating receptor CD94/NKG2C. NKG2A (also NKG2B) contains an ITIM motif

in the cytoplasmic tail. On the other hand, NKG2C (and NKG2E/H) is coupled to the adaptor DAP12<sup>24</sup> (**Figure 4**).

CD94/NKG2A and CD94/NKG2C are constitutively expressed by subsets of human NK cells,  $\gamma\delta$  and  $\alpha\beta$  T lymphocytes; a minor proportion of NK cells may co-express both receptors. Data regarding the mechanisms regulating the transcription of different NKG2 genes is scarce. NKG2A transcription in NK cells is regulated by GATA3<sup>25</sup>. During NK cell development, IL-15 and IL-21 facilitate the differentiation of mature CD94/NKG2A<sup>+</sup> NK cells<sup>26,27</sup>. In mature NK cells, the presence of IL-2 and IL-15 as well as IFN- $\alpha$  increases the expression of CD94/NKG2A, at least in part by inducing transcription from the distal promoter of the *CD94* gene<sup>28</sup>. Moreover, IL-12 can transiently induce the expression of NKG2A in CD94/NKG2C<sup>+</sup> NK cells, providing a potential negative regulatory feedback mechanism<sup>29</sup>.

The natural ligand for CD94/NKG2A and CD94/NKG2C is the non-classical MHC class-I molecule HLA-E in humans<sup>30–32</sup> and its homologue Qa1 in mice<sup>33</sup>. HLA-E consists of three non-covalently bound components: a heavy chain, the  $\beta_2$ -microglobulin subunit, and a nonameric peptide, usually derived from residues 3 to 11 of the highly conserved signal sequences of classical MHC class-I molecules as well as HLA-G. Loading of these nonamer peptides into HLA-E molecule requires their proteasomal trimming and is dependent on the transporter associated with antigen processing (TAP) which translocates them from the cytosol to the lumen of the endoplasmic reticulum (ER)<sup>34</sup>. HLA-E is widely distributed among various tissues, exhibits relatively low surface expression, and has limited polymorphism. Two non-synonymous alleles of HLA-E have been found, HLA-E\*0101 and HLA-E\*0103. HLA-E\*0101 (HLA-E<sup>R</sup>) has an arginine in position 107 of the  $\alpha 2$  domain whereas this residue is replaced by a glycine in HLA-E\*0103 (HLA-E<sup>G</sup>). These two alleles are present at a frequency of nearly 50% in different populations. Surface expression levels of HLA-E<sup>G</sup>

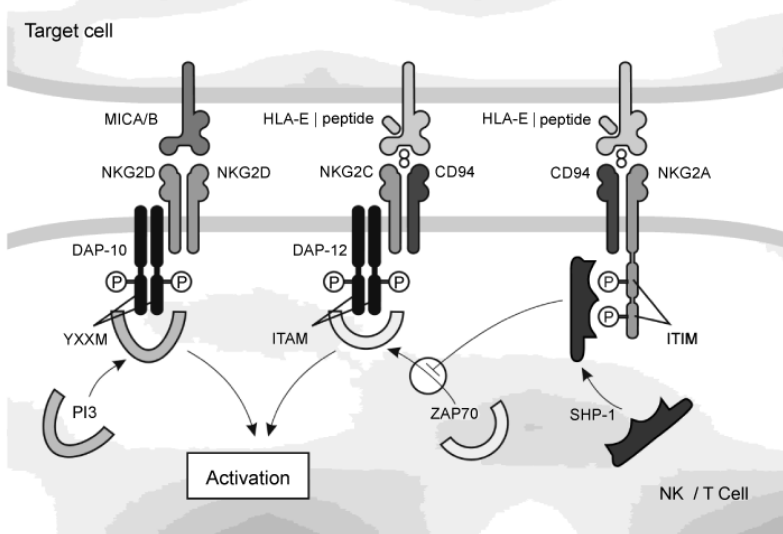
exceed that of HLA-E<sup>R</sup>, correlating with a relative higher peptide binding affinity of the latter<sup>35</sup>. Generally, HLA-E is recognized by CD94/NKG2C with lower affinity than by CD94/NKG2A, although the affinity of CD94/NKG2 receptors for HLA-E is modulated by the peptide associated to HLA-E<sup>36</sup>. Thus, HLA-E recognition by CD94/NKG2A allows NK cells to indirectly monitor the biosynthesis of classical MHC class-I molecules.

In addition to signal sequence peptides derived from MHC class-I molecules, HLA-E can bind peptides from other self- or pathogen-derived proteins which may be recognized by CD94/NKG2 receptors (e.g. ATP-binding cassette transporter multidrug resistance associated protein 7 (MRP7)<sup>37</sup>, heat shock protein<sup>38</sup> or a peptide from the Epstein-Barr virus (EBV))<sup>39</sup>. Remarkably, human cytomegalovirus (HCMV)<sup>40</sup>, HCV<sup>41</sup> and HIV<sup>42</sup> provide peptides capable of stabilizing HLA-E on the infected cell surface, inhibiting NK cell responses through CD94/NKG2A.

A homozygous deletion of the *NKG2C* gene (officially designated *KLRC2*) has been described in ~4% individuals in different populations<sup>43,44</sup> ranging from 0.7% to 10% in a Mexican and Gambian cohorts respectively<sup>45,46</sup>

- **NKG2D**

Unlike other members of the NKG2 family, NKG2D is expressed as a disulfide-linked homodimer. In humans, NKG2D associates with DAP-10 adaptor leading to NK cell activation through the recruitment of PI3K and Grb2<sup>47</sup> (**Figure 4**). Cellular ligands of NKG2D include MHC class-I chain-related gene (MIC)-A, MIC-B and UL16-binding proteins (ULBP)1-6, up-regulated under stress conditions and expressed in some tumor and infected cells<sup>48</sup>. NKG2D ligands can be induced by genotoxic stress and stalled DNA replication, conditions that activate DNA damage checkpoint pathways<sup>49</sup>.



**Figure 4.** Schematic representation of NKG2 receptors and their ligands. Iwaszko et al. J. Transplant Technol Res 2012<sup>50</sup>.

#### 1.4.2 Immunoglobulin-like receptors

- **FcγRIII-A (CD16A)**

The family of Fc receptors for IgG (FcγRs) is widely expressed through the hematopoietic system and includes FcγRI, FcγRII-A, FcγRII-B, FcγRII-C, FcγRIII-A, FcγRIII-B and FcγRIV. FcγRIII-A or CD16A is constitutively expressed in most CD56<sup>dim</sup> NK cells, and recognizes the crystallizable fragment (Fc) region of G1 and G3 immunoglobulins (IgG1 and IgG3) promoting cytotoxicity and cytokine production. CD16A consists of a ligand-binding α-chain and requires the FcεRIγ (abbreviated as FcRγ) or CD3ζ adaptor molecules for expression and signaling<sup>51</sup>.

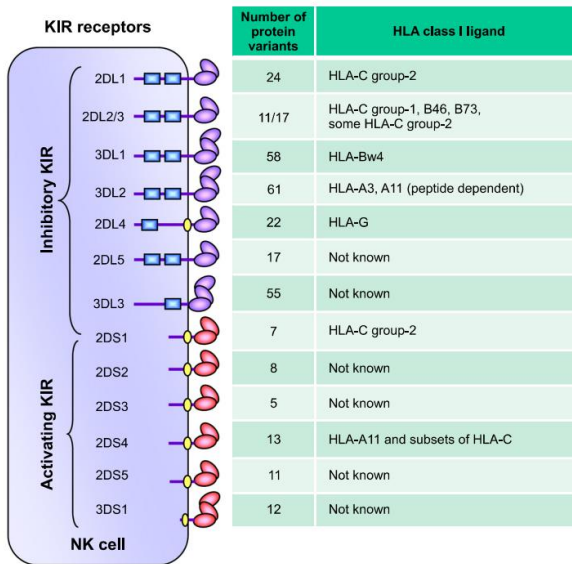
- **Killer Immunoglobulin-like receptors (KIR)**

Killer Ig-like receptors (KIRs) are polymorphic surface receptors expressed by NK cells and a subset of T lymphocytes. KIRs are encoded by a family of fifteen genes and two pseudogenes clustered on the leukocyte receptor complex in chromosome 19q13.4. The number of *KIR* genes may

substantially vary between individuals and, accordingly, over 30 KIR haplotypes have been thus far defined in different human populations<sup>52</sup>.

KIR can be divided into inhibitory and stimulatory receptors. Inhibitory KIR are characterized by a long cytoplasmic tail (L) containing an inhibitory ITIM whereas activating KIR have a short cytoplasmic tail (S) and bear a charged transmembrane residue (Lys/Arg) which facilitates their coupling to DAP12 adaptor. The only exception is KIR2DL4 which has both activating and inhibitory signaling domains. According to structural characteristics, KIR receptors display two (KIR2DL) or three (KIR3DL) Ig-like extracellular domains<sup>52</sup> (**Figure 5**).

Two major groups of KIR haplotypes can be found in human populations. KIR A haplotypes contain nine genes (3DL3-2DL3-2DP1-2DL1-3DP1-2DL4-3DL1-2DS4-3DL2), encoding for inhibitory receptors and the KIR2DS4 activating molecule. KIR B haplotypes may encode for different combinations of inhibitory and activating KIR including KIR2DL5. Among Caucasian populations, both A and B haplotypes are found with similar frequency<sup>53</sup>. KIR receptors recognize structural motifs shared by sets of classical MHC class-I molecules. A dimorphism at position 80 of HLA-C defines two groups of KIR ligands: MHC-C1 allotypes have an asparagine residue (Asn80) and are recognized by KIR2DL2 and KIR2DL3 receptors whereas MHC-C2 allotypes have a lysine residue (Lys80) and are preferentially recognized by KIR2DL1. A sequence dimorphism in the C-terminal region of the HLA-B  $\alpha$ 1-helix (Bw4) determines ligand-binding specificity for inhibitory KIR3DL1, whereas KIR3DL2 has been reported to interact with HLA-A3/11<sup>52</sup>.



**Figure 5.** Activating and inhibitory KIR receptors and their ligands. Rajalingam et al. Korean J. Hematol. 2011<sup>54</sup>.

- **Leukocyte Immunoglobulin-like Receptor B1 (LILRB1)**

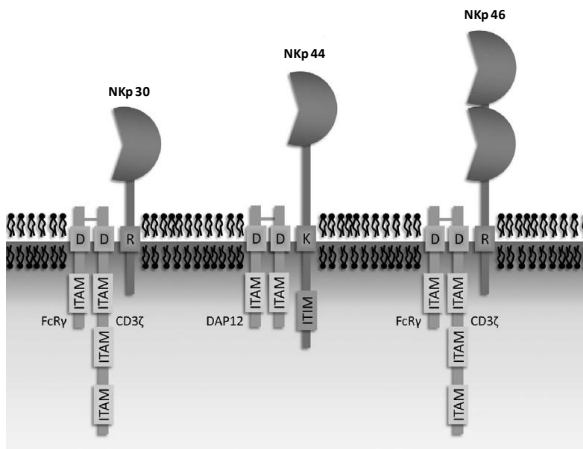
NK cells may express the inhibitory Leukocyte Immunoglobulin-like Receptor B1 (LILRB1, ILT2, LIR1, CD85j) which recognizes a conserved region in the  $\alpha 3$  domain of a wide spectrum of classical and non-classical MHC class-I molecules. LILRB1 comprises an extracellular region with four Ig-like domains (D1-D4) and a cytoplasmic tail containing four immunoreceptor tyrosine-based inhibition motifs (ITIM) which recruit the SHP-1 tyrosine phosphatase. LILRB1 is clonally expressed in NK cells at late differentiation stages<sup>55</sup>.

- **Natural Cytotoxicity Receptors**

Natural cytotoxicity receptors (NCR) include NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKP30 (NCR3; CD337). NCRs are type I transmembrane proteins, containing Ig-like extracellular domains. Their transmembrane domain contains a positive-charged amino acid that facilitates coupling to CD3 $\zeta$  or Fc $\epsilon$ R $\gamma$  adaptors. They were described by their preferential expression on NK cells and their ability to induce NK cell cytotoxicity upon cross-linking with specific monoclonal antibodies (mAbs)

in redirected killing assays<sup>56</sup>. Yet, NCR expression has also been described in some ILC and T cell subsets<sup>57</sup>. **NKp46** is a 46-kDa molecule constitutively expressed by the majority of NK cells. The *NKP46* gene maps on chromosome 19q13.42 in the LRC telomeric to the KIR locus. The receptor is formed by two N terminal C2 type Ig domains and a transmembrane region that can bind CD3 $\zeta$  or FcR $\gamma$  adaptors<sup>58</sup>. **NKp30** is a 30-kDa protein expressed at different levels by all mature NK cells. The *NKP30* gene is located on chromosome 6p21.1 centromeric to MHC class-I. NKp30 presents a single Ig extracellular domain and can bind CD3 $\zeta$  and CD3 $\zeta$ /FcR $\gamma$  adaptors<sup>59</sup>. **NKp44** is a 44-kDa molecule formed by a single V-type Ig extracellular domain that unlike the other NCR, is expressed only on activated NK cells and interacts with DAP12 adaptor through a lysine in the transmembrane region<sup>60</sup>. NKp44 expression has been also reported in subsets of decidual and tonsil NK cells *in vivo*<sup>61,62</sup>. The *NKP44* gene is also located on chromosome 6p21.1 (**Figure 6**).

Although NCRs have been shown to participate in the recognition of various tumor-derived cell lines, their natural ligands remain incompletely defined. Viral, parasite, bacterial-derived ligands and decoy molecules have been identified. The recognition of HCMV tegument protein pp65 by NKp30 resulted in NK cell inhibition. Moreover, influenza hemagglutinins can interact with NKp30, NKp44 and NKp46. Vimentin, a cellular ligand expressed on *Mycobacterium tuberculosis* infected cells, can bind to NKp46 triggering NK cell activation. In contrast, cell-wall components of *M. tuberculosis* can bind NKp44, although no NK cell activation could be detected upon incubation with the bacteria<sup>63</sup>.



**Figure 6:** Natural Cytotoxicity receptors. Kruse et al. Immunol Cell Biol. 2014<sup>63</sup>.

## 2. HUMAN HERPESVIRUS

Herpesviridae constitute a large family of DNA viruses that includes three subfamilies: the *Alphaherpesvirinae* subfamily which includes human herpes simplex virus types 1, and 2 and varicella zoster virus; the *Betaherpesvirinae* subfamily that comprises the human cytomegalovirus and human herpes virus 6 and 7 and the *Gammaherpesvirinae* subfamily that includes Epstein-Barr virus and Kaposi's sarcoma herpesvirus (KSV). Herpesviruses contain linear double-stranded DNA, are enveloped, and undergo latent and lytic lifecycles. In order to successfully coexist within the host and disseminate at the population level, herpesviruses have developed several strategies to escape from innate and adaptive immune responses<sup>64</sup>. HCMV and EBV will be described in more detail in the next sections.

### 2.1 Human Cytomegalovirus

HCMV, also known as human herpesvirus (HHV)-5, is a  $\beta$ -herpesvirus infecting all human populations with a high variable prevalence (50 to 100%) depending on socioeconomic factors and age. Acute HCMV infection is generally asymptomatic in immunocompetent hosts. The virus persists in a life-long latent state undergoing occasional subclinical reactivations that allow transmission. In immunocompromised individuals, HCMV infection,

reactivation or reinfection may have important clinical implications. Vertical transmission during pregnancy may cause congenital infection in 0.5-2% newborns, eventually leading in some cases to severe sensorineural sequelae. Viral excretion in milk may cause early postnatal infection, which may be symptomatic particularly in premature infants. Moreover, immunosuppression in organ allograft and hematopoietic stem-cell transplant patients favors HCMV reinfection/reactivation. Based on epidemiologic studies, HCMV has also been related with the development of atherosclerosis and immunosenescence<sup>65-67</sup>.

HCMV (150-200nm) is formed by an icosahedral nucleocapsid containing a double-stranded linear DNA genome of 230-kb with at least 170 open reading frames (ORF). The nucleocapsid is surrounded by the tegument or matrix enclosed by a lipid bilayer displaying a variety of viral molecules<sup>67</sup>.

HCMV can infect a broad spectrum of cells, including endothelial, epithelial, smooth muscle and glial cells, fibroblasts, neurons, hepatocytes, trophoblasts, monocytes/macrophages, and dendritic cells. Cells of myelomonocytic origin are considered the main viral reservoir. The permissiveness of cells for active viral replication is directly related to their differentiation state. Virus reactivation is a key step in the pathogenesis of HCMV infection and can occur in response to immunosuppression, inflammation, infection or stress<sup>68</sup>.

### **2.1.1 NK cell response to HCMV**

HCMV infection triggers innate as well as adaptive humoral and cellular mediated immunity. CD8<sup>+</sup> T cells recognize virus-derived antigenic peptides presented by MHC class-I molecules on infected cells and are essential to restrain HCMV replication and prevent disease. Specific antibodies recognizing viral proteins can neutralize their interactions with cellular receptors, avoiding viral entry into target cells and activating the complement system. On the other hand, antibodies specific for viral molecules exposed

on the infected cell surface can be recognized by phagocytic and NK cells triggering antibody-dependent cellular cytotoxicity (ADCC)<sup>69</sup>.

Several observations evidenced the role of NK cells in limiting the severity of disease caused by a range of virus, including HCMV. In accordance with the higher susceptibility to murine cytomegalovirus (MCMV) of NK cell-deficient mice, patients with NK cell deficiencies have recurrent herpesviral infections<sup>70,71</sup>.

HCMV has developed several evasion strategies to counteract NK and T cell-mediated immune pressure. A number of viral proteins (US2, US3, US6, US10 and US11) downregulate surface expression of MHC class-I molecules on the infected cell to evade HCMV-specific CD8<sup>+</sup> T cell responses, increasing their susceptibility to NK recognition<sup>72</sup>. On the other hand, the viral MHC class-I-like UL18 protein is a high affinity ligand for the LILRB1 inhibitory receptor<sup>73</sup>, and a peptide derived from the UL40 viral glycoprotein is capable of stabilizing HLA-E on the infected cell surface, engaging the CD94/NKG2A inhibitory receptor<sup>74</sup>, thus preventing activation of LILRB1<sup>+</sup> and NKG2A<sup>+</sup> NK cell subsets respectively. Moreover, HCMV has evolved several mechanisms to interfere with the expression of ligands for activating NKR on infected cells. HCMV UL16 and UL142 glycoproteins hamper the export of NKG2D ligands to the surface of infected cells<sup>75,76</sup>. Moreover, UL141 inhibits the expression of CD155 and CD112 ligands of the activating NK cell co-receptor DNAM-1<sup>77-79</sup>. Finally, HCMV encodes for micro RNAs that target transcripts of NKG2D ligands for degradation<sup>80</sup>.

### **2.1.2 HCMV-induced reconfiguration of the NK cell receptor repertoire**

Immunological memory is considered a feature of adaptive immunity. Recently, some observations are challenging this conventional view and increasing evidences suggest that prior exposure to specific stimuli can also stably reshape the configuration of the NK compartment.

NK cell “memory” was first observed in MCMV-resistant mice, in which NK cells bearing Ly49H, a C-type lectin-like activating receptor that specifically recognizes m157 MCMV protein, undergo a preferential expansion coinciding with acute primary infection<sup>81–83</sup>. A fraction of these cells remained in circulation for more than one month after infection and demonstrated enhanced cytotoxicity and cytokine production upon re-challenging with MCMV, a behavior reminiscent of T cells memory<sup>84</sup>.

In humans, Gumá et al. presented the first evidence supporting that healthy HCMV seropositive (HCMV<sup>+</sup>) individuals had increased frequencies of NKG2C<sup>+</sup> NK cells as compared to seronegative (HCMV<sup>-</sup>) subjects. Expansions of NKG2C<sup>+</sup> NK cells occurred independently of their KIR haplotype, HLA-E dimorphism and past infections such as EBV and herpes simplex virus (HSV)-1<sup>85</sup>. Subsequent studies corroborated and expanded this finding by showing that NKG2C<sup>+</sup> NK-cell expansions associated to HCMV in early childhood<sup>86,87</sup>, in children with symptomatic congenital infection<sup>88</sup>, as well as in recipients of solid organ or hematopoietic cell transplants during episodes of primary HCMV infection or reactivation owing to immunosuppression<sup>89–91</sup>.

NKG2C<sup>+</sup> NK cell expansions have also been observed in patients with several acute and chronic viral infections, including Hantavirus<sup>92</sup>, Chikungunya virus<sup>93</sup>, HIV<sup>94</sup>, Hepatitis B Virus<sup>95</sup>, and EBV<sup>96</sup>, systematically associated to HCMV co-infection, highlighting the imprint of this virus in the distribution of peripheral blood NK cell subsets.

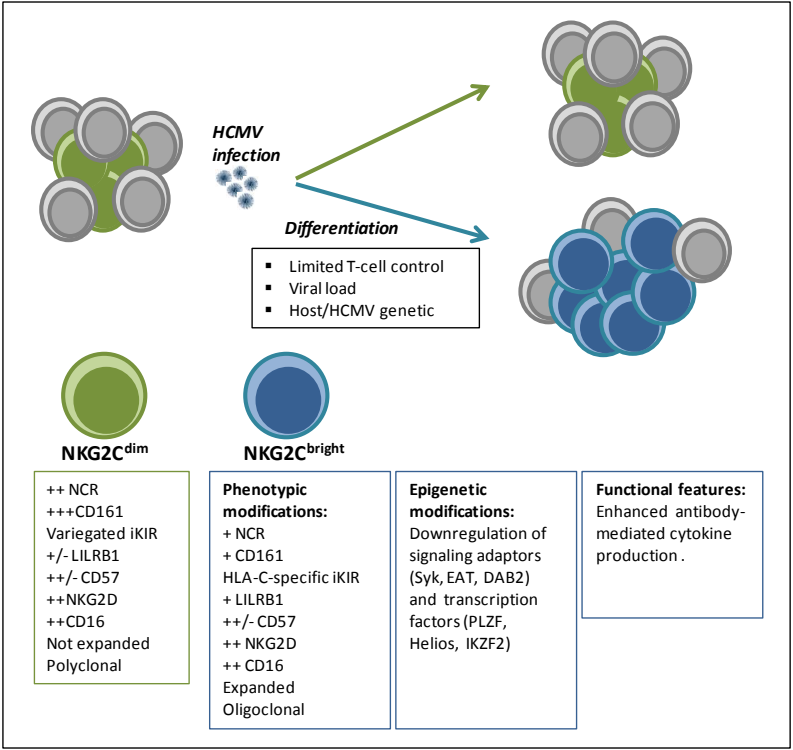
HCMV-induced NKG2C<sup>+</sup> NK cell expansion is quite variable, being undetectable in some HCMV<sup>+</sup> individuals and constituting >50% of the NK cell compartment in others. A limited T cell control, the viral load, together with virus and host genetic factors might underlie the variability in the magnitude of the HCMV-induced proliferation/differentiation process<sup>97</sup>. Regarding their phenotypic characteristics, HCMV-induced NKG2C<sup>+</sup> NK

cells display high surface levels of CD94/NKG2C activating receptor (NKG2C<sup>bright</sup>), lack NKG2A and bear lower levels of surface NKp46, NKp30 and CD161 while maintaining the expression of NKG2D and CD16 as compared to conventional CD56<sup>dim</sup> NK cells (i.e. NKG2A<sup>+</sup> cells and NKG2C<sup>+</sup> NK cells in HCMV<sup>-</sup> individuals). Expression of late differentiation markers such as CD57 and LILRB1 is variable, likely reflecting the coexistence of distinct NKG2C<sup>bright</sup> NK cell differentiation stages<sup>85,97</sup>. In this regard, HCMV infection has been recently associated to the appearance of NK cells deficient for the FcR $\gamma$  adaptor<sup>98</sup>. Generally, FcR $\gamma$  loss appears confined to the expanded NKG2C<sup>bright</sup> NK-cell population. Similar to the gradual acquisition of terminal differentiation markers, down-regulation of the FcR $\gamma$  adaptor points towards a sequential differentiation from NKG2C<sup>bright</sup> FcR $\gamma$ <sup>+</sup> to NKG2C<sup>bright</sup> FcR $\gamma$ <sup>-</sup> NK cells. Indeed, HCMV-induced adaptive NK cell differentiation involves a gradual epigenetic down-regulation of several signaling adaptors (e.g Syk, EAT-2 and DAB2) and transcriptions factors (e.g. PLZF, Helios and IKZF2) leading to a gene expression profile reminiscent to that of memory T cells<sup>99,100</sup> (**Figure 7**).

Little is known about the molecular and cellular mechanisms involved in HCMV-induced adaptive NKG2C<sup>+</sup> NK cell differentiation and expansion. The participation of CD94 and IL-15 in NKG2C<sup>+</sup> NK cell expansion was originally proposed by pioneering studies by Gumá et al. showing that HCMV-infected fibroblasts could support NKG2C<sup>bright</sup> NK cell expansion *in vitro*<sup>101</sup>. These observations have been recently validated highlighting the role of NKG2C, CD94 and HLA-E molecules together with monocyte-produced IL-12 in NKG2C<sup>bright</sup> NK cell proliferation<sup>102</sup>. Indeed, the participation of NKG2C receptor in the expansion of NKG2C<sup>+</sup> NK cells in response to HCMV is indirectly supported by observations showing the influence of NKG2C gene dose in the relative and absolute NKG2C<sup>+</sup> NK cell numbers in a cohort of HCMV congenitally infected infants<sup>88</sup>.

NKG2C<sup>bright</sup> NK cells are functionally mature, display a predominant inhibitory KIR specific for self HLA-C<sup>85,87,103</sup> and mediate cytotoxicity and cytokine production, efficiently responding against MHC class-I-defective tumor cell lines *in vitro*. In the context of infections, NKG2C<sup>bright</sup> NK cells display enhanced effector functions in ADCC responses against HCMV- and HSV-infected targets *in vitro*<sup>104–106</sup>, yet showing limited responses upon direct recognition of infected cells. The importance of NKG2C<sup>bright</sup> NK ADCC proficiency in keeping HCMV reactivation at bay is envisaged.

Remarkably, the NKR redistribution associated to HCMV infection remains stable in steady state along years. Whether the lifelong persistent reconfiguration of the NK cell repertoire can influence the individual immune response to other pathogens e.g. herpesviruses, deserves attention.



**Figure 7.** Phenotypic, epigenetic and functional features of the NKG2C<sup>+</sup> NK cell subsets in relation with HCMV infection.

## 2.2 Epstein-Barr virus

Epstein-Barr virus, also known as human herpes virus-4 (HHV-4), is a  $\gamma$ -herpesvirus with a double-stranded DNA genome of 172 Kb, codifying for approximately 100 genes. EBV is carried by >90% of adults worldwide. B cells are main targets of EBV infection, yet the pathogen can also infect T, NK and epithelial cells<sup>107</sup>. Primary EBV infection usually occurs within the first three years of life by oral transmission through saliva and is generally asymptomatic<sup>108</sup>. In adolescents and adults, primary EBV infection may present as infectious mononucleosis (IM), an acute and self-limiting lymphoproliferative disorder<sup>109</sup>. EBV is also associated with several malignances of B cell origin including Burkitt Lymphoma<sup>110</sup> and Hodgkin disease<sup>111</sup>. In rare cases, EBV infects T or NK cells, associated to the development of T cell and NK cell lymphomas<sup>112,113</sup>. EBV is also associated with non-lymphoid malignances, such as nasopharyngeal carcinoma<sup>114</sup> and EBV<sup>+</sup> gastric adenocarcinoma<sup>115</sup>. On the other hand, a number of observations point out a role of EBV in the pathogenesis of some autoimmune diseases (i.e. multiple sclerosis and rheumatoid arthritis)<sup>116</sup>.

### 2.2.1 EBV virology

Following oral transmission, EBV replicates in the oropharyngeal epithelium as well as in tonsillar B cells. B cell targeting by EBV involves the binding of the major outer membrane glycoprotein gp350/220 (BLLF1)<sup>117</sup> to the complement receptor 2 (CR2 or CD21). Receptor-mediated internalization requires the binding of a second glycoprotein, gp42 to MHC class-II molecules on B cells<sup>118</sup> which, thereafter, initiates the fusion process involving the core complex gH/gL/gp42<sup>119</sup>. Nasopharyngeal epithelial cells can also be infected by virus transfer from infected or virus-coated B cells<sup>120</sup>, contributing to sustain EBV lytic replication. Primary lytic infection leads to virus shedding in the epithelium and initiates a latent, transforming infection, leading to the expansion of infected B cells and their appearance in blood.

Although many of these proliferating B cells are eliminated by the immune response, some survive as long-lived memory B cells down-regulating viral antigen expression. EBV lytic lifecycle is divided into three phases of regulated gene expression (immediate-early, early and late) and depends on the viral DNA polymerase to accomplish with linear viral DNA replication. Immediate-early gene products, such as BZLF1 and BRLF1, act as transactivators of the viral lytic program. Early gene products (i.e. BNLF2a) are involved in nucleotide metabolisms and DNA replication, including the viral DNA polymerase. Late viral genes encode for structural proteins (i.e. VCA, gp350/220) and immunoevasins (i.e. BCRF1). Structural proteins are assembled forming viral particles into which the viral DNA is packaged prior to the release of infectious virions<sup>107</sup> **(Table 1)**.

Latency is the state of persistent viral infection without active viral production. At least three different EBV latency programs have been identified through the characterization of gene expression patterns in different EBV-infected cells. Early upon lytic cycle, EBV enters into Latency 3 or the Growth program (EBNA1, EBNA2, EBNA3, EBNA4, EBNA5, EBNA6, LMP1 and LMP2A, LMP2B, EBERs), a gene pattern expressed in lymphoblasts, characteristic of IM and of the majority of cases of post-transplant lymphoproliferative diseases **(Table 2)**. This latency program drives B cell proliferation as an alternative mechanism of expanding the infected cell pool. *In vitro*, this program is established in B-lymphoblastoid cell lines (LCL) following infection and growth transformation of resting B cells by EBV. Latency 2 or the Default Program (EBNA1, LMP1 and LMP2A, LMP2B, EBERs) is detected in germinal-center B cells, in Hodgkin's lymphomas as well as in T/NK cell lymphomas. Latency 0 and Latency 1, also termed Latency Program are restricted to memory B cells, which are the long-term reservoir of EBV and is also found in Burkitt's lymphoma. This program is characterized by the lack of expression of any viral gene or expression of the weakly immunogenic LMP2 and EBERs,

required for mitotic segregation of the viral episome during cell division<sup>121</sup>. EBV episome in memory B cells is replicated once at the S-phase in synchronization with the host genome and delivered to daughter cells in mitosis. In contrast to lytic replication, episomal replication during the latent phase occurs via host DNA polymerase. Physiologic events such as antigen stimulation or terminal differentiation of memory B cells into plasma cells might initiate EBV reactivation into replicative cycle producing new viral particles that can infect naïve B cells replenishing the pool of virus-infected cells within the body<sup>122</sup>.

**Table 1:** Representative immediate-early, early and late genes expressed during EBV lytic cycle<sup>123</sup>.

Gene products	Description
<b>Immediate-early</b>	
<b>BZLF1</b>	• Transactivators of the viral lytic program.
<b>BRLF1</b>	
<b>Early</b>	
<b>BHRF1</b>	• Similar to human Bcl-2 proto-oncogene with antiapoptotic activity.
<b>BNLF2a</b>	• Role in immunoevasion, reducing cell surface MHC class-I levels.
<b>BALF2</b>	• Single-stranded DNA-binding protein.
<b>BBLF4</b>	• Helicase.
<b>BSLF1</b>	• Primase.
<b>BBLF2/3</b>	• Primase-associated factor.
<b>BALF5</b>	• DNA polymerase.
<b>BMRF1</b>	• DNA polymerase processivity factor.
<b>BGLF4</b>	• Serine/threonine kinase.

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**Late**

- |                            |   |
|----------------------------|---|
| <b>BCRF1</b>               | • Homologue of the human IL-10 with anti-inflammatory properties.   |
| <b>BNRF1</b>               | • Major tegument protein with unknown function.   |
| <b>BVRF2</b>               | • Scaffold protein for the assembly of capsids leading to the formation of infectious viral particles.  |
| <b>BILF1</b>               | • Downregulates expression of HLA-A and HLA-B on the surface of infected cells but not HLA-C.   |
| <b>BXLF2 and<br/>BKRF2</b> | • gH and gL homologues respectively. Implicated in viral particle penetration.  |
| <b>BDLF3</b>               | • Ubiquitination and proteasomal dependent downregulation of MHC class-I and II molecules.  |
| <b>BALF4</b>               | • Encodes gp110. Highly conserved homologue of the abundant envelope glycoprotein gB of other herpesvirus which is involved in virus-cell fusion.   |
| <b>BLLF1</b>               | • Encodes gp350/220, a large, heavily glycosylated protein and the most abundant glycoprotein in virion and virus-infected cell surfaces. The N-terminal region of gp350/220 binds the CD21 receptor expressed in B-cells triggering EBV internalization. |
-

**Table 2:** EBV-encoded genes expressed during latency<sup>121</sup>.

Gene products	Description
<b>EBNA1</b>	<ul style="list-style-type: none"> <li>• Required for viral replication.</li> <li>• Increases Bcell survival.</li> </ul>
<b>EBNA2</b>	<ul style="list-style-type: none"> <li>• Essential transactivator for cellular transformation.</li> <li>• Regulates several viral gene expression (i.e. LMP1 and LMP2A).</li> </ul>
<b>EBNA-LP</b>	<ul style="list-style-type: none"> <li>• Interacts with EBNA2.</li> <li>• Essential for <i>in vitro</i> B-cell transformation.</li> </ul>
<b>EBNA3s (A, B, C)</b>	<ul style="list-style-type: none"> <li>• Transcription factors that regulate viral gene expression.</li> </ul>
<b>LMP1</b>	<ul style="list-style-type: none"> <li>• Inhibits B cell apoptosis by up-regulation of Bcl-2 anti-apoptotic protein.</li> <li>• Viral oncogene.</li> <li>• Resembles to CD40, providing both growth and differentiation signals.</li> </ul>
<b>LMP2A</b>	<ul style="list-style-type: none"> <li>• Mimics B cell receptor signaling.</li> <li>• Induces the expression of genes involved in cell cycle induction, inhibition of apoptosis and suppression of cell-mediated immunity.</li> </ul>
<b>EBER-1 andEBER2</b>	<ul style="list-style-type: none"> <li>• EBV encoded small RNAs.</li> <li>• Induce the secretion of IL-10 stimulating infected B cell growth.</li> </ul>

### 2.2.2 NK cell response to EBV

The role of T lymphocytes, particularly CD8<sup>+</sup> T cells, in the control of EBV infection is well established<sup>124</sup>. Strong and persistent T cell responses specific for both latency-associated and lytic gene products have been evidenced in IM patients as well as in healthy long-term EBV carriers. On the other hand, EBV specific antibody responses develop following primary infection and have been considered as functionally relevant for eliminating infected cells and neutralizing virus infectivity<sup>125</sup>. Specific antibodies targeting antigens expressed along EBV lytic and latent lifecycle have been identified. In particular, gp125/110 (BALF4) and gp350/220 (BLLF1) are immunodominant determinants of the VCA complex<sup>126</sup>, anti-EBNA1 responses dominate during lifelong latent virus carriage<sup>127</sup> and antibodies recognizing latent membrane proteins LMP1 and LMP2 are detectable in a small proportion of healthy carriers<sup>128</sup>.

Beyond the recognized role of adaptive immunity, several observations support the contribution of NK cells in the control of EBV infection. A role for NK cells in the early control of EBV infection is indirectly supported by the fact that patients with genetic defects leading to loss or impairment of NK cell differentiation and function are prone to complications associated with EBV<sup>129,130</sup>. Moreover, experimental humanized immune system (HIS) mice reconstituted with NK cell-depleted human immune cells display low control over EBV infection leading to enhanced symptoms, resembling infectious mononucleosis and EBV-associated lymphomagenesis<sup>131</sup>.

Studies in IM patients show an increase in peripheral blood NK cell numbers owing to the preferential proliferation and differentiation of CD56<sup>dim</sup> NKG2A<sup>+</sup> KIR<sup>-</sup> NK cells, which respond to EBV-infected B cells in lytic cycle *in vitro*<sup>132</sup>. On the other hand, EBV asymptomatic infection early in life has been associated with increased adaptive NKG2C<sup>+</sup> NK cell numbers in

HCMV and EBV co-infected individuals compared to CMV<sup>+</sup> EBV<sup>-</sup> children<sup>96</sup>.

According to *in vitro* studies, the IFN- $\gamma$  response produced by tonsillar CD56<sup>bright</sup> CD16<sup>-</sup> NK cells prevents B cell infection, delays latent antigen expression and results in decreased B cell proliferation early after EBV infection<sup>133,134</sup>. Former studies already revealed the susceptibility of B cells undergoing EBV lytic infection to NK cell recognition. Both, down-regulation of MHC class-I molecules as well as up-regulation of ULBP1 and Nectin2, ligands for NKG2D and DNAM-1 activating receptors respectively, contribute to the recognition of EBV-lytically infected B cell lines by NK cells<sup>135</sup>. Nonetheless, B-LCL sensitivity to NK cell-mediated cytotoxicity is limited to the early lytic phase and reversed along lytic cycle progression by the expression of the anti-apoptotic vBcl-2 protein (BHRF1)<sup>136</sup>. Latently infected *in vitro* transformed B cells express high HLA class-I surface levels and are poorly recognized by NK cells.

As other herpesvirus, EBV has developed countermeasures to avoid NK cell recognition. Viral BILF1, targets a broad range of MHC class-I molecules for degradation while maintaining HLA-C expression. BILF-1-dependent degradation of HLA-A and HLA-B types dampens presentation of viral antigens to cytotoxic CD8<sup>+</sup> T cells while the maintenance of surface HLA-C protects infected cells against NK cell recognition by engaging KIR2DL inhibitory receptors<sup>137</sup>. On the other hand, EBV infected cells secrete modified exosomes containing immunosuppressive molecules such as galectin 1 and 9, viral LMP1 and FasL capable of inhibiting NK and T cell activation<sup>138–140</sup>.

Aside from direct cytotoxicity, former studies evidenced the existence of specific antibodies capable of triggering NK cell-mediated ADCC responses against EBV-infected cells. EBV encoded glycoproteins gp350/220 and gp125 can serve as ADCC targets and specific antibodies against the latent

antigen LMP1 have also been described<sup>141–144</sup>. Despite the putative importance of ADCC in the subsequent control of EBV persisting infection, this immune effector mechanism remains largely unexplored. Nonetheless, the possibility of adoptively transfer NK cells in combination with specific antibodies to manage EBV infections in leukopenic patients has been envisaged<sup>145</sup>.



## Chapter 2

*Aims*

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HCMV infection induces the differentiation and persistent expansion of adaptive NK cells characterized by the expression of CD94/NKG2C C-type lectin NK cell receptor (NKR), which efficiently mediate antibody-dependent effector functions against HCMV-infected cells. We hypothesize that this pattern of immune response is primarily driven by a cognate interaction of the activating NKR with HCMV-infected cells. The mechanisms underlying the adaptive development of NKG2C<sup>+</sup> NK cells remain elusive and their putative role in the response to other infections deserves attention. In this work the following aims have been addressed:

- To assess the influence of *NKG2C* copy number on the HCMV-induced redistribution of the NK-cell receptor repertoire.
- To characterize the EBV-specific antibody-dependent NK cell response and the involvement of adaptive NKG2C<sup>+</sup> NK cells.



## **PART II**

## **RESULTS**



### Chapter 3

*NKG2C* zygosity influences CD94/NKG2C receptor function and the NK-cell compartment redistribution in response to human cytomegalovirus.

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# ***NKG2C* zygosity influences CD94/*NKG2C* receptor function and the NK cell compartment redistribution in response to human cytomegalovirus**

**Running title:** Influence of *NKG2C* copy number and CMV on the NK cell compartment

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## Chapter 4

Antibody-dependent NK cell activation differentially targets EBV-infected cells in lytic cycle and bystander B lymphocytes bound to viral antigen-containing vesicles.

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**Running title:** ADCC in response to EBV

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**PART III**  
**DISCUSSION AND CONCLUSIONS**



## Chapter 5

### Discussion

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NK cells are innate effectors involved in the control of viral infections and tumor cell surveillance by means of cytotoxicity and cytokine secretion, sharing common features with CD8<sup>+</sup> T lymphocytes. NK cell contribution to the control of infections by herpesviruses (e.g. HCMV and EBV) is indirectly reflected by the heightened susceptibility in patients with NK cell deficiencies<sup>71,129</sup>. Observations evidencing the ability of CMV to alter the configuration of the NK cell compartment by promoting the differentiation and expansion of NK cell subsets with enhanced effector potential have challenged the classical dichotomy between adaptive and innate immunity. In the seminal work by Guma M et al, seropositivity for HCMV appeared associated to the expansion of mature NKG2C<sup>+</sup> NK cells in healthy blood donors ruling out the relationship with other genetic (ie. KIR haplotype, HLA-E dimorphism) and environmental variables (i.e. HSV and EBV infections)<sup>85</sup>. A number of studies have confirmed the association expanding the knowledge on HCMV-induced NKG2C<sup>+</sup> NK cells<sup>97</sup>. Nonetheless, crucial questions including the molecular and cellular mechanisms underlying NKG2C<sup>+</sup> NK cell expansion, their contribution to HCMV control and the putative consequences of HCMV-induced reconfiguration of the NK cell receptor repertoire in the response to other viral infections or tumor surveillance remain open.

The study presented in the first part of this thesis addresses the influence of *NKG2C* gene dose on the redistribution of the NK-cell compartment in response to HCMV infection. A relatively frequent deletion of the *NKG2C* gene determines the existence of three genotypes (*NKG2C*<sup>+/+</sup>, *NKG2C*<sup>+/del</sup> and *NKG2C*<sup>del/del</sup>) in populations of different ethnical origin<sup>43–46</sup>. A precedent work by Noyola et al<sup>88</sup>, revealed an association between the *NKG2C* genotype, NKG2C<sup>+</sup> NK cell numbers and CD94/NKG2C surface levels in a small cohort of children with past congenital infection. Higher absolute numbers of NKG2C<sup>+</sup>, NKG2A<sup>+</sup> and total NK cells were detected in *NKG2C*<sup>+/+</sup> as compared to *NKG2C* hemizygous cases. The particular

characteristics of the cohort (e.g. closeness to infection, intra-utero viral transmission and relatively limited sample size) prompted an analysis in healthy adults.

In our study, NKG2C<sup>+</sup> NK cell expansions related to HCMV infection were identified based on their high expression of surface CD94/NKG2C in the absence of CD94/NKG2A (NKG2C<sup>bright</sup>), and distinguished from NKG2C<sup>+</sup> NK cell populations with lower receptor levels and variable NKG2A co-expression (NKG2C<sup>dim</sup>), which were also detected in HCMV<sup>-</sup> subjects. As compared to NKG2A<sup>+</sup> and NKG2C<sup>dim</sup> NK-cell subsets, HCMV-induced NKG2C<sup>bright</sup> NK cells displayed a phenotypic profile of mature NK cells, including low NCR and CD161 expression as well as high proportions of CD57<sup>+</sup>, LILRB1<sup>+</sup> and KIR<sup>+</sup> cells, in line with the original observations by Gumá et al<sup>85</sup>. HCMV-induced expansions of differentiated NKG2C<sup>bright</sup> NK cells were detected to a variable extent in approximately half of HCMV<sup>+</sup> individuals, with similar frequencies in *NKG2C*<sup>+/+</sup> and *NKG2C*<sup>+/-del</sup> subjects, indicating that factors other than *NKG2C* copy number (e.g time of primary infection, efficiency of specific T-cell responses, viral/host genetics) might determine the differentiation of adaptive NK cells. However, our results substantiated an association between *NKG2C* gene dose and steady-state NKG2C<sup>+</sup> NK cell numbers in young healthy HCMV<sup>+</sup> individuals. On the average, *NKG2C*<sup>+/+</sup> individuals presented increased proportions and absolute numbers of NKG2C<sup>+</sup> NK cells as compared to *NKG2C*<sup>+/-del</sup> subjects, independently of HLA-E allelic dimorphism. HCMV-induced NKG2C<sup>+</sup> NK cell expansion was unrelated to NKG2A<sup>+</sup> and total NK cell numbers, indicating that broader changes in the NK cell compartment previously observed in children with congenital infection were likely influenced by the closeness to primary infection.

Increased proportions of NKG2C<sup>+</sup> T lymphocytes were also originally reported to associate with HCMV<sup>+</sup> serology<sup>85</sup>. In contrast to the observations in NK cells, the *NKG2C* genotype appeared unrelated to the

proportions of CD3<sup>+</sup> NKG2C<sup>+</sup> cells in peripheral blood, likely reflecting a different regulation of NKR expression in the T-cell lineage. The role of CD94/NKG2C<sup>+</sup> T cells during HCMV infection remains unclear and deserves further attention.

The expansion of NKG2C<sup>+</sup> NK cells occurs following infection as indicated by studies in congenital and perinatal HCMV infection as well as in transplant recipients<sup>86,88–91</sup>. After infection control, NKG2C<sup>+</sup> NK cells persist in elevated numbers, as illustrated by the steadiness of the NK cell profiles in HCMV<sup>+</sup> individuals along a 5 year follow-up. Cross-sectional studies indirectly support that the magnitude of NKG2C<sup>+</sup> NK cell expansions is rather determined during primary infection, remaining stable along life, without increasing among elderly populations<sup>45,146,147</sup>. Yet, whether the *NKG2C* genotype may influence the long-term stability of the NK cell repertoire reconfiguration associated to HCMV in the elderly remains uncertain.

Recent studies have disclosed that HCMV-induced adaptive NK cells gather epigenetic modifications leading to the downregulation of specific signaling adaptors (i.e., Syk, Eat2 and FcRγ). Expression profiling of these molecules have led to the identification of FcRγ adaptor-negative NK cell subpopulations uncoupled from NKG2C expression in HCMV<sup>+</sup> individuals<sup>99,100</sup>. A recent work from our lab addressed the relationship between the *NKG2C* genotype and the distribution of NKG2C<sup>+</sup> and FcRγ-deficient NK cells in HCMV<sup>+</sup> individuals<sup>148</sup>. Data supported an association between *NKG2C* copy number and distinct distribution patterns of adaptive NK cells in HCMV<sup>+</sup> subjects. The average larger expansions of NKG2C<sup>bright</sup> NK cells in *NKG2C*<sup>+/+</sup> subjects usually contained moderate proportions of FcRγ<sup>-</sup> NK cells whereas the smaller NKG2C<sup>bright</sup> NK-cell pool in *NKG2C*<sup>+/*del*</sup> individuals accumulated greater proportions of FcRγ<sup>-</sup> NK cells. Of note, NKG2C<sup>-</sup> FcRγ<sup>-</sup> NK cells with an adaptive phenotype were more frequently detected in *NKG2C*<sup>+/*del*</sup> and *NKG2C*<sup>*del/del*</sup> individuals, further supporting that

NKG2C<sup>bright</sup> NK cell expansion and FcR $\gamma$  loss can occur independently. Studying the dynamics of additional epigenetic changes in relation to HCMV infection and the *NKG2C* genotype deserves attention requiring larger cohorts.

Conventionally, CD56<sup>dim</sup> NKG2A<sup>+</sup> KIR<sup>-</sup> NK cells are considered to differentiate into polyclonal CD56<sup>dim</sup> NKG2A<sup>-</sup> KIR<sup>+</sup> cells<sup>10</sup>. Such view should be revised by defining the origin of differentiated CD56<sup>dim</sup> NKG2C<sup>+</sup> NKG2A<sup>-</sup> KIR<sup>+</sup> cells expanded in response to HCMV. Further studies on the molecular mechanisms regulating CD94/NKG2C receptor expression in NK cells are warranted.

CD94/NKG2C is a heterodimeric C-type lectin-like receptor whose expression and signaling are dependent on the ITAM-containing DAP12 adaptor. CD94/NKG2C engagement triggers Ca<sup>2+</sup> mobilization and tyrosine kinase-dependent signaling pathways leading to cytotoxicity, cytokine production, and proliferation<sup>24,31</sup>. Co-culture with HCMV-infected fibroblasts promoted the proliferation of NKG2C<sup>bright</sup> NK cells, in a CD94/NKG2C-dependent manner<sup>101,102</sup>, indirectly supporting the involvement of a cognate interaction between the receptor and a putative viral ligand, present in infected cells, in the expansion and differentiation of NKG2C<sup>bright</sup> NK cells. Mature NK cells display low responsiveness to soluble cytokines such as IL-12 and IL-18<sup>95</sup>. Accordingly, a higher stimulation threshold for IL-15-induced proliferation was evidenced for NKG2C<sup>bright</sup> in comparison to NKG2C<sup>-</sup> and NKG2C<sup>dim</sup> NK cells. Interestingly, engagement of CD94/NKG2C, either by crosslinking with an agonistic antibody or upon HLA-E recognition, induced a vigorous proliferation of NKG2C<sup>bright</sup> NK cells in response to IL-15 stimulation, thus supporting that their clonal expansion was regulated by receptor signaling, in resemblance to T cells. In this context, the relation between *NKG2C* gene dose and the density of surface CD94/NKG2C receptor, both in HCMV<sup>-</sup> as well as in HCMV<sup>+</sup> individuals (NKG2C<sup>dim</sup> and NKG2C<sup>bright</sup> NK cells) could

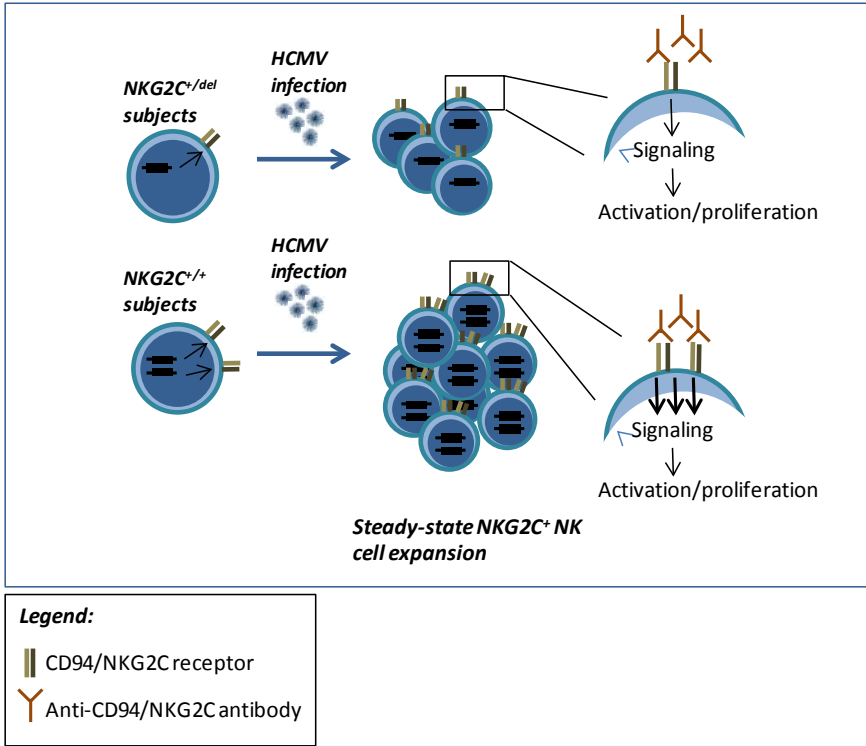
become relevant, as evidenced by our data showing how surface receptor levels had a direct impact on the magnitude of early signaling events (i.e.  $\text{Ca}^{2+}$  mobilization), protein-synthesis independent NK cell function (i.e. degranulation) as well as IL-15-dependent proliferation upon CD94/NKG2C engagement. A greater NKG2C-induced signaling in NKG2C<sup>bright</sup> NK cells from NKG2C<sup>+/+</sup> individuals might enhance downstream events regulating gene expression, proliferation and/or survival. Overall, these results predict that NKG2C<sup>dim</sup> NK cells from NKG2C<sup>+/+</sup> individuals would be more efficiently activated under limiting expression of the “putative” viral ligand for NKG2C. Eventually, an optimal activation through CD94/NKG2C in NKG2C<sup>+/+</sup> individuals might underlie the reduced proportion of NKG2C<sup>bright</sup> NK cells with late differentiation phenotype (FcR $\gamma$  loss), whereas a suboptimal CD94/NKG2C receptor activation in NKG2C<sup>+/-del</sup> individuals, could favor a relative increase of terminally differentiated FcR $\gamma$ <sup>-</sup> NKG2C<sup>bright</sup> cells. Alternatively, as discussed next, lower numbers of NKG2C<sup>bright</sup> NK cells in hemizygous individuals could associate to a reduced control of viral reactivation, indirectly promoting NKG2C<sup>bright</sup> NK cell late differentiation.

The response pattern of NKG2C<sup>bright</sup> NK cells to HCMV infection is reminiscent to that of murine Ly49H<sup>+</sup> NK cells, which specifically recognize m157 MCMV glycoprotein<sup>81,82</sup>, undergoing a sequential expansion and differentiation to persist in the circulation, contributing to an efficient control of subsequent re-infection<sup>84</sup>. Though the asymptomatic behavior of HCMV reactivation in healthy individuals hinders the analysis of immune mechanisms related with infection control in this setting, few observations indirectly support the anti-viral role of adaptive NKG2C<sup>+</sup> NK cells in HCMV infection. On one hand, a NKG2C<sup>+</sup> lymphocytosis paralleling the reduction in blood viral titers during an acute HCMV infection in a patient with a severe combined immunodeficiency, indirectly supported the ability of NKG2C<sup>bright</sup> NK cells to restrict viremia, at least partially, in the absence

of T cells<sup>149</sup>. On the other hand, a recent study in kidney transplant recipients revealed an association between high pre-transplant levels of adaptive NKG2C<sup>+</sup> NK cells and a lower risk of post-transplant HCMV infection (Redondo et al, submitted), supporting a protective role for this adaptive NK cell subset. It is of note that HCMV<sup>+</sup> *NKG2C<sup>del/del</sup>* individuals control this viral infection. *Yet*, in two independent studies, higher proportions of HCMV-specific CD8<sup>+</sup> T cells with an effector memory phenotype<sup>150</sup> and elevated HCMV-specific immunoglobulin G titers<sup>45</sup> were respectively associated with *NKG2C<sup>del/del</sup>* as compared to *NKG2C<sup>+/+</sup>* and *NKG2C<sup>+/-</sup>* HCMV<sup>+</sup> individuals, suggesting a more pronounced adaptive response in the absence of the CD94/NKG2C receptor.

We hypothesize that an inefficient control of the primary HCMV infection facilitates the differentiation and proliferation of NKG2C<sup>bright</sup> NK cells, generating a pool of mature, long-lived adaptive NK cells that may contribute to viral control, boosted upon viral re-infection or re-activation<sup>151</sup>. Accordingly, it is conceivable that *NKG2C<sup>+/+</sup>* individuals might control HCMV infection more efficiently than hemizygous individuals, resulting from a better response of their NKG2C<sup>+</sup> NK cells upon CD94/NKG2C receptor engagement.

In summary, our results pinpoint *NKG2C* copy number as a host genetic factor which partially contributes to the variability in the magnitude of NKG2C<sup>bright</sup> NK cell expansions in HCMV<sup>+</sup> subjects and further support an active involvement of this receptor in the reconfiguration of the NK cell compartment in HCMV-infected individuals.



**Figure 8. Impact of *NKG2C* gene dose in CD94/NKG2C receptor expression and function.**

Besides phenotypic changes, *NKG2C*<sup>bright</sup> NK cells show greater granzyme B levels and proinflammatory cytokine production (TNF- $\alpha$  and IFN- $\gamma$ ) following CD94/NKG2C or CD16 engagement<sup>104,105</sup>. In the second part of this thesis, we explored ADCC responses towards another common  $\gamma$ -herpesvirus such as Epstein-Barr virus, analyzing the participation of HCMV-induced *NKG2C*<sup>+</sup> NK cells.

Although the relevance of NK cells in the response to EBV is being progressively considered<sup>152,153</sup>, and the importance of ADCC is increasingly being appreciated in the context of other viral infections (e.g. HIV, Influenza)<sup>154,155</sup>, the study of antibody-dependent NK cell-mediated responses against EBV infection is limited to some early works<sup>141,142,144</sup>. Our investigation revisited this issue, showing that EBV-specific antibodies present in immune sera can trigger a vigorous NK cell degranulation and

cytokine production against EBV<sup>+</sup> targets, largely exceeding NK cell direct recognition and overcoming inhibition by HLA class-I expression levels in EBV<sup>+</sup> cells.

In agreement with previous studies, the NK cell response to EBV<sup>+</sup> infected cells was relatively modest and directed against cells in lytic cycle<sup>135</sup>. The latter were also preferentially targeted and eliminated by antibody-dependent NK cell cytotoxicity accompanied by the production of TNF- $\alpha$  and IFN- $\gamma$  with pro-inflammatory and antiviral effects<sup>133,134</sup>. Thus, upon primary infection and the development of a specific antibody response, ADCC may become an important mechanism for controlling EBV reactivation and dissemination together with specific T lymphocytes. The possibility that a deficient CD16-dependent antibody mediated response may underlie chronic active EBV infections should be envisaged.

Our data also evidenced that along the lytic cycle, EBV infected cells released viral antigen-containing vesicles and viral particles capable of binding to cells in latent phase and bystander non-infected B lymphocytes involving the interaction of the viral glycoprotein gp350/220 with CD21. Of note, despite promoting antibody-dependent NK cell degranulation and TNF- $\alpha$  secretion, primary B cells coated with gp350-containing vesicles appeared rather resistant to NK cell cytotoxicity and induced minimal levels of IFN- $\gamma$  production. We hypothesize that gp350<sup>+</sup> vesicles might act as a shield diverting the action of the NK cell cytolytic machinery (i.e. perforin) and impairing its direct action on the plasma membrane, thus explaining the dissociation observed between antibody-mediated NK cell activation and target cell killing. Remarkably, NK cell activation in this context was uncoupled from the anti-viral effect of IFN- $\gamma$  production while boosting a pro-inflammatory TNF- $\alpha$  response. It is of note that a poor IFN- $\gamma$  secretion dissociated from degranulation and TNF- $\alpha$  production was also observed upon NK cell activation of Rituximab treated primary B cells, suggesting that accessory signals required for an optimal IFN- $\gamma$  production are missing in

this setting. Among different possibilities, it has been shown that NK cell cytotoxicity and IFN- $\gamma$  secretion in response to IgG stimulation is particularly dependent on LFA-1 engagement as opposed to TNF- $\alpha$  production<sup>156,157</sup>. Differences in ICAM expression in primary B cells as compared to EBV<sup>+</sup> infected B cells in lytic cycle could underlie the TNF- $\alpha$  skewed response. Moreover, BZLF1 inhibits tumor necrosis factor receptor 1 (TNFR1) signaling in infected B cells<sup>158,159</sup>. Thus, rather than contributing to infection control, TNF- $\alpha$ -mediated inflammation might contribute to the pathogenesis of chronic active EBV infection, including the development of hemophagocytic lymphohistiocytosis (HLH)<sup>160</sup>.

Further studies are warranted to comparatively analyze the different outcomes upon antibody-dependent NK cell activation against primary B cells incubated with gp350<sup>+</sup> vesicles or viral particles, respectively. Approaching this aspect would require obtaining preparations of EBV virions free of non-infectious vesicles, a technically challenging process. It is conceivable that fusion of infectious EBV particles with the B cell plasma membrane may transfer envelope glycoproteins (e.g gp350) transiently enabling the anti-viral action of NK cell-mediated ADCC. Receptor-mediated EBV internalization requires the binding of a second glycoprotein, gp42 to MHC class II molecules on B cells<sup>118</sup> which initiates the fusion process involving the core complex gH/gL/gp42<sup>119</sup>. Thus, differences in the molecular composition of non-infectious gp350<sup>+</sup> vesicles and the virion envelope may qualitatively influence the antibody-dependent NK cell response.

Vallhov et al. demonstrated that gp350-containing exosomes competitively blocked EBV viral particle attachment to CD21, exerting a protective effect at this early stage of the B cell infection process<sup>161</sup>. Our data, introduces a novel perspective showing the capacity of gp350-containing vesicles to misdirect ADCC responses, a process that would dampen NK cell anti-viral capacity. It is difficult to ascertain whether the release of gp350<sup>+</sup>

noninfectious vesicles would have a beneficial or detrimental effect on EBV infection control *in vivo*. However, effective competition with infective viral particles would likely require high concentrations of CD21-bound vesicles, thus potentially magnifying NK cell activation and the corresponding pro-inflammatory response while subverting the antiviral function.

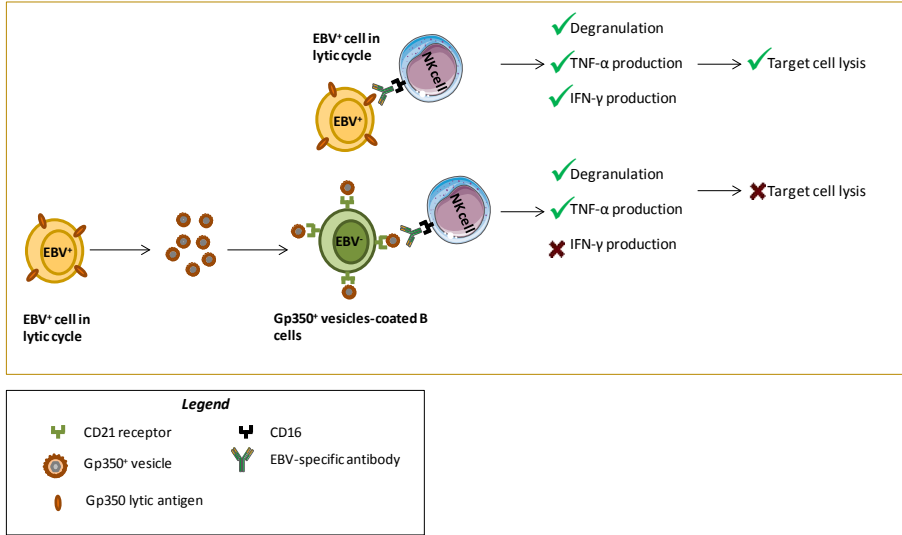
In line with former results using HCMV- and HSV-infected cells<sup>104–106</sup>, NKG2C<sup>bright</sup> NK cells displayed comparable degranulation yet enhanced cytokine production upon antibody-dependent recognition of EBV-infected cells, regardless of HLA class-I expression levels. Thus, HCMV-induced adaptive NK cells could contribute to the control of EBV reactivation through the secretion of IFN- $\gamma$  as previously reported<sup>133,134,162</sup>. Further studies are required to explore the effect of TNF- $\alpha$  in EBV reactivation. On the other hand, it is conceivable that TNF- $\alpha$  efficiently produced by adaptive NKG2C<sup>bright</sup> NK cells in response to stimulation by immunocomplexes formed by gp350<sup>+</sup> non-infectious vesicles and virions<sup>105</sup>, might contribute to systemic inflammation associated to EBV infection.

The participation of other immune cells bearing CD16 and other Fc $\gamma$ R (i.e. TCR $\alpha\beta$ <sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup> cytolytic T lymphocytes subsets and myelomonocytic cells) in antibody-dependent responses against EBV infected cells and the influence of gp350<sup>+</sup> vesicles deserves attention. Genetic factors such as CD16A and IgG allotypes epistatically interact modulating antibody-dependent NK cell activation against HSV-infected cells as well as the susceptibility to HCV clinical reactivation<sup>106</sup>. It is plausible that CD16 and IgG allotypes might also qualitatively modulate the antibody-mediated response to EBV infection.

Identification of the viral antigens on the infected cell surface which could potentially become ADCC targets could improve vaccination strategies to control EBV infection. Few viral glycoproteins such as gp350<sup>142</sup> and gp110<sup>141</sup> have been described as a potential targets for ADCC responses. On the

other hand, EBV latent phase antigens with reported surface expression (i.e. LMP1 and LMP2) could represent suitable targets for antibody-dependent NK cell activation and, despite their low immunogenicity, anti-LMP1 antibodies have been reported in some individuals<sup>143</sup>.

In summary, our data support that antibody-dependent NK cell activation plays an important role in the control of EBV, overriding the relative resistance of infected B cells to direct NK cell activity and, on the other hand, suggest that the release of gp350<sup>+</sup> vesicles might favor viral immune evasion /dissemination.



**Figure 9. Differential antibody-mediated NK cell responses against EBV<sup>+</sup> infected cells in lytic cycle and gp350<sup>+</sup> vesicles-coated B cells.**



## **Chapter 6**

### Conclusions

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1. *NKG2C*<sup>+/+</sup>, *NKG2C*<sup>+/*del*</sup>, and *NKG2C*<sup>*del/del*</sup> genotype frequencies were similar to those reported in other cohorts and comparable in HCMV<sup>+</sup> and HCMV<sup>-</sup> young healthy donors.
2. *NKG2C*<sup>bright</sup> adaptive NK cells were detected in both *NKG2C*<sup>+/+</sup> and *NKG2C*<sup>+/*del*</sup> HCMV<sup>+</sup> individuals, consistent with a major role of the viral infection.
3. A relation of *NKG2C* copy number with the magnitude of the steady state redistribution of the NK cell compartment induced by HCMV was observed.
4. *NKG2C* zygosity appeared unrelated with the increase of *NKG2C*<sup>+</sup> T cell numbers detected in HCMV<sup>+</sup> individuals, suggesting a different regulation of the NKR expression in the T-cell lineage.
5. The influence of *NKG2C* copy number on *NKG2C*<sup>+</sup> NK cell numbers was independent of HLA-E allelic dimorphism.
6. *NKG2C* gene dose was directly associated with CD94/*NKG2C* surface expression levels as well as with receptor-triggered NK cell activation and IL-15-dependent proliferation.
7. Altogether, these results indirectly supported an active role of the CD94/*NKG2C* receptor in the development of adaptive NK cells in response to HCMV infection.
8. EBV-specific serum antibodies efficiently triggered NK cell-mediated effector functions against EBV-infected B cells in lytic cycle, overriding their relative resistance to direct NK cell activation.
9. Adaptive *NKG2C*<sup>+</sup> NK cells displayed enhanced antibody-dependent cytokine production against EBV-infected cells.

10. Adsorption of gp350<sup>+</sup> vesicles/viral particles, released by EBV-infected cells, to primary B lymphocytes efficiently triggered antibody-mediated NK cell degranulation and TNF- $\alpha$  secretion, but induced minimal target cell damage and IFN- $\gamma$  production.
11. Altogether this set of data supported that antibody-dependent NK cell activation plays an important role in the control of EBV, suggesting that the release of gp350<sup>+</sup> vesicles might partially divert the response favoring viral immune evasion.





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## ANNEX 1

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## ANNEX 2

### ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
CLP	Common lymphoid progenitor
CR2	Complement receptor 2
DAP	DNAX adapter protein
DCs	Dendritic cells
EBV	Epstein-Barr Virus
Fc	Fragment crystallizable
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HSC	Hematopoietic stem cell
HSV	Herpes simplex virus
IE	immediate-early
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cells
IM	Infectious mononucleosis
ITAM	immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibition motifs
KIR	Killer Immunoglobulin-like receptors
KSV	Kaposi's sarcoma herpesvirus
LCL	Lymphoblastoid cell lines
LILRB1	Leukocyte Immunoglobulin-like Receptor B1
LMP	Latent membrane proteins
LTi	Lymphoid tissue inducer
mAbs	Monoclonal antibodies
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptors
NK	Natural killer cells
NKR	Natural killer receptor
ORF	open reading frames
PI3K	Phosphatidylinositol 3-kinase
TAP-1	Transporter associated antigen processing-1
TGF- $\beta$	Transforming growth factor- $\beta$
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
ULBP	UL16 binding protein

## ANNEX 3

### LIST OF PUBLICATIONS

➤ **Publications included in the thesis:**

1. **López-Montañés, M**, Alari-Pahissa E, Sintés J, Martínez-Rodríguez JE, Muntasell A, López-Botet M. Antibody-dependent NK cell activation differentially targets EBV-infected cells in lytic cycle and bystander B lymphocytes bound to viral antigen-containing vesicles. Submitted. 2016.
2. Muntasell A, **López-Montañés M**, Vera A, Heredia G, Romo N, Peñafiel J, Moraru M, Vila J, Vilches C, López-Botet M. *NKG2C* zygosity influences CD94/NKG2C receptor function and the NK-cell compartment redistribution in response to human cytomegalovirus. *Eur J. Immunol.* 2013 Dec;43(12):3268-78.

➤ **Publications not included in the thesis:**

3. Crespo M, Yelamos J, Redondo D, Muntasell A, Perez-Saéz MJ, **López-Montañés M**, García C, Torio A, Mir M, Hernández JJ, López-Botet M, Pascual J. Circulating NK-cell subsets in renal allograft recipients with anti-HLA donor-specific antibodies. *Am J Transplant.* 2015 Mar;15(3):806-14.
4. 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.



