

Regulation of Natural Killer and CD4<sup>+</sup>T cell function by  
NKG2 C-type lectin-like receptors

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Doctoral Thesis UPF - 2009

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## **ACKNOWLEDGEMENTS**

I would like to acknowledge the following people, who in many different ways have significantly contributed to the work presented in this thesis: Ana Angulo, Bea Bellosillo, Tamara Brckalo, Esteve Caramés, Eugenia Corrales, Oscar Fornas, Mónica Gumá, Gemma Heredia, Giuliana Magri, Noemí Marina, José Martínez, Esther Menoyo, Aura Muntasell, Neus Romo and Medya Shikhagaie. To all of them, thanks for you help and support.

I am also grateful to my thesis director, for everything I had the opportunity to learn.

And very specially to my parents, who always encouraged me in my scientific career.



## THESIS ABSTRACT

This work is centered on the study of the NKG2 C-type lectin-like receptors on NK and CD4+T cells. We provide evidence supporting that CD4+T cells specific for Human Cytomegalovirus (HCMV) may express different NK cell receptors, and demonstrate that the C-type lectin-like receptor NKG2D is expressed on cytotoxic CD4+T cells with an effector/memory phenotype, enhancing their TCR-dependent proliferation and cytokine production. A second part of the work is centered on the study of the CD94/NKG2 receptors on NK cells. We show that NKG2A can be induced on NKG2C+ NK cells upon activation with rIL-12 or when cocultured with HCMV-infected dendritic cells, and that NKG2A expression inhibits the response of NKG2C+NK clones against HLA-E-expressing targets, providing a potential regulatory feedback mechanism to control cell activation. Altogether, our results support that expression of NKG2 C-type lectin like receptors may be shaped during the course of viral infections, providing mechanisms to finely regulate both NK and CD4+T cell functions.

## RESUM DE LA TESI

Aquesta tesi es centra en l'estudi dels receptors lectina de tipus C NKG2 en cèl·lules *Natural Killer* i T CD4+. Demostrem que les cèl·lules T CD4+ específiques pel Cytomegalovirus Humà poden expressar diferents receptors NK, i que el receptor lectina tipus C NKG2D s'expressa en cèl·lules citotòxiques i de memòria, potenciant la proliferació i secreció de citocines depenent del *TCR*. La segona part d'aquesta tesi es centra en l'estudi de l'expressió dels receptors CD94/NKG2 en cèl·lules NK. Mostrem com l'expressió de CD94/NKG2A s'indueix en cèl·lules CD94/NKG2C+ estimulades amb IL-12 o cultivades amb cèl·lules dendrítiques infectades pel Cytomegalovirus Humà, i que l'expressió de CD94/NKG2A inhibeix la resposta de clons NK CD94/NKG2C+ envers dianes HLA-E+, constituint un possible mecanisme de *feedback* negatiu per controlar l'activació cel·lular. En resum, els nostres resultats demostren que l'expressió dels receptors lectina tipus C NKG2 pot ser modificada durant les infeccions víriques constituint un possible mecanisme per regular la resposta tant de cèl·lules NK com T CD4+.



## PREFACE

Original observations supporting that some lymphocytes were able to kill tumor cells without any prior antigenic sensitization, led to the definition of *Natural Killer (NK)* cells in the early 70s. Since then, researchers have worked to unravel the biological role of NK cells in the context of the immune response to infections and cancer, and how their effector functions are regulated. Key aspects of NK cell biology have been discovered along the past 15 years. NK cell subsets with different phenotype and function have been identified, their interactions with other cells of the immune system have been unraveled, and clinical applications based on this knowledge are currently being envisaged. The major progress lies on the identification of a set of surface receptors employed by NK cells as sensors to discriminate pathological targets, selectively triggering their effector functions. Our work provides original insights on how some of these molecules may regulate the response not only of NK cells but also of some T lymphocyte subsets to pathogenic stimuli



# CONTENTS

Thesis Abstract		i
Preface		iii
<b>PART I</b>	<b>INTRODUCTION AND AIMS</b>	
Chapter 1	Introduction	7
	1.1 Natural Killer cell biology	9
	1.1.1 Development and distribution of NK cells	9
	1.1.2 Activation and Effector functions	11
	1.2. Natural Killer cell Receptors	14
	1.2.1 MHC-I mediated regulation of NK cell function: the “missing-self hypothesis”	14
	1.2.2 NK cell receptor signalling	16
	1.2.3 NK cell receptor repertoire	18
	1.2.4 NK cell receptors on T cells	30
	1.3. Immune responses to Human Cytomegalovirus	33
	1.3.1 The Human Cytomegalovirus	33
	1.3.2 The immune response to HCMV	35
	1.3.3 NK cell receptors in the response to HCMV	40
Chapter 2.	Aims	45



<b>PART II</b>	<b>NK CELL RECEPTOR EXPRESSION ON CD4+T CELLS</b>	
Chapter 3	Expression and function of NKG2D on CD4+T cells specific for Human Cytomegalovirus. <i>Eur J Immunol</i> 2006; 36(12):3198-206	51
Chapter 4	Expression of NK cell receptors in CD4+T-Large Granular Lymphocytosis. <i>Manuscript in preparation</i>	77
<b>PART III</b>	<b>CD94/NKG2 RECEPTORS IN THE REGULATION OF NK CELL FUNCTION</b>	
Chapter 5	IL-12 dependent inducible expression of the CD94/NKG2A inhibitory receptor regulates CD94/NKG2C+ NK cell function. <i>J Immunol</i> 2009 182 (2)	91
Chapter 6	Expansion of NKG2A+ NK cells upon stimulation of PBMC with autologus Epstein-Barr virus infected B cells. <i>Preliminary results</i>	125
<b>PART IV</b>	<b>DISCUSSION AND CONCLUSIONS</b>	
Chapter 7	Discussion	141
Chapter 8	Conclusions	151
<b>ANNEX 1</b>	References	157
<b>ANNEX 2</b>	Abbreviations	181



**PART I**

**INTRODUCTION AND AIMS**



Chapter 1

Introduction

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## 1.1 NATURAL KILLER CELL BIOLOGY

In 1975, Rolf Kiessling, Hans Wigzell (Karolinska Institute, Sweden) and Ronald Herberman (NCI-NIH, USA) described Natural Killer (NK) cells as lymphocytes displaying spontaneous (natural) cytotoxicity against tumors. Since then, a large number of studies have focused on unravelling the role of NK cells in immune responses. It has become clear that NK cells can directly contribute to the innate immune defence against tumors, viruses and other microbial pathogens, but also regulate and promote adaptive immune responses. NK cells have a heterogeneous repertoire of surface activating receptors that allow them to react to different stimuli such as microbial products, cytokines, chemokines, and inducible molecules expressed on infected and transformed cells. Inhibitory receptors specific for MHC class I molecules play a key role in the ability of NK cells to discriminate between normal and pathological cells. Following activation, they can directly kill target cells, produce cytokines that restrict viral replication, or prime other cells of the immune system <sup>1-4</sup>.

### 1.1.1 DEVELOPMENT AND DISTRIBUTION OF NK CELLS

NK cells are derived from haematopoietic stem cells (HSC), and the bone marrow is considered the main site of NK cell generation in adults, providing cytokines, growth factors and stromal cells necessary for NK cell development.<sup>3</sup> Early experiments in mice proved that bone marrow ablation seriously compromised NK cell development <sup>5,6</sup>, whereas NK cell numbers are not affected in athymic nude mice, patients with Di George syndrome or mice and humans suffering from splenectomy <sup>7-10</sup>.

Human NK cells develop from CD34<sup>+</sup> haematopoietic progenitors. Transcription factors involved in development of NK cells include Ikaros, ETS1 and PU.1 and ID proteins (inhibitors of DNA binding) <sup>3</sup>. NK cell precursors (NKP) were first identified in the adult mouse bone marrow. These cells could

## Chapter 1

give rise to NK cells but not to B, T, myeloid or erythroid cells.<sup>11</sup> Human bone marrow NKPs are negative for CD16, CD56 and other NK-cell receptors, but express IL-2R $\beta$  and IL-15R $\alpha$  and respond ex-vivo to IL-2 and IL-15. In fact, IL-15 is essential for NK cell development as shown by the fact that absence of IL-15 signaling in humans or mice causes severe reduction in NK cell numbers<sup>12</sup>. It is thought that the interaction of the c-kit and flt-3 receptors with their ligands in the bone marrow stromal cells synergises with IL-15 to enhance NK cell development<sup>13-16</sup>. Maturation of NKPs can be subdivided into further steps defined by the acquisition of receptors. First, NKR-P1 receptors are expressed, followed by CD94-NKG2A and lastly KIR (see next chapter for a more detailed description on NK cell receptors)<sup>3</sup>. This orderly acquisition of inhibitory receptors has also been analysed in patients recovering from haematopoietic stem cell transplantation, where NK cells are mainly NKG2A+ KIR- early after reconstitution, and later begin to acquire KIR<sup>17</sup>.

Mature NK cells, characterised by the presence of CD56 and the lack of CD3, are exported to the periphery and are found in blood, liver and spleen more abundantly, though they are also present in lymph nodes, and can migrate into inflamed tissues and tumors<sup>18</sup>. Two different human NK cell subsets have been described according to the surface levels of the CD56 molecule, the 140KDa isoform of neural cell adhesion-molecule (NCAM) expressed by NK and T cell subsets. The two NK cell subsets differ in their proliferative response, cytolytic capacity, cytokine production and NK cell receptor and adhesion molecule expression. Regarding NK cell receptor distribution, CD56bright cells are NKG2A+ but lack CD16, ILT2 and only a low proportion express KIR. By contrast, CD56dim NK cells are CD16+ and contain variable proportions of ILT2+, KIR+ and NKG2A+ cells. CD56bright NK cells produce higher levels of cytokines whereas CD56dim NK cells are more potent killers<sup>19</sup>.

Interestingly, some studies described the existence of a subset of CD56- NK cells. These cells are negative for CD56, CD3, CD4, TCR, CD19 and CD14, but

express CD16 and other NK cell receptors. They were initially described in the context of HIV infection <sup>20,21</sup>, though studies in our laboratory show variable proportions of this subset in peripheral blood of healthy subjects (Romo N, unpublished). Further studies are needed to evaluate the role of the CD56- NK cell subset.

### 1.1.2 ACTIVATION AND EFFECTOR FUNCTIONS

NK cell effector functions include cytotoxicity and cytokine secretion. NK cells can directly kill tumors, virus-infected cells, and IgG-coated cells (through Fc $\gamma$ R III), but can also promote an inflammatory response, control viral replication, favour Th1 polarization and modulate haematopoiesis through secretion of different cytokines and chemokines <sup>22,23</sup>. IFN $\gamma$  and TNF $\alpha$  produced by NK cells directly contribute to the control of several infections, and NK cells can also produce chemokines like MIP-1 $\beta$ , MIP-1 $\alpha$  and RANTES to attract other inflammatory cells <sup>24</sup>. Transcripts for certain cytokines and cytolytic mediators (perforin and granzyme) are constitutively expressed on resting NK cells <sup>25,26</sup>. Yet, to become activated, they need to be triggered through engagement of specific receptors on their cell surface, or by exposure to cytokines secreted by other cells such as type I IFN, IL-15, IL-12 and IL-18 <sup>1</sup>.

The CD56bright subset represents only around 10% of NK cells in peripheral blood, but the majority in secondary lymphoid organs. CD56bright cells have limited cytolytic capacity but are more potent producers of cytokines such as IFN $\gamma$ , TNF $\alpha$ , LT $\beta$ , GM-CSF, IL-10, IL-5 and IL-13 as compared to CD56dim cells. CD56bright cells have a limited ability to spontaneously kill target cells, however, some studies have shown that they can acquire efficient killing capacity after stimulation with IL-2 or IL-12 <sup>27</sup>. IFN $\gamma$  is considered the prototypic NK cell cytokine, and is usually produced after stimulation with IL-12 and IL-1, IL-2, IL-15, IL-18 or upon engagement of activating NKRs <sup>19</sup>. CCR7 expression drives CD56bright cells to the lymph nodes, where co-stimulation with T cell-derived

## Chapter 1

IL-2 and DC-derived IL-12 results in the production of large amounts of IFN $\gamma$ , which in turn can further activate APCs to up-regulate MHC-I expression and favor Th1 polarization<sup>28,29</sup>. IFN $\gamma$  also activates macrophage-mediated killing and has antiproliferative effects on viral and malignant-transformed cells, for instance suppressing herpes viral gene expression<sup>18</sup>, or limiting B cell transformation by Epstein-Barr virus<sup>30</sup>. The CD56bright subset represents around 10% of total NK cells in peripheral blood. This population proliferates in the presence of activated myeloid immature DC and produces high amounts of IFN $\gamma$ . In fact, when NK cells are cultured in the presence of LPS-activated DC, proliferation and IFN $\gamma$  secretion is mainly confined to the CD56bright subset<sup>31</sup>.

The majority of NK cells in peripheral blood are CD56dim NK cells. They are characterised by having a higher cytolytic capacity, and secrete lower levels of IFN $\gamma$ . CD56dim cells also express CD16 (Fc $\gamma$ RIII), which can bind to the constant (Fc) region of immunoglobulins and trigger lysis of IgG-coated cells, a mechanism called antibody-dependent cellular cytotoxicity (ADCC)<sup>32</sup>. In peripheral tissues, the inflammatory response to pathogens is characterised by the release of various cytokines and chemokines by DCs, endothelial cells, macrophages, neutrophils, fibroblasts, mast cells and eosinophils. Circulating NK cells express chemokine receptors that respond to these stimuli (CXCR1 and CX3CR1) promoting NK cell extravasation and activation. However, a number of studies have reported the presence of CD56brightCD16- rather than CD56dimCD16+ NK cells at sites of inflammation<sup>32,33</sup>. Myeloid DC release IL-12 and IL-15 after activation by various pathogens, enhancing NK cell-mediated cytotoxicity and IFN $\gamma$  secretion<sup>34</sup>. On the other hand, IL-4 secretion by resident mast cells and eosinophils may inhibit cytokine production and cytotoxicity of NK cells in peripheral tissues, since incubation of NK cells *in vitro* with IL-4 has been shown to inhibit their response to target cell lines<sup>35,36</sup>.

Importantly, as will be discussed later, activation of NK cells also occurs through engagement of specific activating receptors by ligands present on target cells <sup>37-39</sup>. Moreover, adhesion molecules like LFA-1 and CD2 have also been shown to participate in NK cell activation <sup>40,41</sup>.

The main pathway of NK cell cytotoxicity depends on perforin and granzymes, but also on FAS-L and TRAIL <sup>42</sup>. Perforin and granzymes are released by NK cells into the synapse causing target cell death, and have been implicated in killing of tumors <sup>43,44</sup> and virus-infected cells <sup>45-48</sup>. TRAIL has been shown to contribute to NK cell mediated immune responses against tumors and encephalomyocarditis <sup>49-51</sup>. Engagement of Fas receptors by Fas-ligand on NK cells has also been involved in the rejection of some tumors <sup>52</sup>.

A number of studies have focused on the so called NK/DC cross-talk, a concept that includes different mechanisms leading to reciprocal regulation of both cell types. As mentioned, dendritic cells can activate NK cell effector functions through secretion of different cytokines. Myeloid and plasmacytoid DC promote NK cell mediated cytotoxicity through secretion of type I IFN in response to different microbial stimuli, and IFN $\gamma$  production by NK cells is triggered by IL-12 secretion and cell-cell contact with DC <sup>53</sup>. Monocyte-derived DC (moDC) have also been reported to activate NK cells through direct cell contact <sup>54-56</sup> and secretion of cytokines <sup>57-60</sup>. On the other hand, NK cells can also activate and promote DC maturation, in a process involving IFN $\gamma$  and TNF $\alpha$  secretion and cell-cell contacts <sup>56</sup>. Several studies have been published showing that NK cells can kill immature moDC through the engagement of the NK cell activating receptor NKp30 when high NK/DC ratios are used in vitro <sup>61</sup>, and has been proposed as a mechanism to eliminate immature DC (iDC) in favor of mature DC. However, in studies using myeloid and plasmacytoid iDCs instead of moDC, no NK cell-mediated killing was observed <sup>53</sup>. A recent publication shows that BAT3 (HLA-B-Associated Transcript-3) is released by immature DC and binds NKp30, triggering NK cell cytotoxicity and killing of iDC <sup>62</sup>. Mature DC

## Chapter 1

appeared resistant to NK cell lysis, predictably by HLA-E engagement of the NKG2A inhibitory receptor <sup>56,61</sup>.

It seems clear that NK cells can promote Th1 responses through activation of APC, but it has also been shown that NK cells may directly activate CD4+T cell responses. Human activated NK cells, like T cells, express MHC-II molecules, and in different experiments NK clones were shown to present antigen via MHC class II to CD4+T cells <sup>63</sup>. In addition, NK cells can express different ligands for costimulatory receptors involved in T cell activation. IL-12 and IL-15 induce expression of CD86 on NK cells, and engagement of some NK cell activating receptors induces surface expression of the OX40-ligand, that binds OX40 in activated CD4+T cells. Zingoni et al. showed that receptor-activated NK cells co-stimulate TCR-induced proliferation and cytokine production of autologous CD4+T cells through OX40-OX40L and CD28-CD86 <sup>64</sup>. NK-T cell interactions could take place both in the parafollicular region of the lymph nodes and in peripheral organs such as the liver, where NK cells and T cells have been shown to accumulate under pathological conditions (i.e. MCMV infection)

## 1.2 NATURAL KILLER CELL RECEPTORS

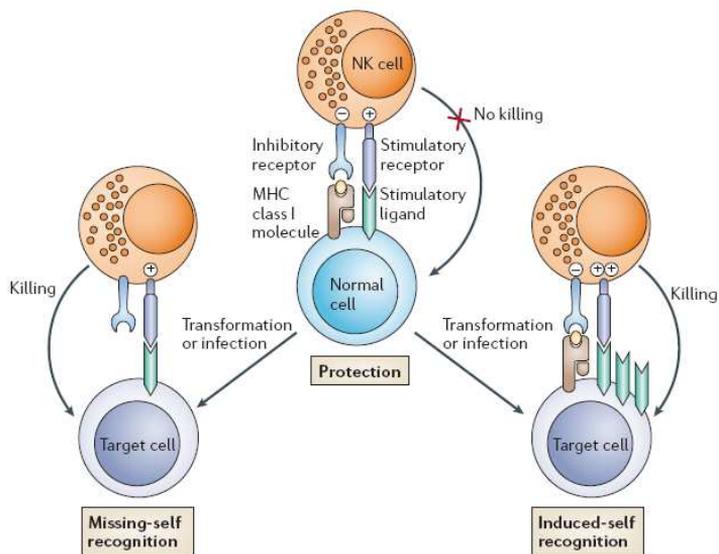
### 1.2.1 MHC CLASS I-MEDIATED REGULATION OF NK CELL FUNCTION: THE “MISSING-SELF HYPOTHESIS”

T and B cells possess a single type of antigen receptor that dominates their development and activation. Signals initiated through these antigen receptors are modulated by engagement of costimulatory molecules. In contrast, NK functions are controlled by a vast combination of inhibitory and activating receptors <sup>65</sup>. Inhibitory receptors prevent autoreactivity against normal autologous cells by binding self MHC-I molecules <sup>66</sup>. This basic mechanism was first proposed as the “missing-self hypothesis” by Karre et al. when they observed that MHC-I expression protected tumor cells against NK cell activation. On the other hand,

NK cells express activating receptors that trigger cytokine secretion and/or cytotoxicity against transformed or infected cells upon engagement with specific ligands. NK cells display a wide variety of activating receptors, allowing them to detect the presence of stress-induced molecules (ULBP and MIC) on tumors and virus-infected cells, pathogen associated molecular patterns (PAMPs), or specific pathogen-derived molecules (i.e. the MCMV m157 protein or the influenza virus hemagglutinin) <sup>67-69</sup>. However, ligands for some activating receptors still remain unknown, and it remains an intriguing question why some activating receptors bind self-MHC-I molecules (see discussion below).

Activation of NK cell functions is controlled by the balance between inhibitory and activating signals. As mentioned, engagement of inhibitory receptors on NK cells by self MHC-I molecules allows recognition of normal cells and prevents autoreactivity. Downmodulation of MHC-I may happen in tumors and some virus-infected cells, and serves as an escape mechanism of T cell responses. When MHC-I is lost or down-regulated, NK cells are no longer inhibited through their MHC-I specific inhibitory receptors and become able to lyse target cells<sup>38</sup>. However, even if MHC-I expression is lost, activation and killing may not take place. For instance, NK cells do not lyse erythrocytes, which lack MHC-I molecules, or other cell types with low MHC-I expression like hepatocytes or fibroblasts. It is believed that NK cells can be inhibited by molecules other than MHC-I, like CEACAM1 (carcinoembryonic antigen cellular adhesion molecules 1), but also that activating receptors need to be engaged by specific ligands on target cells to trigger effector functions <sup>70</sup>.

Thus, the response of an NK cell against a target will ultimately be conditioned by the balance between inhibitory and activating ligands present on the target cell and by the influence of cytokines (Figure 1).



**Figure 1:** Regulation of NK cell function by NK cell inhibitory and activating receptors <sup>71</sup>

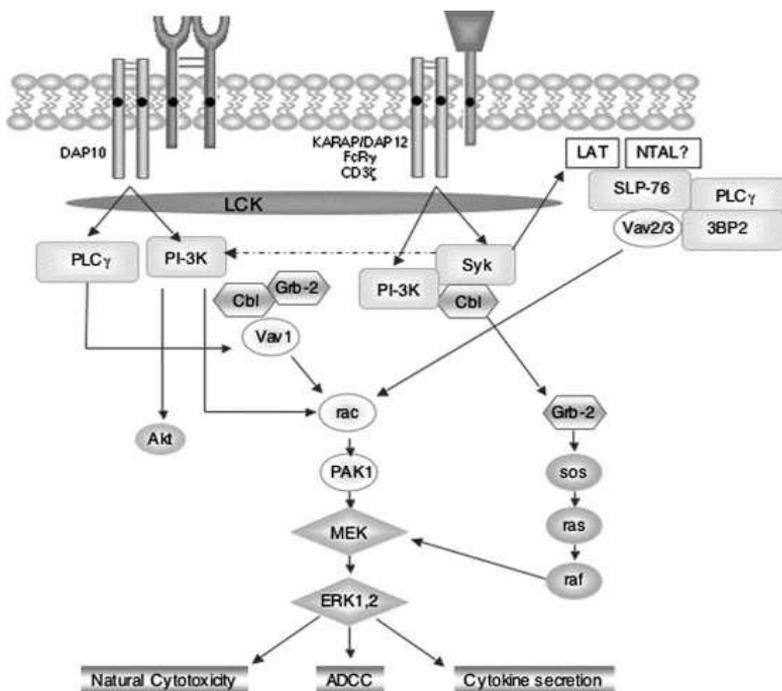
NK cells were predicted to acquire at least one inhibitory receptor during differentiation to prevent autoimmunity <sup>72</sup>. Though recent studies have detected peripheral blood NK cells which lack expression of inhibitory NKR <sup>73</sup>, it has been proposed that NK cells missing expression of NKG2A and KIR represent functionally immature NK cells with low cytotoxic capacity and IFN $\gamma$  production<sup>74</sup>.

### 1.2.2 NK CELL RECEPTOR SIGNALLING

Activating receptors are coupled to adaptor molecules that transduce activating signals. Several adaptor proteins exist that bind to different receptors through an aspartate residue in their transmembrane region, which interacts with an Arginin or Lysine residue present on the receptor. CD3 $\xi$ , Fc $\epsilon$ RI $\gamma$ -chain or DAP12

adaptors bear in their cytoplasmic tail *immunoreceptor tyrosine-based activation motifs* (ITAM). When the receptor is engaged, ITAMs are phosphorylated by Src-family kinases and become able to bind ZAP70 and/or Syk tyrosine kinases. Downstream events include among others phosphorylation of SLP-76, 3BP2, Shc, p85 PI3-kinase, c-Cbl, phospholipase C (PLC)- $\gamma$ 1 and PLC- $\gamma$ 2, recruitment of Grb2, Vav-1 and Vav-2, a raise of intracellular Ca<sup>2+</sup> levels, and the activation of Rho, Ras, p38 mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK). DAP 10 (*DNAX activating protein of 10 kDa*) constitutes a different adaptor molecule containing the YxxM motif. It can be phosphorylated by Src-family kinases or Jak3, and binds either the p85 subunit of the PI3K or the Grb2 adaptor, activating signals through Vav1, Rho, GTPases and PLC $\gamma$ . Activation of PI3K leads to AKT phosphorylation, that exerts anti-apoptotic effects <sup>75-78</sup> (figure 2).

At a molecular level, the role of inhibitory receptors is to block the intracellular cascade initiated by activating signals. Inhibitory receptors contain in their cytoplasmic tail *immunoreceptor tyrosine-based inhibitory motif* (ITIM) whose canonical sequence is V/IxYxxL/V. Upon receptor engagement, they become phosphorylated by Src family kinases and are then able to bind tyrosine phosphatases containing SH2 domains (i.e SHP-1, SHP-2 or SHIP). SHP-1 and SHP-2 dephosphorylate different proteins coupled to activating receptors (i.e Src or syk kinases and adaptor proteins), and SHIP dephosphorylates inositol phosphates <sup>77-79</sup>, all leading to blocking of the activating cascade.



**Figure 2:** Signaling cascades downstream of NK cell activating receptors bound to DAP10, DAP12, CD3ξ and FcεRI γ-chain <sup>78</sup>.

### 1.2.3 THE NK CELL RECEPTOR REPERTOIRE

NK cells express a wide variety of activating and inhibitory receptors. Yet, not all of them are designated NK cell receptors (NKR), either because they were not initially described on NK cells and/or they show a much broader distribution in other cell types (i.e. TLRs, FcγR III). NKR can be classified in two different groups according to their extracellular structure: C-type lectin-like receptors and immunoglobulin-like receptors. This chapter will focus on the description of NKR, but other NK-cell associated receptors will also be briefly reviewed. The specific implication of these molecules in the response to Human Cytomegalovirus will be discussed in the next chapter.

## C-TYPE LECTIN-LIKE NK CELL RECEPTORS

## – CD94/NKG2 receptors

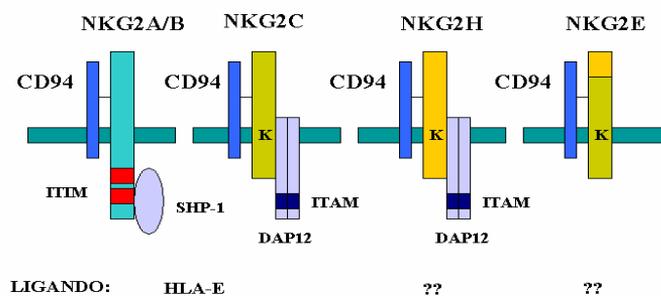
CD94 and NKG2 are type II integral membrane glycoproteins that contain an extracellular C-type carbohydrate recognition domain (CRD) and are encoded in chromosome 12 together with other members of the C-type lectin superfamily (NKG2D, CD69, NKR-P1A). CD94-NKG2 heterodimers are selectively expressed by NK cells and a subset of cytotoxic T lymphocytes<sup>80</sup>. CD94 binds to NKG2 receptors through disulfide bridges and is required to stabilize their surface expression, though it may also form surface homodimers<sup>81,82</sup>.

CD94/NKG2 receptors comprise five different molecules coded by three different genes: NKG2A, NKG2C and NKG2E<sup>83</sup>. Alternative splicing of A and E gives rise to isoforms NKG2B and NKG2H respectively<sup>84</sup>. CD94/NKG2 receptors show a high degree of homology in their ligand-binding domains; however, NKG2A/B present longer cytoplasmic tails containing ITIM, whereas NKG2C and –E/H have short intracellular regions and associate to the DAP12 adaptor molecule forming triggering receptors<sup>85-89</sup>. The NKG2F receptor, also coded by an adjacent gene, does not bind to CD94, and is only expressed intracellularly; though it has been shown to interact with DAP12, its function remains unknown<sup>90</sup>. The CD94/NKG2A inhibitory receptor is coupled to SHP tyrosine phosphatases through the NKG2A ITIM. Signalling via CD94/NKG2A has been shown to inhibit CD16-dependent activation of syk and ERK<sup>91,92</sup>. By contrast, engagement of the CD94/NKG2C activating receptor by specific mAb triggers a tyrosine kinase dependent pathway and activates p56lck, ZAP-70, PI3K and PLC $\gamma$ 1<sup>87,93</sup>. So far, functional studies have been performed in cells expressing either NKG2A or NKG2C, yet transcripts for both receptors have been detected in some clones<sup>94</sup>.

Both CD94/NKG2A and CD94/NKG2C, as well as CD94/NKG2E, bind to the non-classical HLA-E molecule, which presents peptides derived from the

## Chapter 1

leader sequence of other MHC-I molecules <sup>95-98</sup>. This is considered a sensitive mechanism evolved to survey the normal biosynthesis of HLA class I molecules, a process that can be hampered in certain virus-infected and tumor cells. The association of peptides to the HLA-E molecule depends on the presence of a Met residue in position 2. This Met is not present in every HLA allotype, and in fact, signal sequences derived from most HLA-A and HLA-C alleles but only from a subset of HLA-B molecules are able to bind to HLA-E. Nonamers presented by HLA-E may influence CD94/NKG2 mediated recognition by modulating the affinity of the receptor for its ligand. In a number of experiments, HLA-E loaded with different HLA leader sequence peptides was recognized by CD94/NKG2C+ NK clones less efficiently than by CD94/NKG2A+ cells <sup>99</sup>, and a lower affinity of the activating receptor was later confirmed <sup>100</sup>. The ability of CD94/NKG2 receptors to discriminate among different HLA-E/peptide complexes might also influence NK and T-cell reactivity against allogeneic and virus-infected cells. On the other hand, two HLA-E alleles have been described, distinguished by a single sequence dimorphism at position 107, glycine (E\*0101) or arginine (E\*0103), that do not appear to affect the affinity of HLA-E for the CD94/NKG2 receptors <sup>98</sup>.



**Figure 3:** The family of CD94/NKG2 C-type lectin-like receptors

## – NKG2D

NKG2D, also named KLRK1 (killer cell lectin like receptor, subfamily K, member 1), is a conserved lectin-like receptor encoded in human chromosome 12 and constitutively expressed by NK cells, CD8+  $\alpha\beta$ T cells,  $\gamma\delta$ T cells, and murine macrophages. The NKG2D gene is clustered together with the genes coding for the CD94/NKG2 receptors described above, yet it bears little homology with the CD94/NKG2 receptors. The human NKG2D receptor complex is an hexamer, formed by an NKG2D homodimer associating with two DAP10 homodimers. In mice, NKG2D exists in long (NKG2D-L) and short (NKG2D-S) isoforms, which are generated by alternative splicing. Whereas NKG2D-L only pairs with DAP10, NKG2D-S can couple to either DAP10 or DAP12. Human NKG2D can only pair with DAP10, leading to activation through PI3K and Grb2. Both molecules are required to induce Ca<sup>2+</sup> influx after crosslinking of DAP10, although they cannot bind simultaneously to the same DAP10 chain since their binding sites overlap. Grb2 has been proposed to be recruited to the synapse either by direct binding to DAP10 or by recruitment of Sos1-Vav1-Grb2 complexes to PI3P-rich sites generated by activated PI3K at the immune synapse. The events downstream of PI3K and Grb2-Vav1 in the NKG2D signalling pathway are less well defined. Phosphorylation of the kinases Jak2, Akt, MEK1/2 and Erk, but not Jnk or p38 has been detected in assays using recombinant NKG2D ligands, but how PI3K and Grb2 activation leads to phosphorylation of these kinases is not yet known <sup>1,76,101</sup>.

NKG2D was reported to function as an activating receptor in redirected killing assays and in experiments using soluble ligands <sup>101,102</sup>. However, other studies have shown that engagement of the receptor alone fails to induce cytokine secretion <sup>102</sup>, but costimulates KIR-dependent cytokine production and NCR-mediated killing of tumor cells <sup>103,104</sup>. On the other hand, recent data suggests that IL-15 stimulates Jak3 phosphorylation of DAP10 and that this process is necessary to enable the NKG2D receptor to trigger killing <sup>105</sup>. Whether NKG2D

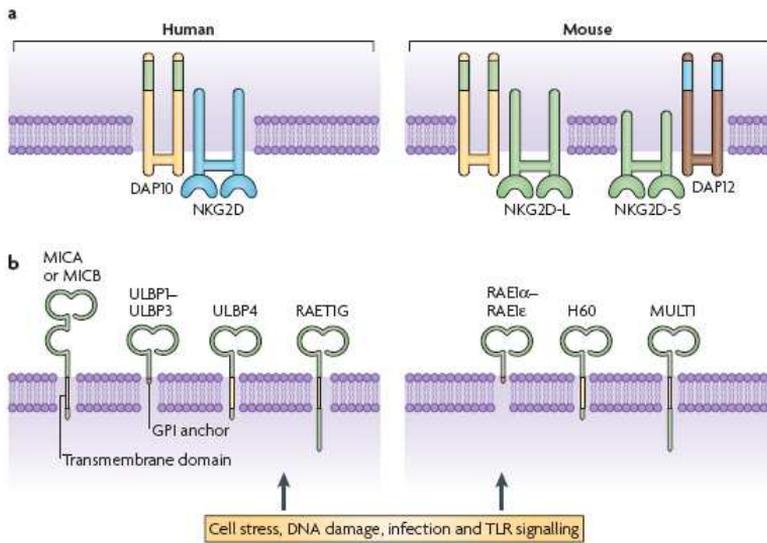
## Chapter 1

is able to trigger effector functions by itself or it requires additional signals is still under debate.

Human NKG2D binds stress-inducible molecules MICA, MICB, ULBP1, ULBP2, ULBP3, ULBP4 and RAET1G. All ligands have MHC-I-like  $\alpha 1$  and  $\alpha 2$  domains that mediate binding to NKG2D. They are highly polymorphic, over 70 distinct alleles have been identified for the MIC genes <sup>67</sup>, and different MIC alleles have been associated to a number of diseases <sup>106,107</sup>. MICs are expressed in some virus infected cells, are frequently found in tumors including lung, kidney, ovarian, prostate, gastric, colon carcinomas or melanomas (as well as tumor cell lines), but have been also detected in basal conditions or under the influence of proinflammatory stimuli in some normal tissues (i.e. the intestinal epithelium).

NKG2D has been involved in the response of NK cells to MIC+ tumors in humans and mice <sup>108,109</sup>, and has been shown to protect from tumor initiation <sup>110</sup>. On the other hand, the release of soluble MIC by some tumors may cause the downregulation of NKG2D on the surface of NK and T cells impairing target recognition <sup>111-113</sup>. NKG2D expression has been also shown to be modulated by the action of different cytokines. IL-12 was shown to increase NKG2D levels <sup>114</sup> whereas TGF $\beta$ , IFN $\gamma$  and IL-21 have been shown to downregulate NKG2D expression <sup>115-118</sup>.

On the other hand, NKG2D has been implicated in the response to influenza-infected DC or EBV-infected cell lines undergoing active viral replication <sup>119,120</sup>. Interestingly, NKG2D ligands MICA/B and ULBP1-3 are induced on HCMV-infected cells <sup>67</sup>, and the receptor has been involved in the response to the pathogen.



**Figure 4:** NKG2D and its MHC-I like ligands in human and mice <sup>67</sup>

#### IMMUNOGLOBULIN-LIKE NK CELL RECEPTORS

##### – Killer Immunoglobulin-like receptors (KIR)

Members of the killer immunoglobulin-like receptor family (KIR) were the first human NKR to be characterized. They are coded by genes located in chromosome 19q13.4, include 15 different members, and different allelic variants have been described<sup>121</sup>. The KIR family comprises different pairs of activating and inhibitory receptors that differ in their cytoplasmic tail. They are called KIR2D or KIR3D depending on the number of Ig-like domains on their extracellular region. Inhibitory receptors have a long cytoplasmic tail containing ITIM and are classified as L (KIR2DL and KIR3DL), while activating receptors have a short tail, are classified as S (KIR2DS and KIR3DS) and are coupled to the DAP12 adaptor protein <sup>122-124</sup>.

## Chapter 1

Studies of KIR genotypes demonstrated variations in the KIR gene content depending on the individual. Based on these studies, two major groups of KIR haplotypes have been described. The A haplotype contains KIR2DL1, -2DL3, -3DL1, -3DL2 and -3DL3 and most include KIR2DS4 and KIR2DL4 as the only activating KIRs. B haplotypes contain different combination of inhibitory and activating KIR and most are characterised by the presence of KIR2DL5. In caucasians, A and B haplotypes are found in comparable frequencies. Along differentiation, different NK cell clones may express different combinations of KIR genes <sup>125</sup>.

Ligands for several KIRs have been defined, and every receptor appears to recognize a set of classical HLA class I molecules. KIR2D receptors recognize HLA-C while KIR3D recognize HLA-A and B. It is believed that the peptides bound to MHC-I may influence the recognition by KIRs <sup>122</sup>. Some pairs of activating and inhibitory receptors may recognize the same ligand, but activating KIR interact with MHC-I molecules with lower affinity than their inhibitory counterparts.

### – Immunoglobulin-like transcript 2 (ILT2)

ILT2 (LIR1, CD85j, LILRB1) is an inhibitory receptor that belongs to the family of immunoglobulin-like transcripts (ILT), also termed leukocyte Ig-like receptors (LIRs). These genes are centromeric to the KIR gene cluster on chromosome 19, and are characterized by the presence of two or four extracellular Ig-like domains. ILT2, ILT3, ILT4, ILT5 and LIR8 possess long cytoplasmic tails containing ITIMs and thus constitute inhibitory receptors, while others display a charged transmembrane residue and may have an activating function by coupling to the ITAM-bearing Fc $\epsilon$ R $\gamma$  chain. ILTs are preferentially expressed by myeloid cells, and ILT2 is the only member of the family expressed by both myeloid and lymphoid cells, including B lymphocytes and NK and T cell subsets<sup>126,127</sup>.

ILT2 has been shown to recognise a broad spectrum of classical and non-classical MHC-I proteins, interacting with a conserved region of their  $\alpha 3$  domain. Recognition by ILT2 of HLA class I molecules inhibits NK and T cell-mediated cytotoxicity, and mAb-ligation of ILT2 also hampered receptor-induced  $\text{Ca}^{2+}$  mobilization in B cells and macrophages <sup>127,128</sup>.

### – Natural Cytotoxicity Receptors

Natural cytotoxicity receptors comprise the NKp46, NKp30 and NKp44 molecules. NKp46 (NCR1) maps on chromosome 19q13.42 in the Leucocyte Receptor Complex telomeric to the KIR family, NKp44 (NCR2) maps on chromosome 6p21.1 centromeric to the MHC, and NKp30 (NCR3) maps to chromosome 6p21.32 within the MHC <sup>129</sup>. NCRs are expressed by NK cells, but NKp44 is only detected upon activation. They belong to the Ig superfamily but each present a different structure and associate with different ITAM-containing adaptor proteins. NKp46 has two C2-type Ig-like domains in the extracellular region and associates to the CD3 $\xi$  and the Fc $\epsilon$ RI  $\gamma$ -chain adaptor proteins. NKp30 and NKp44 have a single V-type domain and the first binds to the CD3 $\xi$  chain while NKp44 binds to DAP12. Crosslinking of NKp30 and NKp46 receptor leads to  $\text{Ca}^{2+}$  influx, cytotoxicity and cytokine production <sup>130 131</sup>.

These receptors have been mainly involved in lysis of tumor cells by still poorly defined ligands. Yet, NKp46 and NKp44 were described to bind influenza virus haemagglutinins (HA), and NK cells were shown to be activated by recognition of HA on virus-infected cells via NKp46 <sup>69,132</sup>. An NKp44 fusion protein was reported to bind to HIV-infected CD4+T cells <sup>133</sup>, and NKp30 has been involved in the immune response to HCMV, as will be further described later. On the other hand, the BAT3 protein has been shown to bind NKp30 and activate NK cell cytotoxicity when released on exosomes by tumor cells or immature DC <sup>62</sup>.

## Chapter 1

### OTHER NK CELL ASSOCIATED RECEPTORS

NKp80 (KLRF1, Killer cell lectin-like receptor subfamily 1) is an activating receptor expressed by NK and CD56<sup>+</sup> T cells <sup>134</sup>. It lacks charged residues in its transmembrane domain and thus is not associated to any known adaptor protein. Yet, NKp80 stimulates NK cell cytotoxicity and induces Ca<sup>2+</sup> influx upon stimulation by specific mAbs <sup>135</sup>. The ligand for NKp80 was recently described. NKp80 was shown to bind the myeloid specific activating receptor AICL, and AICL-NKp80 interactions were shown to promote NK cell-mediated cytotoxicity of malignant myeloid cell lines <sup>136</sup>.

KLRG1 belongs to the C-type lectin-like family of receptors and contains cytoplasmic ITIM, that when phosphorylated recruit SHIP and SHP-2 but not SHP-1. It is expressed by subsets of CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as NK cells, and is expressed on most EBV and HCMV-specific CD8<sup>+</sup>T cells. It has been shown to bind N- and E-cadherins and inhibit TCR-mediated activation of T cells <sup>137,138</sup>.

Human and murine NK cells have been shown to express several members of the SLAM-family of receptors, namely 2B4, NTB-A and CRACC in humans. 2B4 binds CD48, whereas NTB-A and CRACC function through homotypic interactions. Receptors of the SLAM family function as adhesion molecules between APC and T cells during antigenic presentation, and 2B4, NTB-A and CRACC have been shown to trigger NK cell cytotoxicity when stimulated with specific mAb in redirected killing assays. They do not bind to any adaptor proteins through the transmembrane domain, but may associate to intracellular molecules including SAP and other SAP-family members (EAT-2 and ERT) through cytoplasmic immunotyrosine-based switch motifs (ITSMs). Subsequent recruitment of Fyn activates protein tyrosine phosphorylation of different components of the activating cascade. Interestingly, mutations in SAP have been linked to the development of the X-linked lymphoproliferative disease (XLP), ultimately caused by a defective response to Epstein-Barr virus infection <sup>139,140</sup>.

DNAM-1 is a co-stimulatory adhesion molecule expressed on different leukocytes including NK cells. It contains a putative binding site for Grb2 in its cytoplasmic domain, which activates the Ras pathway <sup>141</sup>. It associates with LFA-1, and binds to Polyoma virus receptor (PVR) and to Nectin-2. DNAM-1 has been shown to function as an adhesion molecule enhancing NK cell activation and to be involved in tumor rejection <sup>142-144</sup>.

It was recently described that human NK cells can express several Toll-like Receptors such as TLR3 and TLR9 <sup>68,145,146</sup>. TLR3 enables recognition of dsRNA while TLR9 recognises unmethylated CpG from bacterial and viral DNA. It has been shown that stimulation through both receptors together with DC-secreted IL-12 induces the release of IFN $\gamma$  and TNF $\alpha$  by NK cells <sup>68</sup>.

**Table 1:** Activating and Inhibitory NK cell receptors: signaling, ligands and function\*.

Activating receptors on NK cells			
Receptor	Signaling	Ligand	Function
KIR2DS/3DS	DAP12-ITAM	HLA class I	?
KIR2DL4	?	HLA-G soluble	Trophoblast induced vascular remodeling ?
CD94/NKG2C	DAP12-ITAM	HLA-E	NK cell response to HCMV?
Fc $\gamma$ RIII (CD16)	TCR $\epsilon$ /FcR $\gamma$ -ITAM	IgG	Elimination of Ab-coated cells (ADCC)
NKG2D	DAP10-YxNM	ULBP, MICA/B	Surveillance of stressed cells
NKp30	TCR $\epsilon$ /FcR $\gamma$ -ITAM	BAT3, pp65	Response to tumors/ immature DC
NKp44	DAP12-ITAM	Haemagglutinins	Response to tumors/ Influenza virus/ HIV
NKp46	TCR $\epsilon$ /FcR $\gamma$ -ITAM	Haemagglutinins	Response to tumors/ Influenza virus
2B4	ITSM	CD48	Interaction with haematopoietic cells
CRACC	ITSM	CRACC	Interaction with haematopoietic cells
NKp80	?	AICL	NK cell-myeloid crosstalk
DNAM	?	PVR and Nectin-2	Adhesion and costimulation/ HCMV
LFA-1	?	ICAM	Recruitment and activation during inflammation
CD2	?	CD58	Interaction with haematopoietic and endothelial cells

## Chapter 1

Inhibitory receptors on NK cells			
Receptor	Signaling	Ligand	Function
KIR2DL/3DL	ITIM	HLA class I alleles	Assess loss of MHC-I alleles
ILT2/LIR1	ITIM	HLA class I	Assess loss of MHC-I expression
CD94/NKG2A	ITIM	HLA-E	Surveillance of MHC-I expression
KLRG1	ITIM	E-, N-, P-Cadherin	Assess loss of tissue integrity
NKR-P1	ITIM	LLT1	?
LAIR-1	ITIM	Collagen	Control activation in extracellular matrix
Siglec-7	ITIM	Sialic acid	?
Siglec-9	ITIM	Sialic acid	?
IRp60 (CD300a)	ITIM	?	?

\*Adapted from Bryceson et al. *Curr Opin Immunol* 2008 <sup>147</sup>

### MHC-I RECOGNITION BY ACTIVATING NKR

As described, several pairs of homologous activating and inhibitory NKR have been shown to bind HLA class I, including KIR and CD94/NKG2. Inhibitory receptors bind MHC-I molecules in order to prevent autoreactivity, but the role of activating receptors binding to HLA-I remains under debate. Several hypotheses have been proposed to interpret the role of MHC-I binding to activating NK cell receptors. The nature of the peptide presented by HLA-I molecules could influence the affinity of the complex for the activating receptors. For instance, NKG2C binds with higher affinity MHC-I molecules bound to the HLA-G-derived nonamer <sup>99,100</sup>, as compared to signal peptides derived from other MHC class I molecules. In a similar way, it is conceivable that pathogen-derived peptides could selectively increase the affinity of the MHC-I complex for the activating NKR, but thus far this has not been proven. On the other hand, activating receptors might bind a non-MHC ligand, either a pathogen derived molecule, as shown for the murine Ly49H and the m157 HCMV protein, or an endogenous ligand induced under stress conditions. Supporting this possibility, KIR2DS4, that binds HLA-Cw4 with low affinity, has been also reported to

recognize a ligand present on MHC-I negative melanoma cells and activate their killing <sup>148</sup>.

#### NK CELL RECEPTORS: STIMULATION OR CO-STIMULATION?

What constitutes an activating receptor or a costimulatory molecule in NK cells has been a matter of debate. Bryceson et al. demonstrated that when agonist mAb are used to cross-link the different activating receptors on freshly isolated human NK cells, only CD16 was able to trigger cytolytic activity and cytokine secretion. Yet, when different pairs or combinations of receptors were simultaneously cross-linked, effector functions were triggered <sup>147</sup>. Other reports claim that at least some NKR, like NKG2D and NKp46, function as single stimulatory units without the need for other costimulatory signals <sup>149,150</sup>. However, some of these studies are ambiguous, as they are based on antibody-mediated redirected killing assays with the murine P815 cell line, that expresses Fc receptors and is used to present mAb to NK cells. These assays may lead to wrong conclusions, since besides engaging of the receptor through presentation of the specific mAb, the P815 cell line itself may express ligands for other stimulatory receptors or adhesion molecules on NK cells. Actually this was proved to be the case for NKG2D, which was shown to act in concert with NKp46 stimulation by a ligand present on the P815 cell line <sup>104</sup>. On the other hand, a number of studies have analysed the stimulating function of NKR by blocking the killing of tumor cell lines with specific mAb. Such systems do not allow to discriminate whether the receptor is actually signalling together with other costimulatory molecules, because blocking of a single component may result in abrogation of killing <sup>149,150</sup>.

The ability to activate NK cell effector functions may also vary under the influence of cytokines. As mentioned, individual engagement of NKG2D, NKp46, DNAM and 2B4 receptors on resting NK cells was not sufficient to stimulate efficient cell lysis in P815 redirected killing assays. By contrast, when

## Chapter 1

NK cells were previously treated with IL-2, triggering of the receptors alone was enough to induce cytotoxicity <sup>151</sup>.

Thus, when considering the function of NKR in NK cells, a precise description and critical assessment of the experimental conditions used are warranted.

### 1.2.4 NATURAL KILLER CELL RECEPTORS ON T CELLS

Naïve T cells need co-stimulatory signals together with TCR-signals to complete full activation. This is accomplished by engagement of CD28 and CD27 by CD80, CD86 and CD70 on antigen-presenting cells. Memory and effector T cells are characterised by the lack of CD28 and CD27 expression, but in contrast express other co-stimulatory molecules such as 4-1BB and ICOS that enhance their activation <sup>152</sup>. Some NK cell receptors have been also detected in subpopulations of T cells, and are believed to modulate TCR-dependent signals. In general, T cells expressing NKR have an effector-memory phenotype characterised by the lack of CCR7, lack of co-stimulatory CD27 and CD28 molecules, and increased proportions of perforin+ cells. Furthermore, they show a restricted oligoclonal TCR repertoire, suggesting that they are originated as a result of extensive clonal expansions <sup>137</sup>.

#### NKR EXPRESSION ON CD8+T CELLS

NKR have been mainly reported in the CD8+T cell compartment, though NKG2D is the only NKR constitutively expressed by these cells. It functions as a costimulatory molecule enhancing TCR-dependent activation <sup>149</sup> and has been implicated in the response of CD8+T cells to HCMV <sup>153</sup>. Yet, NKG2D has been described to trigger CD8+ T cell activation without TCR engagement after stimulation with IL-15 <sup>154</sup>, and interestingly, a recent study shows that the NKG2D activating pathway is coupled to IL-15 signaling <sup>105</sup>. Moreover, IL-15 also upregulates NKG2D expression on CD8+T cells <sup>155</sup>. On the other hand, Lanier et al. reported that engagement of NKG2D is not sufficient to co-

stimulate TCR-dependent signals neither in naïve nor effector T cells, and suggested that NKG2D may function as a costimulatory or activating molecule only in certain T cell subpopulations or under the influence of cytokines <sup>156</sup>.

Inhibitory KIR and ILT2 are also expressed on subsets of CD8+T cells from healthy blood donors and, moreover, ILT2 has been detected in influenza virus, EBV, HIV-1 and HCMV-specific CD8+T cells <sup>157,158</sup>. KIR and ILT2 are acquired after antigenic stimulation, along differentiation to effector and memory cells. KIR+T cells are usually mono or oligoclonal, reflecting antigen-specific expansions. The TCR rearrangement of ILT2+ T cells is more variegated, suggesting that its expression may precede acquisition of KIR and is differently regulated <sup>159</sup>. Both inhibitory KIR and ILT2 have been shown to downmodulate TCR-dependent signals <sup>160,161</sup>. It is of note, that stimulatory KIR expression has been also detected in CD8+T cells <sup>162,163</sup>.

The lectin-like receptor CD94/NKG2A has been detected in CD8+ $\alpha\beta$ +T cells and  $\gamma\delta$ +T cells. In contrast to KIR and ILT2, NKG2A expression is induced on T cells after treatment with IL-15, IL-12 or TGF $\beta$ , and its ligation impairs TCR-dependent activation <sup>164-166</sup>. The CD94/NKG2C receptor is expressed in peripheral blood CD8+ $\alpha\beta$ +T cells,  $\gamma\delta$ +T cells and rarely on CD4+T cells. CD94/NKG2C+T lymphocytes generally display a TCR $\alpha\beta$ +CD8+CD56+CD28- phenotype, and the receptor is capable of directly activating the response of a subset of CD8+ $\alpha\beta$ T cells expressing DAP12 <sup>167</sup>. The antigen specificity of NKG2C+T cells is uncertain, but increased proportions were found in HCMV+ individuals. Yet, most HCMV-specific CTL identified with the HLA-A\*0201/pp65 tetramers did not express NKG2C <sup>168</sup>.

NCRs were first identified on NK cells and their expression was suggested to be restricted to this lymphocyte subset. However, several studies have reported NCR expression on T cells. NKp30, 44 and 46 were shown to be induced by IL-

## Chapter 1

15 in umbilical cord CD8+CD56+T cells and expression of NKp44 and NKp46 was detected in intraepithelial CTL cells from celiac patients <sup>169 170</sup>.

### NKR EXPRESSION ON CD4+T CELLS

Reports of NKR expression on CD4+T cells were scarce and most studies focused on pathological conditions where increased frequencies of NKR+CD4+T cells were observed.

NKG2D+CD4+T cells have been reported in different diseases, and NKG2D expression can be induced on CD4+T cells in vitro by IL-15 <sup>171</sup>. Expression of the receptor was observed on CD28-CD4+T cells of patients with rheumatoid arthritis (RA). RA synoviocytes were shown to express MIC, and the NKG2D+CD4+T cell population was suggested to participate in the autoimmune response<sup>171</sup>. NKG2D+CD4+T cells have been also related to Crohn's disease (CD), as their frequency is increased in peripheral blood and in the intestinal mucosa of CD patients, and they were shown to produce IFN $\gamma$  and kill MICA+ targets<sup>172</sup>. Increased frequency of NKG2D+CD4+T cells has also been reported in patients with MIC+ tumors, and patients with HTLV-1 (Human T cell lymphotropic virus type I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a chronic inflammatory disease of the central nervous system that resembles multiple sclerosis <sup>173 174</sup>.

Van Bergen et al. reported small numbers (~0.2%) of KIR+ CD4+T cells in peripheral blood of adult healthy individuals, increasing with age <sup>175</sup>. Most cells showed preferential expression of KIR2DL2/KIR2DL3/KIR2DS2 (as detected with common GL183 mAb), and lacked CCR7, CD27 and CD28 expression. Expansion of the KIR+CD28-CD4+T cell subset has been also observed in RA patients, and proposed to result from chronic antigenic stimulation <sup>176,177</sup>. These cells preferentially expressed the activating KIR2DS2 and were suggested to contribute to the T-cell mediated autoimmune response. Expression of DAP12 in CD4+ T cells is heterogeneous at a clonal level. It was demonstrated that

triggering of KIR2DS2 alone was sufficient to activate cytotoxicity and IFN $\gamma$  production in T cells expressing DAP12, whereas in the absence of DAP12, KIR2DS2 only costimulated TCR-mediated IFN $\gamma$  production<sup>178</sup>. Association of KIR2DS2 with DAP10 has not been demonstrated, but triggering of the receptor in DAP12- clones lead to activation of the JNK pathway, that complements TCR mediated signals through activation of the ATF2 and c-Jun transcription factors<sup>178,179</sup>. KIR expression has also been reported in peripheral blood CD4+CD28-T cells of patients with acute coronary syndromes and KIR3DL2 has been identified as a phenotypic marker of Sézary cells<sup>180,181</sup>.

Saverino et al. reported intracellular expression of ILT2 in all CD8+ and CD4+ T cells. However, surface expression on peripheral blood CD4+T cells was confined to a minor subpopulation<sup>161</sup>. The receptor was able to inhibit TCR mediated activation and was also shown to partially inhibit the response of CD4+T cell clones to tuberculosis antigen (PPD)-presenting cells<sup>182</sup>.

Few studies have focused on the expression of NKG2A and NKG2C receptors on CD4+T cells. Yet, significant NKG2A expression was detected on CD4+T cells after persistent (15 days) CD3/TCR stimulation; under this conditions, transcription of other NKG2 genes was also induced, but no expression of the receptors could be detected on the cell surface<sup>183</sup>.

### **1.3 IMMUNE RESPONSES TO HUMAN CYTOMEGALOVIRUS**

#### **1.3.1 THE HUMAN CYTOMEGALOVIRUS**

Human cytomegalovirus is a member of the human herpesvirus family, which includes herpes simplex virus 1 and 2, Epstein-Barr virus, varicella-zoster virus, and human herpesvirus 5-8. It infects with high prevalence all human populations, with a variable percentage of infection ranging from 50 to 100% depending on the socioeconomic status of the population<sup>184</sup>.

## Chapter 1

The virus is usually acquired during childhood, and after asymptomatic or mild primary infection it remains in a life-long latent state. Periodical reactivation of the virus results in shedding and spread to additional hosts, and increased viral DNA and specific T cells have been detected in peripheral blood during these periods. In immunocompetent individuals reactivations can be controlled, and the virus returns back to the latent phase. However, large numbers of specific T cells can also be found in peripheral blood during latency periods, reflecting the impact of the virus burden on the T cell compartment <sup>185</sup>.

Immunocompromised individuals are unable to control viral replication and the virus can spread to most organs and tissues as a result of reactivation, primary infection or reinfection. In this context, transplant recipients undergoing immunosuppressive therapy and HIV-infected patients can be severely affected by HCMV infection. In transplant recipients, HCMV infection can cause pneumonia, hepatitis or graft failure, and HIV infected patients commonly develop retinitis. In addition, HCMV is the leading viral cause of congenital disorders such as hearing loss, chorioretinitis or mental retardation. On top of that, the infection has been considered a contributing factor to atherosclerosis and coronary restenosis following angioplasty. <sup>186</sup>

The virus DNA codes for more than 200 ORF <sup>187</sup>. According to their sequential expression during viral replication, genes can be divided into immediate early (IE), early (E), and late (L). The predominant proteins critical for virion production are envelope proteins gB, gH, gM and gL and matrix proteins pp65 and pp28 <sup>188</sup>. HCMV infects different cell types including fibroblasts, endothelial cells, epithelial cells, muscle cells and haematopoietic cells. However, cells of myelomonocytic origin are considered the main viral reservoir in vivo. The virus is thought to reactivate upon differentiation of monocytes into macrophages, thus allowing productive HCMV replication and dissemination. Different mechanisms have been proposed to be responsible for CMV reactivation, including stress through catecholamines and the cAMP pathway, inflammation

through TNF $\alpha$  and NF $\kappa$ B signaling or by prostaglandins also through the cAMP pathway. The result is always the activation of the CMV immediate early enhancer (IE-1), which is responsible for the initiation of viral replication<sup>188</sup>. Experiments are usually performed with fibroblasts, the most permissive cell type in vitro, by infecting with the laboratory strains AD169 or Towne. Yet, a clinical isolate-derived strain (TB40/E) allows for infection of endothelial and myeloid cells with variable efficiency<sup>189</sup>.

Studies performed in mice, infected by strains of murine Cytomegalovirus (MCMV), have revealed the importance of both the innate and the adaptive immune response in the control of the infection. On the other hand, the virus has evolved to escape immune recognition through various mechanisms, counteracting both T cell and NK cell mediated immune responses<sup>23,190,191</sup>.

### **1.3.2 THE IMMUNE RESPONSE TO HCMV**

#### T CELL RESPONSES TO HCMV

During primary infection, specific CD4<sup>+</sup> T cells are the first to be identified in peripheral blood shortly after detection of CMV DNA, followed by specific antibodies and ultimately by CD8<sup>+</sup> T cells. In adult healthy individuals, the T cells specific for HCMV antigens represents as many as ~4% of total peripheral blood T cells and ~10% of the memory T cell pool<sup>192,193</sup>. pp65, gB and IE1 were considered to constitute the immunodominant antigens, eliciting the majority of T cell responses<sup>194-197</sup>, however a recent study suggests a broader specificity for other viral antigens. Using intracellular IFN $\gamma$  staining upon stimulation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells with HCMV-derived 15mer peptides, responses could be detected against around 21 different ORFs, and the proportion of T cells responding to a specific antigen correlated with its abundance in viral particles<sup>193</sup>.

The virus has evolved to minimize T cell recognition down-modulating both MHC-I and MHC-II molecules on infected cells, and different viral proteins

## Chapter 1

involved in such mechanism have been identified (US2, US3, US6, US11). US3 is an immediate-early protein that retains MHC-I molecules in the ER <sup>198</sup>. US6 is expressed late in the viral cycle and interferes with the transport of peptides into the ER by binding to TAP <sup>199</sup>. US2 and US11 are expressed early and late and bind MHC-I molecules in the ER targeting them for translocation to the cytoplasm and degradation <sup>200</sup>. Remarkably, these viral molecules were shown to preserve HLA-E expression <sup>201</sup>. On the other hand, US2 and US3 may also interfere with the expression of MHC-II molecules <sup>202</sup>.

### – The role of CD4+T cells

CD4+T cells are crucial in initiating and maintaining an appropriate adaptive immune response. They are critical for activating cytotoxic T cells and for the generation of specific antibodies through activation of B cells. They have been shown to activate DC through CD40L-CD40 interactions, which are then able to prime CD8+ cells through a cross-presentation mechanism that initiates the cytotoxic response. The role of CD4+T cells in maintaining CD8+T cell numbers and function is less well understood, and has been attributed to the production of IL-2. It has been reported that the absence of CD4+ cells during the primary response impairs the appropriate development of CD8+ memory cells. On the other hand, CD4+T cells have been shown to activate B cells through ligation of CD40, resulting in the secretion of high-affinity virus-specific antibodies <sup>203,204</sup>. Interestingly, in patients suffering from symptomatic primary infection, appearance of antibody and specific CTL in peripheral blood occurs before that of specific CD4+T cells responses, which are delayed and can only be detected after antiviral treatment <sup>204</sup>.

Many reports have studied the phenotype of the CD4+ and CD8+T cell pool specific for human cytomegalovirus. CD4+T cells are divided in central- and effector-memory cells depending on the expression of the chemokine receptor CCR7, which directs lymphocytes to secondary lymphoid organs. Central-

memory CD4+T cells express CCR7 and provide help to B and CD8+T cells in secondary lymphoid organs through secretion of IL-2. By contrast, CCR7-effector-memory cells travel to peripheral target organs where production of antimicrobial cytokines contribute to direct control of the viral infection <sup>205,206</sup>. In latently infected individuals, peripheral blood HCMV-specific CD4+T cells have been described as effector-memory cells. Most lack CD28 and CD27 co-stimulatory molecules, are negative for the CCR7 receptor and express CD45RO<sup>207,208</sup>. The proportion of these cells in peripheral blood is higher in elderly individuals <sup>208</sup>, and they become less dependent on co-stimulation <sup>209</sup>. In peripheral tissues they contribute to direct control of the viral infection through the production of cytokines. They display a Th1 profile, secreting IFN $\gamma$  and TNF $\alpha$ , and may express granzyme B and perforin that confer them cytotoxic potential <sup>208</sup>. CD4+T cell clones specific for several HCMV antigens are cytolytic<sup>210</sup> and in fact, murine cytomegalovirus can be controlled in the absence of CD8+T cells <sup>211</sup>.

*CD4+T Large Granular Lymphocytosis (LGL):*

Two interesting studies have been recently published that demonstrate a role for HCMV in the development of CD4+T cell large granular lymphocytosis <sup>212,213</sup>. Lymphoproliferative disorders of T large granular lymphocytes (LGL) are characterised by the presence of unusual large numbers of peripheral blood monoclonal T cells. Mechanisms involved in the development of these disorders are unclear, and the clinical course is usually asymptomatic or associated with cytopenias, autoimmune manifestations, neuropathies or recurrent infection. A recent study analysed a group of 36 patients with monoclonal TCR $\alpha\beta$ + /CD4+ T-LGL lymphocytosis. From these, 15 presented Vb13.1+TCR expansions and showed identical HLA-DRB1\*0701+ genotype, as well as a common aminoacid motif in the CDR3-TCR-Vb sequence <sup>212</sup>. These results suggested the potential involvement of a common antigen in driving lymphoproliferation observed in these patients. Another publication demonstrated that HLA-DRB\*0701 is able to

## Chapter 1

present a peptide derived from glycoprotein B, a Human Cytomegalovirus antigen, eliciting a CD4+ response of Vb13.1+ clones <sup>214</sup>. Moreover, the first group recently demonstrated that Vb13.1+ CD4+T cells from LGL patients recognise and respond to HCMV antigens <sup>213</sup>. These results directly implicate HCMV as the source of the antigenic stimulus responsible for driving the Vb13.1+ CD4+T lymphoproliferations.

### – The role of CD8+T cells

The use of HLA-tetramers and peptide stimulation assays have revealed that ~1-2% of peripheral blood CD8+T cells are specific for HCMV, a number that raises up to 10% in elderly individuals, as also observed for CD4+T cells <sup>192,215</sup>. Most phenotypic studies revealed a close similarity between the phenotype of the CD8+ and the CD4+T cell compartment. CD8+T cells are predominantly CCR7, CD27 and CD28 negative, show a clonally restricted TCR repertoire and present high levels of intracellular perforin, consistent with an effector-memory phenotype <sup>216</sup>. Expression of ILT2 has been detected on HCMV-specific CD8+T cells <sup>159</sup> whereas studies regarding expression of NK cell receptors on HCMV-specific CD4+T cells were missing so far.

### NK CELL RESPONSES TO HCMV

NK cells have been proven essential to counteract CMV infection. A patient selectively lacking NK cells was reported to suffer life-threatening illness after infection with HCMV and recently, a patient lacking functional T cells was reported to partially recover from primary HCMV infection apparently through the involvement of an NK cell mediated response <sup>217,218</sup>.

Many studies have been performed in mice, illustrating the importance of the NK cell response to MCMV. NK cell-deficient mice have higher susceptibility to CMV infection <sup>219</sup>. During viral infections type I IFNs are produced in the host, and this accounts for increased NK cell activation and killing. At the same time,

IFN $\alpha$  stimulation induces the production of IL-15 by DC, a potent growth factor for NK cells<sup>220,221</sup>. In this way, viral infection results in NK-cell proliferation and recruitment to the infected tissues<sup>222</sup>. An NK/DC crosstalk also takes place during MCMV infection. In this regard, plasmacytoid DC produce IFN $\alpha$ , IL-12 and TNF $\alpha$  in response to the virus through engagement of TLR9, and these cytokines trigger NK-cell cytotoxicity and IFN $\gamma$  production *ex vivo*<sup>223,224</sup>. IFN $\gamma$  directly inhibits replication of MCMV<sup>225,226</sup>, and in infected macrophages, it has been shown to act through suppression of IE1 expression<sup>227</sup>. NK cells have also been shown to directly kill MCMV-infected cells through perforin secretion<sup>46,47</sup>.

An important mechanism leading to effective control of MCMV was described some years ago. Some mice strains were shown to effectively counteract MCMV infection whereas others were highly susceptible to the virus. The key was a genetic trait called *cmv1*, present in resistant mice, that was later identified in the gene coding for the Ly49H activating receptor. This receptor was proved to directly bind the viral protein m157, expressed during MCMV infection, and activate NK cell effector functions contributing to clearance of the virus<sup>228,229</sup>.

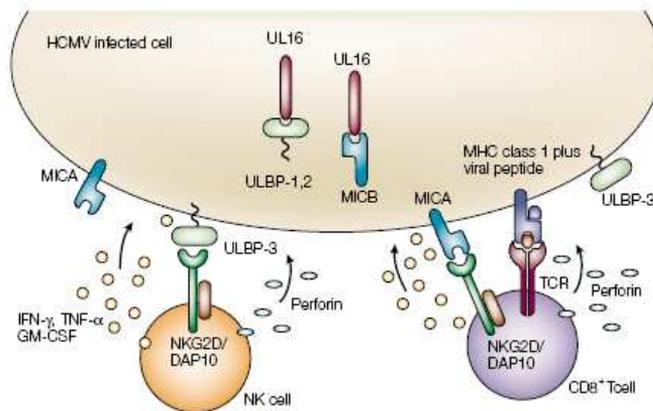
Down-modulation of MHC-I molecules serves as a viral immune evasion mechanism to avoid T cell recognition, but predictably renders infected cells susceptible to NK cell attack as MHC-I specific inhibitory receptors are no longer engaged. Yet, the virus has also evolved to escape NK cell responses, either by engaging inhibitory receptors, or down-regulating the expression of ligands for activating molecules. In this regard, *in vitro* studies with human cytomegalovirus have lead to divergent results. Some studies claim that HCMV-infected fibroblasts are more susceptible to NK cell attack<sup>230</sup>, while others suggest that they are in fact resistant to NK cell lysis<sup>231</sup>. Reports regarding the role of NK cells in the response to HCMV-infected dendritic cells are missing so far, an interesting issue that is currently being addressed in our laboratory (Giuliana Magri, unpublished).

### 1.3.3 NK CELL RECEPTORS IN THE IMMUNE RESPONSE TO HCMV

#### NKG2D

As described before, NKG2D interacts with the stress-inducible molecules MICA/B and ULBPs. NKG2D ligands are expressed by CMV-infected cells, and the interaction of NKG2D with MICA has been shown to participate in the response of HCMV-specific CD8<sup>+</sup> T cell clones to infected HLA-matched fibroblasts expressing NKG2D ligands. However, when HLA-mismatched fibroblasts were used no response was observed, implying that TCR recognition was needed <sup>153</sup>. In mice, interaction of NKG2D with its ligands was shown to contribute to the IFN $\gamma$  response of NK cells to MCMV-infected fibroblasts <sup>232</sup>.

CMV has developed strategies to evade the NKG2D-mediated recognition by preventing expression of NKG2D ligands in human and mice. The HCMV UL16 glycoprotein inhibits surface expression of MICB, ULBP1, ULBP2 and also interacts with RAET1G, thus partially interfering with the NKG2D-mediated response <sup>233</sup>. Upon infection with a UL16 deletion mutant, all ULBP molecules were expressed at the cell surface leading to an increase in NK cell lysis. UL142 has been shown to block the expression of proteins encoded by some MICA alleles. Interestingly, a frequent allele of MICA, MICA\*008, has a premature stop codon that truncates the cytoplasmic domain making it resistant to downregulation by HCMV <sup>234</sup>. Recently, US11 has been reported to retain both MICA and MICB in the ER suppressing their cell surface expression, another mechanism suggested to favor viral immune escape (Aicheler et al. 11<sup>th</sup> Meeting of the Society for Natural Immunity, 2008). An additional mechanism that prevents expression of NKG2D ligands has been proposed, in which HCMV microRNA-UL112 downregulates MICB expression on infected cells <sup>235</sup>.



**Figure 5:** HCMV immune evasion of NKG2D mediated NK and T cell responses<sup>231</sup>.

#### ILT2 (LIR-1, CD85j)

ILT2 interacts with a broad range of MHC-I molecules. Down-modulation of MHC-I proteins by HCMV could dampen inhibition through ILT2, favoring the NK cell attack. Yet, the viral MHC-I-like protein UL18, that binds peptides and associates with  $\beta$ 2-microglobulin, is thought to function as an escape mechanism by engaging ILT2<sup>236,237</sup>. Though surface expression of UL18 has been detected on HCMV-infected fibroblasts<sup>238</sup>, functional studies remain controversial. Some studies reported that UL18 was capable of inhibiting NK cell mediated lysis<sup>239,240</sup>, whereas others have suggested that instead of conferring protection, UL18 increases susceptibility to NK-mediated killing<sup>241</sup>. A recent publication demonstrates that UL18 inhibits ILT2<sup>+</sup> NK cell degranulation, whereas ILT2-NK cells are activated by UL18 by a still undefined mechanism<sup>242</sup>. Studies on T cells also showed that cytotoxicity against HCMV<sup>+</sup> fibroblasts was inhibited by UL18 and ILT2-specific blocking antibodies at late stages of infection<sup>243</sup>. On the other hand, increased ILT2 expression has been associated to a positive serology for HCMV. ILT2 expression was shown to be increased in PBMC from patients

## Chapter 1

undergoing HCMV infection after lung transplantation <sup>244</sup>, and the proportions of ILT2+T cells and NK cells are raised in HCMV+ individuals <sup>168</sup>. Further studies are required to understand the mechanisms controlling ILT2 expression on NK and T cells.

### CD94/NKG2

Increased proportions of NKG2C+NK and T cells have been detected in HCMV+ individuals as compared to seronegative donors <sup>168</sup>. Interestingly, peripheral blood NKG2C+ cells have been shown to include higher proportions of ILT2+ and KIR+ cells, and lower expression levels of NCR (i.e. NKp30 and NKp46). In coculture experiments of PBMC with infected fibroblasts, the NKG2C+NK cell population has been shown to preferentially expand, and this effect could be blocked by an anti-CD94 mAb, suggesting that the receptor may be implicated in the response to HCMV-infected cells <sup>245</sup>. A specific viral ligand may exist that engages the receptor activating the response, as it has been described for the murine Ly49H receptor. Alternatively, a peptide derived from a viral protein and presented through the HLA-E molecule may engage NKG2C with higher affinity than NKG2A. Interaction of NKG2C with HLA-E may as well contribute with other costimulatory signals to enhance the NK cell response. Increased proportions of NKG2C+ cells observed in peripheral blood could as well be the result of alterations in the cytokine network secondary to the viral infection <sup>185</sup>.

HCMV has developed strategies to engage the inhibitory NKG2A receptor. The signal peptide of the viral protein UL40 has been shown to bind to HLA-E <sup>246,247</sup>, engaging NKG2A on NK cells and conferring protection from lysis by NKG2A+ NK cells. Moreover, fibroblasts infected by a UL40-deletion mutant of AD169 HCMV strain were killed by CD94/NKG2A+ primary NK cell lines more efficiently than cells infected with the wild-type virus <sup>248</sup>. To be maintained on the surface, HLA-E should not be targeted by the viral proteins responsible

for downmodulation of other MHC-I molecules. Indeed, US2 and US11 have been shown to preserve HLA-E expression <sup>201</sup>, and presentation of the UL40 nonamer is TAP independent and not affected by UL6 <sup>246</sup>. However, other studies do not support that HLA-E expression is maintained along HCMV infection and contributes to evade NK cell responses <sup>230</sup>.

On the other hand, an endogenous mechanism may operate to enhance NK cell responses. A peptide derived from the HSP-60 protein, expressed under stress conditions, has been shown to stabilize expression of HLA-E but impair binding to NKG2A and NKG2C. The NK cell susceptible cell line K562 was shown to be protected from NK cell killing when transfected with HLA-E and loaded with the signal peptide derived from the HLA-B7 molecule, whereas transfection of HLA-E and loading of HSP-60 conferred no protection to NK cell attack. It has been hypothesised that HSP-60 may compete with endogenous peptides during stress-situations to avoid NK cell inhibition through NKG2A <sup>249</sup>.

### OTHER NK CELL-ASSOCIATED RECEPTORS IMPLICATED IN THE RESPONSE TO HCMV

As described before, the CD155 molecule functions as the poliovirus receptor in humans and binds to the NK cell activating molecule DNAM1 and the adhesion molecule TACTILE (CD96) <sup>143,250</sup>. The HCMV protein UL141 was reported to downmodulate CD155 expression and impair NK cell mediated killing of infected fibroblasts, suggesting that DNAM1 is implicated in the response to HCMV-infected cells <sup>251</sup>.

The HCMV structural protein pp65 has been reported to interact with NKp30 expressed on NK cells. This interaction would promote dissociation of the activating receptor from its adaptor by a still undefined mechanism, thus preventing NK cell activation <sup>252</sup>.



## Chapter 2

### Aims

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This work has been developed in the context of a research project focused on the role of NK cell receptors (NKR) in the immune response to human cytomegalovirus (HCMV) infection. The main objectives have been:

- 1) To explore the expression and function of NKR on CD4+ T cells specific for HCMV.
- 2) To analyse the mechanisms and functional implications underlying the coexpression of CD94/NKG2A and CD94/NKG2C in NK cells.



**PART II**

**NK CELL RECEPTOR  
EXPRESSION ON CD4+T CELLS**



## Chapter 3

### Expression and function of NKG2D in CD4<sup>+</sup> T cells specific for human cytomegalovirus

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*Eur J Immunol* 2006;36:3198-206



## Expression and function of NKG2D in CD4<sup>+</sup> T cells specific for human cytomegalovirus

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### Funded by:

- Ministerio de Educación y Ciencia; Grant Number: SAF2004-07632
- DURSI (Generalitat de Catalunya)

### KEYWORDS

Cytomegalovirus ·KIR ·CD85j ·NKG2D ·T cell

Received: 5 September 2006; Revised: 9 October 2006; Accepted: 20 October 2006

### Abbreviations

aNKR: activating NKR,

HCMV: human cytomegalovirus

KIR: killer immunoglobulin-like receptor

KLR: killer lectin-like receptor

NKR: natural killer receptor

RA: rheumatoid arthritis

ULBP: UL16-binding proteins

### ABSTRACT

The human NKG2D killer lectin-like receptor (KLR) is coupled by the DAP10 adapter to phosphoinositide 3-kinase (PI3 K) and specifically interacts with different stress-inducible molecules (*i.e.* MICA, MICB, ULBP) displayed by some tumour and virus-infected cells. This KLR is commonly expressed by human NK cells as well as TCR $\gamma\delta$ <sup>+</sup> and TCR $\alpha\beta$ <sup>+</sup>CD8<sup>+</sup> T lymphocytes, but it has been also detected in CD4<sup>+</sup> T cells from rheumatoid arthritis and cancer patients. In the present study, we analysed NKG2D expression in human cytomegalovirus (HCMV)-specific CD4<sup>+</sup> T lymphocytes. *In vitro* stimulation of peripheral blood mononuclear cells (PBMC) from healthy seropositive individuals with HCMV promoted variable expansion of CD4<sup>+</sup>NKG2D<sup>+</sup> T lymphocytes that coexpressed perforin. NKG2D was detected in CD28<sup>-</sup> and CD28<sup>dull</sup> subsets and was not systematically associated with the expression of other NK cell receptors (*i.e.* KIR, CD94/NKG2 and ILT2). Engagement of NKG2D with specific mAb synergized with TCR-dependent activation of CD4<sup>+</sup> T cells, triggering proliferation and cytokine production (*i.e.* IFN- $\gamma$  and TNF- $\alpha$ ). Altogether, the data support the notion that NKG2D functions as a prototypic costimulatory receptor in a subset of HCMV-specific CD4<sup>+</sup> T lymphocytes and thus may have a role in the response against infected HLA class II<sup>+</sup> cells displaying NKG2D ligands.

## INTRODUCTION

Some T lymphocyte subsets share with NK cells the expression of inhibitory and activating receptors specific for HLA class I molecules, such as killer immunoglobulin-like receptors (KIR), CD94/NKG2A, CD94/NKG2C and ILT2 (LIR1, CD85j) [1]-[4]. NK cell receptors (NKR) have been detected in TCR $\gamma\delta$ <sup>+</sup> cells as well as TCR $\alpha\beta$ <sup>+</sup> CD8<sup>+</sup> and CD4<sup>+</sup> lymphocytes with an effector-memory phenotype [5]-[8], including virus-specific cytotoxic T lymphocytes (CTL) [9], [10]. The molecular basis regulating NKR expression along T cell differentiation is not completely understood, but there is evidence supporting the involvement of cytokines and TCR-dependent signals, and it has been hypothesized that NKR expression results from chronic antigenic stimulation [6], [11].

Inhibitory NKR (iNKR) are believed to prevent T cell-mediated autoreactivity and counterbalance the action of activating NKR (aNKR) [6], [12]. Most aNKR (*ie* KIR and CD94/NKG2C) are coupled by DAP12 to protein tyrosine kinase (PTK) activation pathways [4] and may trigger or costimulate T cell proliferation and effector functions [13]-[15]. By contrast, the human NKG2D killer lectin-like receptor (KLR) is linked by DAP10 to a phosphoinositide 3-kinase (PI3 K) activation pathway [16], [17]; NKG2D functions as a costimulatory molecule in T cells [18], [19] but may activate NK cells and IL-15-stimulated intraepithelial T lymphocytes in a TCR-independent manner [20], [21].

Human NKG2D interacts with several stress-inducible ligands including MICA, MICB and a family of proteins termed “UL16-binding proteins” (ULBP) that are expressed by some normal tissues, tumour cells and virus-infected cells [16], [19], [22]-[25]. The KLR activates NK cells and costimulates the response of CD8<sup>+</sup> CTL against human cytomegalovirus (HCMV)-infected targets [18], [26]; the identification of immune evasion mechanisms that interfere with surface expression of NKG2D ligands underline its importance in the antiviral response.

### Chapter 3

The UL16 glycoprotein inhibits surface expression of MICB, ULBP1, ULBP2 [22], [27]-[29] and also interacts with RAET1G [24]. The gpUL142 HCMV molecule has been recently reported to down-regulate MICA [30].

Human NKG2D was originally identified on NK cells, TCR $\gamma\delta$ <sup>+</sup> cells and TCR $\alpha\beta$ <sup>+</sup>CD8<sup>+</sup> T lymphocytes, but CD4<sup>+</sup>NKG2D<sup>+</sup> T cells have been described in rheumatoid arthritis (RA) and some cancer patients [31], [32]. Goronzy and Weyand [33] hypothesized that CD4<sup>+</sup> T lymphocytes expressing NKG2D and aNKR represent senescent effector-memory T cells that may contribute to the pathogenesis of RA and other chronic inflammatory disorders [15]. NKG2D might exacerbate RA progression by reacting with its ligands abnormally expressed by the inflamed synovium [31], [33]. On the other hand, stimulation by soluble MIC molecules (sMIC) has been proposed to account for the increased frequencies of CD4<sup>+</sup>NKG2D<sup>+</sup> T cells producing Fas ligand in patients bearing MIC<sup>+</sup> tumours [32]. Recently, CD4<sup>+</sup>NKG2D<sup>+</sup> T cells have been identified in patients with human T cell lymphotropic virus type I (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [34].

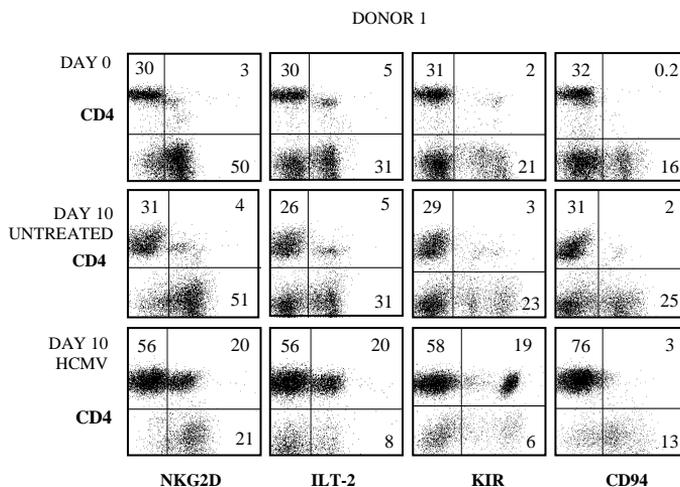
In the present study, we provide the first evidence supporting the notion that a subset of HCMV-specific CD4<sup>+</sup> T cells displays NKG2D. Remarkably, expression of the KLR was not systematically associated with expression of other NKR (*i.e.* ILT2, KIR and CD94/NKG2). Engagement of NKG2D costimulated TCR-dependent proliferation and cytokine production, indicating that the KLR may contribute to the response of a subset of HCMV-specific CD4<sup>+</sup> T lymphocytes against infected HLA class II<sup>+</sup> cells bearing NKG2D ligands.

## RESULTS AND DISCUSSION

### Expression of NKG2D on HCMV-stimulated CD4<sup>+</sup> T cells

Human NKG2D is commonly displayed by NK cells as well as TCR $\gamma\delta$ <sup>+</sup> and TCR $\alpha\beta$ <sup>+</sup>CD8<sup>+</sup> T lymphocytes, but it has also been detected in TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup> cells from RA and cancer patients [31], [32]. In the present study, we analysed the expression of NKG2D in HCMV-specific CD4<sup>+</sup> cells. In agreement with previous reports [35], T cell proliferation was detected when peripheral blood mononuclear cells (PBMC) from healthy HCMV-seropositive (HCMV<sup>+</sup>) blood donors ( $n=9$ ) were cultured in the presence of the virus. Cells recovered from HCMV-stimulated samples were predominantly CD4<sup>+</sup> T lymphocytes ( $84\pm 7\%$ ), which displayed an oligoclonal pattern of TCR rearrangement (data not shown), consistent with the expansion of T cells specific for viral antigens [35]; no response was observed in HCMV-seronegative (HCMV<sup>-</sup>) individuals ( $n=3$ ) (data not shown).

As compared to fresh PBMC and to control samples cultured in the absence of the virus, substantial numbers of HCMV-stimulated CD4<sup>+</sup> lymphocytes displayed NKG2D (Fig. 1); the proportion of CD4<sup>+</sup> T lymphocytes expressing NKG2D on day 10 of stimulation varied widely in different HCMV<sup>+</sup> donors ( $n=9$ ; mean  $\pm$  SD,  $21\pm 15\%$ ; range, 1-75%) and were rare (<4%) in the absence of HCMV stimulation. The expansion of NKG2D<sup>+</sup> cells was comparable upon incubation with different HCMV strains (*i.e.* Towne and AD169) and was undetectable in samples from HCMV-seronegative donors (not shown). Similar results were obtained using purified or UV-inactivated virus preparations (not shown).

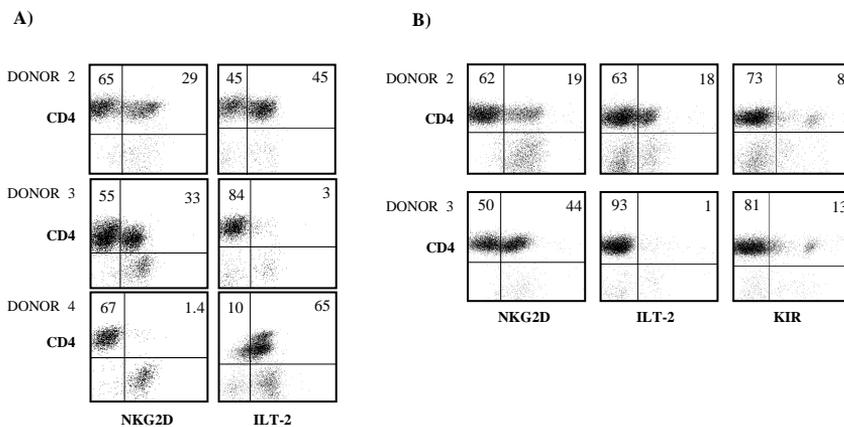


**Figure 1. Expansion of CD4<sup>+</sup>NKG2D<sup>+</sup> T cells in HCMV-stimulated PBMC.** PBMC from a healthy HCMV<sup>+</sup> donor (#1) were cultured for 10 days either alone (untreated) or in the presence of the virus (AD169). Two-colour flow cytometry analysis was carried out staining fresh (day 0) and cultured (day 10) samples with anti-CD4 mAb in combination with BAT221 anti-NKG2D, HP-F1 anti-ILT2, HP-3B1 anti-CD94 mAb or with a mixture of anti-KIR mAb (HP-3E4, CH-L, DX9, 5133).

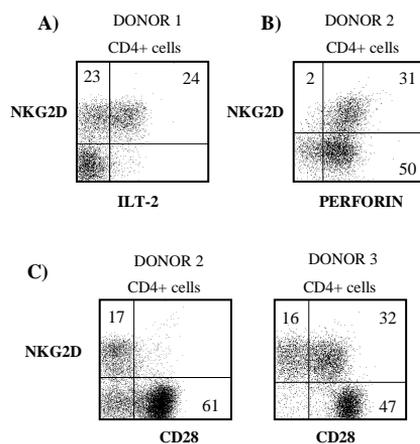
### Distribution of ILT2, KIR and CD94/NKG2 NKR on HCMV-stimulated CD4<sup>+</sup> T cells

Flow cytometry analysis carried out in parallel with a panel of mAb specific for different NKR indicated that ILT2<sup>+</sup> and KIR<sup>+</sup> cells were also increased among HCMV-stimulated CD4<sup>+</sup> T cells (Fig. 1); by contrast, few CD4<sup>+</sup>CD94/NKG2<sup>+</sup> cells were detectable (Fig. 1 and data not shown). Remarkably, the expression of NKG2D, ILT2 and KIR did not systematically coincide. In fact, when expression of NKG2D and the ILT2 inhibitory receptor were compared on HCMV-stimulated CD4<sup>+</sup> cells, distinct distribution patterns were observed. In most cases ( $n=6$ ), CD4<sup>+</sup>ILT2<sup>+</sup> and CD4<sup>+</sup>NKG2D<sup>+</sup> cells were detected (Fig. 2A, donor #2), and three-colour flow cytometry analysis revealed the existence of a

subset coexpressing both molecules (Fig. 3A). However, in some individuals only CD4<sup>+</sup> cells selectively bearing either NKG2D ( $n=1$ ) or ILT2 ( $n=2$ ) were expanded (Fig. 2A, donors #3 and #4). When samples from several representative donors ( $n=5$ ) were reanalysed 4-10 months later, the distribution patterns observed in the first study were reproduced in every case, regardless of the variability in the proportions of recovered NKG2D<sup>+</sup> or ILT2<sup>+</sup> cells; the phenotypes of HCMV-stimulated CD4<sup>+</sup> cells from two individuals (#2 and #3) studied at different time points are shown for comparison (Fig. 2A, B).



**Figure 2. Dissociated expression of NKG2D, ILT2 and KIR in HCMV-stimulated CD4<sup>+</sup> T cells.** Experiments were carried out as described in Fig. 1, comparing the expression of NKG2D, ILT2 and KIR in HCMV-stimulated PBMC from HCMV<sup>+</sup> donors ( $n=9$ ). (A) Data correspond to samples from three individuals (#2, #3 and #4) representative of the different distribution patterns of NKG2D and ILT2 observed. (B) HCMV-stimulated PBMC samples from donors #2 and #3, analysed in assays carried out 6 months later, illustrate the dissociated expression of NKG2D and KIR.



**Figure 3. Expression of ILT2, perforin and CD28 in HCMV-stimulated CD4+NKG2D<sup>+</sup> T cells.** Three-colour immunofluorescence and flow cytometry analysis of HCMV-stimulated cells was carried out as described in the Materials and methods, gating on CD4<sup>+</sup> cells. (A) Cells were sequentially stained with anti-NKG2D (ON72) and allophycocyanin (APC)-conjugated goat anti-mouse Ig, followed by biotin-labelled anti-ILT2 (HP-F1), streptavidin-FITC and CD4-PE. (B) Cells stained with anti-NKG2D and CD4-PE were sequentially fixed, permeabilised and incubated with anti-perforin-FITC. Data correspond to an experiment representative of three performed with different donors. (C) Cells stained by indirect immunofluorescence with anti-NKG2D were subsequently labelled with anti-CD4-FITC and anti-CD28-PE. Data correspond to samples from two donors illustrating the different distribution patterns observed.

The complex diversity of KIR haplotypes and, particularly, the inability of the available mAb to discriminate homologous activating and inhibitory KIR [1] did not allow precise analysis of the relationship between individual members of this receptor family and NKG2D. Yet, two-colour analysis of CD4<sup>+</sup> cells using a mixture of different anti-KIR mAb as described [36] also ruled out coordinated expression of NKG2D with these receptors (Fig. 2B).

The dissociated distribution of NKG2D, KIR and ILT2 in HCMV-specific CD4<sup>+</sup> cells indicates that their expression is differentially regulated. NKG2D was

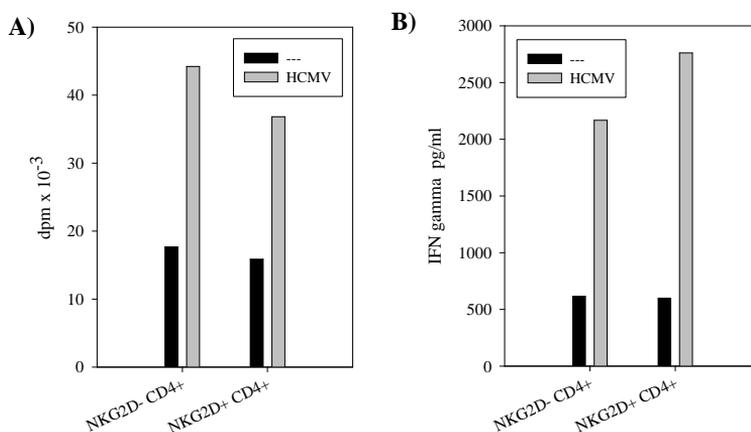
reported to be induced under the influence of cytokines (*i.e.* IL-15) and TCR-dependent stimulation [31], [37]; furthermore, it has been proposed that NKG2D engagement by soluble MIC molecules promotes the expansion of CD4<sup>+</sup>NKG2D<sup>+</sup> cells in cancer patients bearing MIC<sup>+</sup> tumours [32]. KIR and ILT2 were shown to be displayed by effector-memory T cells [6], [8], yet the mechanisms regulating their expression remain poorly defined. CD8<sup>+</sup> T cells bearing inhibitory NKR have been reported to be increased in HIV-infected patients [38]. ILT2<sup>+</sup> T cells have also been observed to be augmented during HCMV infection in transplant patients [39] as well as in HCMV<sup>+</sup> blood donors [36]. ILT2 was detected in HCMV-specific CD8<sup>+</sup> T cells [9], [10] and in CD4<sup>+</sup> T lymphocytes responding to *Mycobacterium tuberculosis* antigens [40]. Our results provide a first indication that ILT2 may also be expressed by HCMV-specific CD4<sup>+</sup> T cells. ILT2 interacts with different HLA class I molecules and with the UL18 HCMV glycoprotein [41], [42]. The regulatory function of ILT2 in the response of CD4<sup>+</sup> T cells against HCMV-infected cells deserves further attention.

#### **CD4<sup>+</sup>NKG2D<sup>+</sup> T cells are CD28<sup>-</sup> or CD28<sup>dim</sup> cytotoxic T lymphocytes**

Additional phenotypic studies using three-colour flow cytometry analysis revealed that CD4<sup>+</sup>NKG2D<sup>+</sup> lymphocytes express perforin (Fig. 3B) and granzyme B (data not shown), thus corresponding to a described subset of HCMV-specific CD4<sup>+</sup> CTL [43]; it is noteworthy that both perforin and granzyme B were also detected in CD4<sup>+</sup>NKG2D<sup>-</sup> cells (Fig. 3B; data not shown). Considering the costimulatory function of NKG2D in CD8<sup>+</sup> cells, we compared its distribution to that of CD28. Both molecules appeared dissociated in HCMV-stimulated CD4<sup>+</sup> lymphocytes (Fig. 3C), but a NKG2D<sup>+</sup>CD28<sup>dull</sup> subset was detectable in some samples (Fig. 3C). These observations suggest that HCMV-specific CD4<sup>+</sup> T cells, previously shown to display an effector-memory phenotype [44], [45], may switch the use of costimulatory receptors, becoming CD28<sup>-</sup> NKG2D<sup>+</sup>.

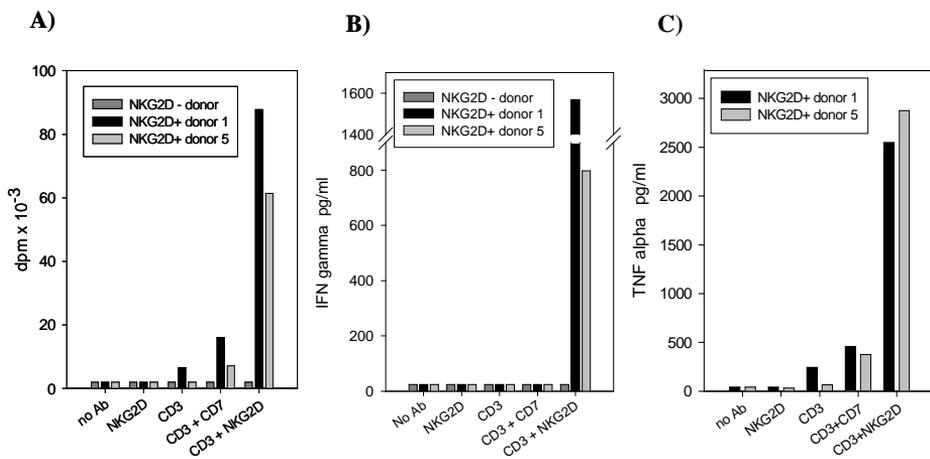
### NKG2D functions as a costimulatory receptor in a subset of HCMV-specific CD4<sup>+</sup> T cells

To verify whether NKG2D<sup>+</sup> cells were specific for HCMV antigens, CD4<sup>+</sup>NKG2D<sup>+</sup> and CD4<sup>+</sup>NKG2D<sup>-</sup> subsets were sorted and restimulated with the virus in the presence of irradiated (40 Gy) autologous PBMC. A specific proliferative response and IFN- $\gamma$  production were detected in both populations (Fig. 4), thus indicating that CD4<sup>+</sup>NKG2D<sup>+</sup> cells represent only a fraction of HCMV-specific T lymphocytes, as predicted by the phenotypic analyses described above. Attempts to grow CD4<sup>+</sup>NKG2D<sup>+</sup> T cell clones were unsuccessful, suggesting that their proliferative capacity is limited, in line with previous studies showing that HCMV-specific T cells undergo replicative exhaustion [46].



**Figure 4. Proliferation and IFN $\gamma$  production by CD4<sup>+</sup>NKG2D<sup>+</sup> and CD4<sup>+</sup>NKG2D<sup>-</sup> T cell subsets in response to HCMV stimulation.** HCMV-stimulated cells were sequentially stained by indirect immunofluorescence with anti-NKG2D and FITC-tagged rabbit anti-mouse Ig, followed by CD4-PE. CD4<sup>+</sup>NKG2D<sup>+</sup> and CD4<sup>+</sup>NKG2D<sup>-</sup> cell subsets were sorted and incubated in 96-well plates ( $10^5$  cells/well) together with autologous irradiated PBL ( $2 \times 10^5$ /well) in the presence or absence of HCMV. (A) Cultures were labelled with <sup>3</sup>HTdR at 48 h and harvested 18 h later. (B) IFN $\gamma$  was measured by ELISA in supernatants harvested after 48 h. Similar results were obtained in two different experiments.

To assess the function of NKG2D, HCMV-stimulated CD4<sup>+</sup> T cells were incubated with suboptimal concentrations of anti-CD3 mAb in the presence or absence of anti-NKG2D mAb. Under these conditions, engagement of the KLR synergized with TCR-dependent signals, efficiently triggering proliferation and cytokine production (Fig. 5). These results support the notion that NKG2D functions as a prototypic costimulatory receptor in HCMV-specific CD4<sup>+</sup> cells. In line with this, the KLR has recently been shown to be coupled to the DAP10 adapter in CD4<sup>+</sup>NKG2D<sup>+</sup> cells derived from cancer patients [32].



**Figure 5. NKG2D costimulates TCR-dependent proliferation and cytokine production in CD4<sup>+</sup> T cells.** HCMV-stimulated T cells (day 10) stained with CD4-PE were sorted. CD4<sup>+</sup> T cells were incubated in 96-well plates (10<sup>5</sup> cells/well) in the presence of the indicated plate-bound antibodies (A) Cultures were labelled with <sup>3</sup>HTdR at 48 h and harvested 18 h later. (B-C) IFN $\gamma$  and TNF $\alpha$  were analysed by ELISA in supernatants harvested at 48 h. Data correspond to samples from two donors containing 25% (donor #1) and 45% (donor #5) CD4<sup>+</sup>NKG2D<sup>+</sup> cells that were independently analysed in different experiments; cells from a third individual that did not display NKG2D were tested as a control (NKG2D<sup>-</sup>).

### Chapter 3

The design of an *in vitro* experimental system suitable to directly study the costimulatory role of NKG2D and other NKR (*i.e.* ILT2) in the response of CD4<sup>+</sup> T cells to HCMV-infected cells is warranted, though some technical limitations must be overcome. It is noteworthy that fibroblasts, which are commonly used to analyse the response to HCMV, do not express HLA class II molecules, and other susceptible cell types (*i.e.* endothelial and hemopoietic cells) are far less permissive to *in vitro* infection [47].

Collectively, our data suggest that CD4<sup>+</sup>NKG2D<sup>+</sup> cells expanding in HCMV-stimulated cultures correspond to virus-specific memory T cells that have acquired NKG2D while losing CD28. By switching the use of costimulatory molecules, CD4<sup>+</sup> cells primed by professional antigen-presenting cells (APC) might efficiently respond to other HLA class II<sup>+</sup> virus-infected cell types. Though most studies have focused on the response of CD8<sup>+</sup> CTL to HCMV antigens [48], CD4<sup>+</sup> T cells specific for epitopes of viral proteins such as pp65 and IE1 have been identified [49]-[51]. Recently, the gB HCMV glycoprotein was shown to be processed *via* the endosomal pathway by non-professional APC, being efficiently presented by HLA class II molecules to CD4<sup>+</sup> T cells [52]. HLA class II expression in different HCMV-infected cell types may be either constitutive (*i.e.* macrophages) or inducible by proinflammatory cytokines, and it is impaired by some virus molecules [53]. On the other hand, NKG2D ligands are displayed in HCMV-infected cells, and immune evasion mechanisms that interfere with their expression indirectly reflect the importance of the KLR in the anti-viral response [18], [22], [27]-[29].

CD4<sup>+</sup>NKG2D<sup>+</sup> T cells were originally described in RA patients [31]. Goronzy and Weyand hypothesized that immunosenescence contributes to the development of RA and proposed that expression of NKG2D and aNKR (*i.e.* KIR2DS2) may lower the activation threshold of senescent CD4<sup>+</sup>CD28<sup>-</sup> T lymphocytes, favouring their participation in the pathogenesis of the disease. NKG2D might exacerbate RA upon interaction with its ligands expressed by the

inflamed synovium [31], [33]. A role for NKR<sup>+</sup> T cells in the development of other chronic inflammatory disorders such as atherosclerosis has also been suggested [15]. It is noteworthy that HCMV infection is considered to be a major contributor to the immunosenescence process [54]. According to our observations, the possibility that the increased numbers of CD4<sup>+</sup>NKG2D<sup>+</sup> T cells found in RA and cancer patients [32] may represent HCMV-specific T cells should be envisaged. Frequent subclinical reactivation of the virus, favoured by the immune dysfunction and/or immunosuppressive therapy in these patients, might account for the increased proportions of CD4<sup>+</sup>NKG2D<sup>+</sup> T cells. On the other hand, the possibility that this T lymphocyte subset may also expand in response to different antigens, including other microbial pathogens, is not ruled out. Regardless of their primary antigenic specificity, NKG2D would enhance the response of potentially autoreactive CD4<sup>+</sup> T cells against non-infected tissues, where expression of its ligands may be either constitutive or inducible by a variety of stimuli [26], [55], [56].

NKG2D has been detected in murine NK cells, CD8<sup>+</sup> T lymphocytes and macrophages, and it has been shown to participate in the immune response against murine cytomegalovirus (MCMV) [19], [57] as well as in the pathogenesis of experimental autoimmune type I diabetes [58]. The expression of NKG2D in murine effector-memory CD4<sup>+</sup> CTL should be carefully reassessed.

### **Concluding remarks**

Our results provide the first evidence supporting the notion that human NKG2D functions as a costimulatory molecule in a subset of HCMV-specific CD4<sup>+</sup> T lymphocytes and thus may contribute to their response against HLA class II<sup>+</sup> virus-infected cell types displaying NKG2D ligands. The possibility that the increased numbers of CD4<sup>+</sup>NKG2D<sup>+</sup> T cells found under some pathological conditions may be primarily specific for HCMV or other microbial pathogens should be envisaged. Further studies are required to explore the putative role of CD4<sup>+</sup>NKG2D<sup>+</sup> cells in the pathogenesis of chronic inflammatory disorders. In

## Chapter 3

this regard, the dissociated expression of NKG2D and inhibitory NKR (*i.e.* ILT2, KIR) in CD4<sup>+</sup> T lymphocytes deserves special attention, as it might increase the risk of autoimmune reactions.

### Materials and methods

#### Subjects

Heparinized blood samples were obtained from healthy adult individuals. Written informed consent was obtained from every donor, and the study protocol was approved by the Ethics Committee (CEIC-Institut Municipal d'Assistencia Sanitaria). As described [36], standard clinical diagnostic tests were used to analyse serum samples from blood donors for circulating IgG antibodies against CMV (Abbot Laboratories, Abbot Park, IL); nine HCMV-seropositive (HCMV<sup>+</sup>) and three seronegative (HCMV<sup>-</sup>) donors were studied.

#### HCMV preparations

As described [59], AD169 and Towne strains of HCMV were propagated in HFF or MRC-5 fibroblast cell lines following standard procedures. Viral titres were determined by standard plaque assays on MRC-5 cells. The AD169 strain of virus was inactivated under UV light as described [59]. The purified AD169 strain of HCMV was obtained from ABI Advanced Biotechnologies Inc. (Columbia, MD).

#### Lymphocyte cultures

Culture medium was RPMI 1640 with Glutamax-I and 25 mM HEPES (Gibco, UK), supplemented with 10% v/v heat-inactivated fetal calf serum (FCS), penicillin (100 U/mL) and streptomycin (10 µg/mL), referred to as complete medium. PBMC obtained from heparinized blood by Ficoll-Hypaque gradient centrifugation (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway) were incubated in 24-well plates (2 × 10<sup>6</sup> cells/well) in complete medium supplemented with 10 U/mL human recombinant interleukin-2 (hrIL-2; Proleukin, Chiron, Emeryville, CA) either alone or in the presence of cell-free HCMV (2 × 10<sup>5</sup>

PFU/well). Cell cultures were maintained at 37°C in a 5% CO<sub>2</sub> 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.

### **Antibodies**

HP-3E4 anti-KIR2DL1/S1/S3, HP-3B1 anti-CD94, HP-F1 anti-CD85j mAb and BAT221 anti-NKG2D were generated in our laboratories [23], [36]. 5.133 anti-KIR3DL1/L2 and KIR2DS4 was provided by Dr. Marco Colonna. Z199 anti-NKG2A and ON72 anti-NKG2D mAb were provided by Dr. Alessandro Moretta (University of Genova, Italy). Dx9 anti-KIR3DL1 mAb was provided by Dr. Lewis Lanier (UCSF, San Francisco, CA). CH-L anti-KIR2DL2/S2/L3 was provided by Dr. Silvano Ferrini (University of Genova, Italy). 3A1 anti-CD7 has been described [60], and anti-NKG2C (MAB1381) was from R&D Systems (Minneapolis, MN). Indirect immunofluorescence analysis was carried out with PE- or FITC-tagged F(ab<sup>2</sup>) rabbit anti-mouse Ig antibodies (Dakopatts, Glostrup, Denmark) or allophycocyanin (APC)-labelled goat anti-mouse Ig (BD Biosciences Pharmingen). The following fluorochrome-tagged mAb were used for multicolour staining: CD4-PE, CD4-FITC, CD28-PE, anti-human Perforin-FITC and FITC-conjugated mouse anti-human Granzyme B (BD Biosciences Pharmingen). HP-F1 was labelled with biotin using EZ-Link Sulfo-NHS-Biotin (Pierce) according to the manufacturer's instructions and was used in combination with streptavidin-FITC (BD Biosciences Pharmingen).

### **Immunofluorescence and flow cytometry analysis**

Multicolour immunofluorescence and flow cytometry analysis was performed as described [36]. Briefly, cells were pretreated with saturating concentrations of human aggregated Ig to block FcR and were then incubated with the individual unlabeled mAb. After washing, samples were labelled either with FITC-tagged F(ab<sup>2</sup>) rabbit anti-mouse Ig antibody (Dako) or allophycocyanin (APC)-labelled

### Chapter 3

goat anti-mouse (BD Biosciences Pharmingen). In some experiments, cells were incubated with biotin-labelled HP-F1 followed by streptavidin-FITC (BD Biosciences Pharmingen). Subsequently, samples were washed and incubated with PE- or FITC-conjugated antibodies (BD Biosciences Pharmingen). Flow cytometry analysis was carried out as described (FACScan, Becton Dickinson, Mountain View, CA).

For multicolour intracellular staining, the BD Cytotfix/Cytoperm Kit was used (BD Biosciences Pharmingen). Briefly, cells were stained with ON72 anti-NKG2D and allophycocyanin (APC)-labelled goat anti-mouse Ig, followed by CD4-PE. Samples were fixed, permeabilised following the manufacturer's instructions and stained with anti-human Perforin-FITC or FITC-conjugated anti-human Granzyme B mAb (BD Biosciences Pharmingen). Cells stained with CD4-PE alone or in combination with BAT221 anti-NKG2D and FITC-tagged F(ab')<sub>2</sub> rabbit anti-mouse Ig, as described above, were sorted under sterile conditions (FACSVantage, Becton Dickinson, Mountain View, CA) and used in functional assays.

#### **Cytokine production and cell proliferation assays**

NKG2D<sup>+</sup>CD4<sup>+</sup> and NKG2D-CD4<sup>+</sup> HCMV-stimulated cells were sorted (FACSVantage, BD), resuspended in complete medium and cultured in 96-well plates (10<sup>5</sup> cells/well) with irradiated (40 Gy) autologous PBMC (2 × 10<sup>5</sup> cells/well), either alone or in the presence of HCMV (10<sup>4</sup> PFU/well). In other experiments, CD4<sup>+</sup> cells were sorted and stimulated with mAb preadsorbed to culture plates as described [13]. Briefly, 96-well plates were coated with sheep anti-mouse Ig (10 µg/mL) overnight at 4°C, washed with PBS and incubated with specific antibodies for 3 h at room temperature. The following mAb were used: 3A1 anti-CD7 (10 µg/mL), SpvT3b anti-CD3 (2 ng/mL) and ON72 anti-NKG2D (50 µL hybridoma supernatant). After washing plates, CD4<sup>+</sup> sorted cells (10<sup>5</sup>/well) were added in complete medium; each experimental condition was set up in triplicate. In every case, supernatants were harvested after 48 h, and

production of IFN- $\gamma$  and TNF- $\alpha$  was analysed by ELISA (Human IFN $\gamma$  Module Set and Human TNF $\alpha$  Module Set; Bender MedSystems, San Bruno CA). As described [13],  $^3\text{H}$ -Thymidine ( $^3\text{HTdR}$ ) was added (1  $\mu\text{Ci}/\text{well}$ ) at 48 h, and cultures were further incubated for 18 h at 37°C; cells were harvested, and  $^3\text{HTdR}$  incorporation was measured in a  $\beta$ -counter (LKB Wallac, Turku, Finland).

### **Acknowledgments**

This work was supported by grants from the Ministerio de Educación y Ciencia (SAF2004-07632 and SAF2005-05633). A. S-B- is supported by a fellowship from DURSI (Generalitat de Catalunya). We are grateful to Dr. Oscar Fornas for advice in flow cytometry analysis as well as to Gemma Heredia and Carmen Vela for technical support.

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## Chapter 4

### Expression of NK cell receptors in CD4+T - Large Granular Lymphocytosis

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*Manuscript in preparation*



## INTRODUCTION

Lymphoproliferative disorders of T large granular lymphocytes are characterised by the presence of unusual large numbers of clonal T cells in peripheral blood. Mechanisms involved in the development of these disorders are unclear, and their clinical course is usually asymptomatic or associated with cytopenias, autoimmune manifestations, neuropathies or recurrent infection.

Together with NK cell large granular lymphocytosis, T-LGLs are considered as lymphoid neoplasms<sup>253</sup>. However, no marker has been found that distinguishes these cells from a subpopulation of normal effector T cells, and it remains under debate whether this T-cell pathology should be considered as a malignancy or rather as a secondary reactive phenomenon<sup>254</sup>. Therefore, most of the current criteria for diagnosis of T-LGL leukaemia are based on the presence of a monoclonal T-cell proliferation and clinical symptoms such as neutropenia or autoimmune manifestations. Among T-LGLs, expansions of clonal CD8+ T cells are more frequently found, whereas CD4+T cell are rare. CD4+T-LGL do not show cytopenias and autoimmune manifestations commonly found in CD8+T-LGL patients, although they frequently have associated neoplasias. The lack of a clear association with autoimmune disorders points out the potential involvement of a non-self antigen in the development of CD4+T-LGLs.

A recent study on CD4+T-LGL patients showed preferential expansion of Vb13.1+ CD4+ T clones among the subjects analysed, and this was associated to identical HLA-DRB1\*0701+ genotype and a common aminoacid motif in the CDR3-TCR-Vb sequence of Vb13.1+ clones<sup>212</sup>. These results suggested the potential involvement of a common antigen in driving lymphoproliferation observed in these patients. Another publication demonstrated that HLA-DRB1\*0701 is able to present a peptide derived from glycoprotein B, a Human Cytomegalovirus immunodominant antigen, and elicit a CD4+ response of Vb13.1+ clones<sup>214</sup>. Moreover, the first group recently demonstrated that

## Chapter 4

Vb13.1+ CD4+T cells from patients with LGL recognise and respond to HCMV antigens <sup>213</sup>. These results directly implicate HCMV as the source of the antigenic stimulus driving Vb13.1+CD4+T lymphoproliferations.

Expanded clonal T cells in LGL patients usually show a memory/effector phenotype being CD8+CD45RA+CD27- and expressing Fas and FasL. CD8+ T cells have been described to commonly express NK cell receptors in healthy donors and interestingly, studies on CD8+ T-LGL patients have shown altered expression of NKR on these cells <sup>254</sup>. Reports on expression of NKR on CD4+ cells from healthy donors are scarce, and descriptions were restricted to pathological conditions such as reumathoid arthritis, cancer and celiac disease. We have described expression of NKG2D, ILT2 and KIR on CD4+ T cells specific for HCMV expanded in vitro from PBMC of healthy donors <sup>255</sup>. In the present study we analysed NK cell receptor expression on CD4+T cells of patients undergoing HCMV-specific CD4+T cell LGL proliferations (Vb13.1+) as well as patients with Vb13.1negative CD4+T cell expansions.

## Material and Methods

### Subjects

Peripheral blood samples were obtained in EDTA tubes by venous puncture from healthy donors and CD4+T-LGL patients. Individuals used as controls for CD4+T cell staining included 15 adult donors, 4 men and 11 women (age range: 23-61 years; mean  $\pm$  SD = 44.1  $\pm$  10.5 years), and those used to assess NKG2C+cell proportions included 159 adult individuals, including 95 men and 64 women (age range: 35–79 years; mean  $\pm$  SD: 55,4  $\pm$  11,9 years). Written informed consent was obtained, and the study protocol was approved by the Comite de Etica e Investigacion Clínica-Institut Municipal d'Assistencia Sanitaria (CEIC-IMAS). 9 CD4+T-LGL patients including 6 women and 3 men (age range: 51-68 years; mean  $\pm$  SD = 59.1  $\pm$  6.4 years) were studied. Peripheral blood and frozen PBMC from patients were obtained from the Haematology

service of the Hospital Universitario Virgen de las Nieves in Granada (Spain), and from the Department of Immunology of the Erasmus Medical Center in Rotterdam (The Netherlands). Informed consent was given by the patients in accordance with the Declaration of Helsinki.

### **Antibodies**

HP-3E4 anti-KIR2DL1/S1/S3, Dx9 anti-KIR3DL1, CH-L anti-KIR2DL2/S2/L3, 5.133 anti-KIR3DL1/L2 and KIR2DS4, HP-3B1 anti-CD94, HP-F1 anti-CD85j, BAT221 anti-NKG2D and Z199 anti-CD94/NKG2A monoclonal antibodies (mAbs) have been previously reported <sup>255</sup> (Saez-Borderias EJI 2006). Indirect immunofluorescence analysis was carried out with allophycocyanin (APC)-labelled goat anti-mouse Ig (BD Biosciences Pharmingen). Anti-NKG2C (MAb1381) was from R&D Systems, Inc. (Minneapolis, MN). Anti-CD4-FITC was from BD Bioscience Pharmingen. Anti-Vb13.1-PE was from Immunotech.

### **Immunofluorescence and flow cytometry analysis**

Immunophenotypic analysis was performed using samples of whole fresh blood or PBMC isolated by centrifugation on Ficoll-Hypaque (Axis-Shield PoC AS, Oslo, Norway) as described <sup>168</sup>. Immunofluorescence staining was performed according to the protocols previously described <sup>255</sup>, and samples were subsequently analyzed by flow cytometry (FACS) (BD LSR; Becton Dickinson).

### **PCR**

The *NKG2C* gene sequence between intron 1 and exon 3 was amplified from total genomic DNA using the following primers:

FNKG2C, 5'-GGCATTGT<sup>\*</sup>TCAACTGTAAATCTGCG-3';

and RNKG2C, 5'-ACCTTTCTGCGTTCTTGTATTTCGG-3'.

PCR amplifications were run at 94°C for 1 minute, 61°C for 30 seconds, and 72°C for 1 minute and 30 seconds for 35 cycles, with a final 10-minute extension at 72°C.

## RESULTS AND DISCUSSION

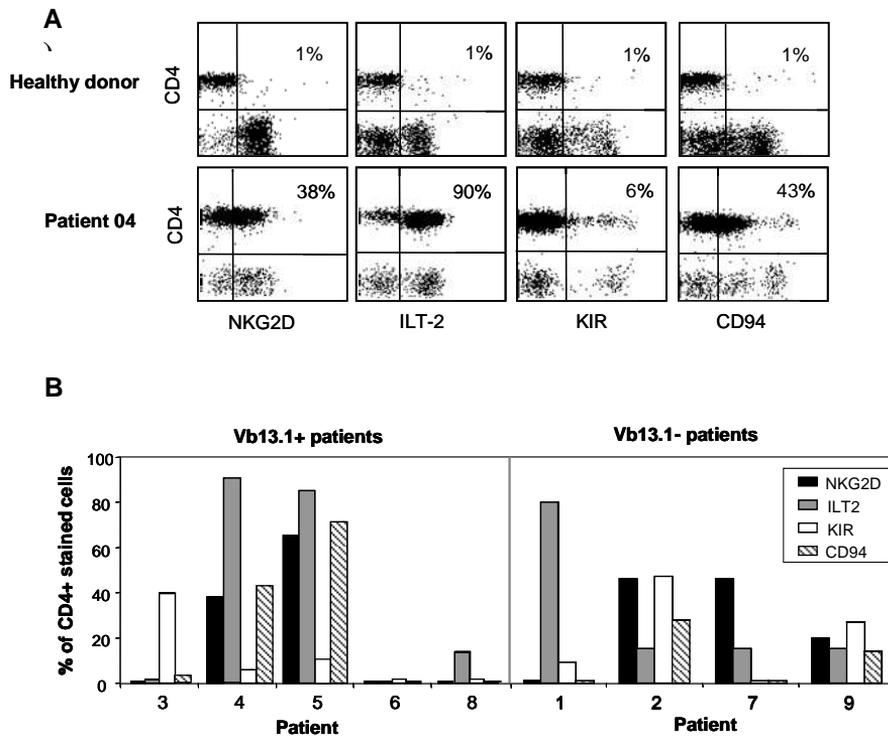
Expanded CD4+ T cells from LGL patients have been reported to display a common phenotype (TCR $\alpha\beta$ + / CD4+ / CD8- / dim / CD57+), with cytotoxic capacity (granzymeB+) and effector/memory markers (CD2+bright, CD7- / +dim, CD11a+bright, CD28-, CD62L-, HLA-DR+) <sup>212,253</sup>. We analysed the expression of NKG2D, ILT2, KIR and CD94 in peripheral blood CD4+ T cells of 9 patients with CD4+T-LGL and 15 adult controls. Analysis of KIR was performed using a cocktail of antibodies against anti-KIR2DL1/S1/S3, anti-KIR3DL1, anti-KIR2DL2/S2/L3, anti-KIR3DL1/L2 and KIR2DS4. Expression of CD56 was also studied, as it is a marker often reported to be associated to CD4+T cell expansions in these patients. Five cases were HLA-DRB1\*0701+ and presented a Vb13.1+CD4+T cell expansion, a subset that has been shown to specifically respond to the gB HCMV antigen in the context of HLA-DR\*0701 <sup>213</sup>.

**Table 1.** Proportions of CD4+, CD56+ and Vb13.1+ cells in peripheral blood of the T-LGL patients

patient	Vb	% CD4+	CD4+	
			%CD56+	%Vb13+
1		73	84	
2		58	20	
3	13.1	55	61	34
4	13.1	90	86	66
5	13.1	77	81	85
6	13.1	84	0	49
7		96	0	
8	13.1	61	26	40
9		46	27	

Numbers correspond to proportion of stained cells.

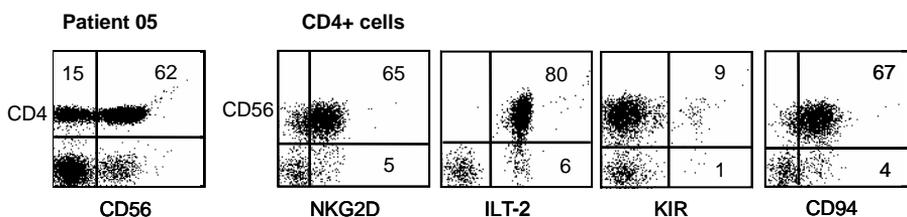
Consistent with previous reports <sup>255</sup>, healthy donors (n=15) presented very low proportions of NKR+ CD4+T cells in peripheral blood (mean±SD, range): NKG2D+ (0.9±0.6, 0-1.9%); ILT2+ (1.6±1.8, 0-5.6%) ; KIR+ (0.4±0.5, 0-1.9%); CD94+ (0.3±0.4, 0-1%). In contrast, 8 of the 9 patients analysed showed high proportions of NKR+ CD4+ T cells (Figure 1). It is of note that, in every case, CD94+ CD4+ T cells were NKG2A- and NKG2C-.



**Figure 1: Increased NKR expression on CD4+T cells from LGL patients.** PBMC from healthy donors and LGL patients were stained for NKG2D, ILT2, KIR and CD94 in combination with CD4 mAb. A) A representative example of NKR expression on CD4+T cells from peripheral blood of an LGL-patient and a healthy control is shown. B) NKR expression on CD4+T cells from 9 CD4+T-LGL patients.

Interestingly, the expression pattern of NKG2D, ILT-2 and KIR differed between individuals (Figure 1), as previously shown for HCMV-specific CD4+ T cells expanded in vitro <sup>255</sup>. The dissociated distribution of NKR indicates that their expression is differentially regulated. Variability in the expression pattern of NKR on HCMV-specific CD4+T cells might affect the CD4+T cell response to the antigenic stimuli. In this regard, we demonstrated that NKG2D functions as a costimulatory receptor in HCMV-specific CD4+ T cells, enhancing proliferation and cytokine secretion upon suboptimal TCR stimulation.

NKR expression on CD8+T cells from healthy donors is often associated to expression of CD56. The functional role of CD56 remains unknown in NK and T cells, and the biological significance of this association is uncertain. CD56 expression has been also described on expanded clonal T cells on most LGL patients <sup>212</sup>. We analysed whether NKR expression was associated to that of CD56 in patients bearing CD56+CD4+T cell expansions. Our results show that NKR are preferentially expressed on CD56+ cells, although the CD56-subset showed a remarkable NKR expression in some cases (Figure 2 and Table 2).



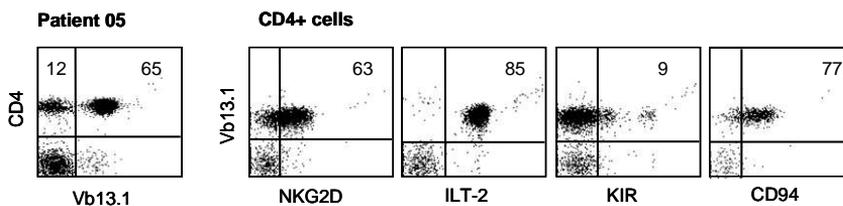
**Figure 2: NKR are preferentially expressed on CD56+CD4+T cells.** PBMC from LGL patients were stained for NKG2D, ILT2, KIR and CD94 in combination with CD4 and CD56. A representative example of the preferential expression of NKR on CD56+CD4+ cells is shown.

**Table 2:** Distribution of NKR on CD56+ and CD56-CD4+ T cells.

Patient	NKG2D		ILT2		KIR		CD94	
	CD56+	CD56-	CD56+	CD56-	CD56+	CD56-	CD56+	CD56-
1	0	0	88	63	10	2	0	0
2	90	52	31	8	88	32	50	19
3	0	0	2	0	60	18	3	0
4	41	17	96	40	5	0	46	18
5	80	21	100	25	12	8	82	21
8	0	0	27	6	4	1	2	1
9	47	11	39	9	60	12	27	8

Numbers correspond to the proportions of the indicated NKR in the CD56+ and CD56-CD4+T cells subsets

Expression of NKR was found in both Vb13.1positive and negative cases (Figure 1B). The nature of the antigens driving expansion of Vb13.1negative T cells remains uncertain, though it cannot be excluded that they may also recognise HCMV antigens. Alternatively, it is likely that expression of NKR on CD4+ T cells is not restricted to HCMV-specific cells and may be also found on T cells specific for other pathogens. By multicolour analysis, a preferential NKR expression was detected on the Vb13.1+CD4+T cell subset (Figure 3 and Table 3), as compared to Vb13.1-CD4+T cells. This indirectly supports that NKR expression is confined to the gB HCMV-specific T cell fraction. These results are in accordance with most reports suggesting that NKR expression on T cells is associated to persistent antigenic stimulation, and expression is restricted to effector/memory cells.



**Figure 3:** NKR are preferentially expressed on Vb13.1+CD4+T cells PBMC from LGL patients were stained for NKG2D, ILT2, KIR and CD94 in combination with CD4 and Vb13.1. A representative example of the preferential expression of NKR on Vb13.1+ cells is shown.

**Table 3:** Distribution of NKR on Vb13.1+ and Vb13.1-CD4+ T cells.

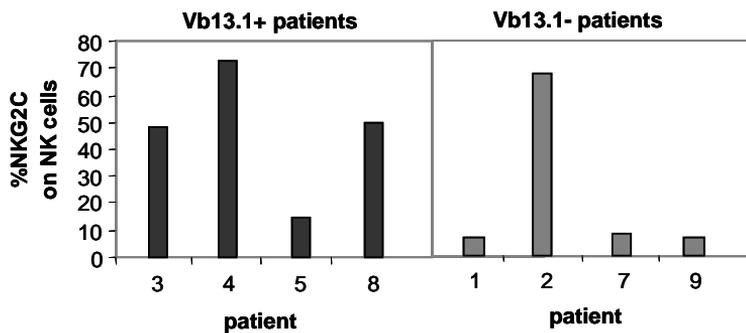
Patient	NKG2D		ILT2		KIR		CD94	
	Vb13.1+	Vb13.1-	Vb13.1+	Vb13.1-	Vb13.1+	Vb13.1-	Vb13.1+	Vb13.1-
3	0	0	0	0	60	30	0	0
4	50	5	89	10	8	1	59	30
5	74	2	100	2	10	2	90	2
6	0	0	0	0	0	0	0	0
8	0	0	28	6	3	1	0	0

Numbers correspond to the proportions of the indicated NKR in the Vb13.1+ and Vb13.1-CD4+T cell subsets.

The mechanisms leading to increased numbers of HCMV-specific CD4+T cells in peripheral blood of T-LGL patients are uncertain. It is plausible that these patients may undergo frequent reactivation of the virus and subsequent chronic antigenic stimulation of virus specific CD4+T cells, unable to effectively control the pathogen. Recently, Guma et al reported increased proportions of NKG2C+ NK and CD8+T cells in HCMV+ individuals, and NKG2C+ NK cells expanded in vitro when cocultured with HCMV-infected fibroblasts<sup>168,245</sup>. Guma et al suggested that a high HCMV-reactivation rate may lead to increased proportions of NKG2C+ NK cells in peripheral blood of healthy donors and HIV patients<sup>256</sup>. The impact of the viral infection on the NKG2C+ compartment observed in vivo was reminiscent of the oligoclonal expansion of antigen-specific T cells with an effector-memory phenotype, and the authors suggested that proportions of NKG2C+ cells present in peripheral blood could be a good parameter to monitor the impact of the infection on the NK and CD8+T cell compartments.

On that basis, we analysed the proportions of NKG2C+ NK cells from peripheral blood of CD4+T LGL patients, to determine whether oligoclonal expansions of HCMV-specific CD4+T cells correlated with increased proportions of NKG2C+ NK cells. Indeed, high proportions of NKG2C+ NK cells were detected on Vb13.1+ patients as compared to HCMV+ healthy donors and most Vb13.1- patients. In healthy seropositive individuals, the mean proportion of NKG2C+ NK cells was 12.2% (mean±SD = 12.2±13.9%,

range = 0.02-80.02%), and only 5% presented  $\geq 45\%$  NKG2C+ NK cells. In contrast, 3 out of 4 Vb13.1+ patients studied bearing the NKG2C gene presented  $\geq 45\%$  of NKG2C+ NK cells. It is of note that patient 06 was missing the NKG2C gene as evidenced by PCR of genomic DNA, and as described for  $\sim 4\%$  of healthy donors <sup>257</sup>. The high proportions of NKG2C+ NK cells observed in these patients would be consistent with a high HCMV reactivation rate and NKR+ Vb13.1+ T cell expansions. Remarkably, the Vb13.1- patient number 2 also showed high proportions of peripheral blood NKG2C+ NK cells, suggesting that HCMV might be also responsible for the CD4+T cell expansion observed in this patient.



**Figure 2: Proportions of NKG2C+ NK cells in peripheral blood of CD4+T-LGL patients.** PBMC from LGL patients were stained for NKG2C in combination with CD3 and CD56 mAb. Proportions of CD3-CD56+ cells stained with NKG2C from patients bearing the NKG2C gene are represented.

### Concluding remarks

Altogether, our results indicate that CD4+T cells from LGL patients show an increased expression of NKR as compared to healthy donors, similar to that detected in HCMV-specific CD4+T cells expanded in vitro from healthy donors<sup>255</sup>. The expansion of NKR+CD4+T cells likely results from chronic

## Chapter 4

antigenic stimulation, and HCMV has been shown to provide the antigenic stimulus driving clonal expansion of Vb13.1+CD4+T cells. Moreover, NKR expression on HCMV-specific Vb13.1+ LGL appeared associated with high proportions of NKG2C+NK cells, consistent with an inefficient control of the viral infection.

**PART III**

**CD94/NKG2 RECEPTORS IN THE  
REGULATION OF NK CELL FUNCTION**



## Chapter 5

IL-12 dependent inducible expression of the CD94/NKG2A inhibitory  
receptor regulates CD94/NKG2C+ NK cell function

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*Journal of Immunology* 2009; Jan 182(2)



**Interleukin-12 dependent inducible expression of the CD94/NKG2A inhibitory receptor regulates CD94/NKG2C+ NK cell function<sup>(2)</sup>**

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**Keywords:** Human, Natural Killer cells, Cell surface molecules, Cytokines.

**ABSTRACT**

The inhibitory CD94/NKG2A and activating CD94/NKG2C killer lectin-like receptors specific for HLA-E have been reported to be selectively expressed by discrete NK and T cell subsets. In the present study, minor proportions of NK and T cells co-expressing both CD94/NKG2A and CD94/NKG2C were found in fresh peripheral blood from adult blood donors. Moreover, CD94/NKG2A surface expression was transiently detected upon *in vitro* stimulation of CD94/NKG2C<sup>+</sup> NK cells in the presence of irradiated allogeneic PBMC or rIL-12. A similar effect was observed upon co-culture of NKG2C<sup>+</sup> NK clones with human cytomegalovirus-infected autologous dendritic cell cultures, and was prevented by an anti IL-12 mAb. NKG2A inhibited the cytolytic activity of NKG2C<sup>+</sup> NK clones upon engagement either by a specific mAb or upon interaction with a transfectant of the HLA class I-deficient 721.221 cell line expressing HLA-E. These data indicate that beyond its constitutive expression by an NK cell subset, NKG2A may be also transiently displayed by CD94/NKG2C<sup>+</sup> NK cells under the influence of IL-12, providing a potential negative regulatory feedback mechanism.

## INTRODUCTION

Natural Killer (NK) cells participate in the innate immune response against microbial pathogens and tumours, exerting cytotoxicity and cytokine production. Triggering of NK cell effector functions depends on the integration of signals delivered by an array of inhibitory and activating receptors. Some inhibitory receptors are engaged by self MHC class I molecules preventing NK cell reactivity against normal autologous cells. Loss of class I molecules and/or expression of ligands for activating receptors render tumours and infected cells susceptible to the NK cell attack (1-4). The human NK cell receptor (NKR) repertoire is defined by the different combinations of receptors acquired by individual NK clones along differentiation, and is conditioned by the genomic diversity at the KIR locus (5,6).

Different NKR gene families (i.e. NKG2, KIR and Ly49) encode for pairs of homologous activating and inhibitory molecules that may interact with the same ligand. Among them, the NKG2A and NKG2C C-type lectin molecules are expressed as heterodimers coupled to CD94, forming receptors with opposite functions (7,8). The inhibitory NKG2A molecule contains cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIM) that recruit SHP-1 and SHP-2 tyrosine phosphatases (9). By contrast, NKG2C is coupled through the DAP12/KARAP adaptor to a tyrosine kinase-dependent pathway, triggering NK cell effector functions (10). Both receptors specifically recognize HLA-E, which presents peptides derived from the leader sequence of other HLA class I molecules (11-13); the affinity of their interaction depends on the sequence of the HLA-E-bound nonamers and appears higher for CD94/NKG2A (14-16). According to recent structural analyses, the CD94 subunit plays a pivotal role in the interaction between CD94/NKG2A and the HLA-E/peptide complex (17,18). In mice, CD94/NKG2 receptors are conserved and recognize the Qa1b class Ib molecule which presents as well MHC class I-derived peptides (19,20).

CD94/NKG2A and CD94/NKG2C are displayed by subsets of human NK cells,  $\gamma\delta$  and  $\alpha\beta$  T lymphocytes (7,8,21). Differences in the expression pattern of other NK cell receptors have been noticed comparing NKG2A+ and NKG2C+ NK cells (22,23). CD94/NKG2A appears detectable at relatively early stages of NK cell differentiation, preceding the expression of KIR (24,25); by contrast, this inhibitory receptor may be displayed by T cells with an effector/memory phenotype and is inducible in vitro under the influence of some cytokines (26-28). Little information is currently available on the expression of NKG2C during NK and T cell differentiation (24,29). In this regard, increased numbers of circulating NKG2C+ NK cells have been associated to a positive serology for human cytomegalovirus (HCMV) in healthy individuals and aviremic HIV-1 infected patients (22,23,30). Furthermore, an expansion of CD94/NKG2C+ cells upon in vitro interaction with HCMV-infected fibroblasts was reported (31). Altogether these observations support the hypothesis that NKG2C+ cells may play a role in the response to HCMV, and that the lifelong persistent viral infection may shape the NKR repertoire. Functional characterization of CD94/NKG2 receptors has been reported in NK and T cell subsets selectively bearing NKG2A or NKG2C (9,10,32-34). Yet, these genes may be co-transcribed at the clonal level in some NK and T cells (35,36), and both proteins have been detected together at the surface of decidual and peripheral blood CD56bright NK cells (37). The functional implications resulting from co-expression at the single cell level of activating and inhibitory NKR specific for the same ligand are unknown.

In the present study, minor NK and T cell subsets co-expressing CD94/NKG2A and CD94/NKG2C were found in fresh blood samples from adult donors. Moreover, we provide evidence that, under the influence of IL-12, NKG2A may be transiently displayed by CD94/NKG2C+ NK cells, inhibiting their cytolytic activity against target cells bearing HLA-E. Such expression pattern is reminiscent of that observed for the CD28 and CTLA-4 leukocyte

receptors, where the inhibitory molecule induced upon cell stimulation serves as a negative regulatory feedback mechanism (38).

## **MATERIAL AND METHODS**

### **Subjects**

Peripheral blood samples were obtained in EDTA tubes by venous puncture from 195 adult individuals, including 104 men and 91 women (age range: 18–79 years; mean  $\pm$  SD: 49,2  $\pm$  17,2 years; median: 50 years). Written informed consent was obtained, and the study protocol was approved by the Comité de Ética e Investigación Clínica-Institut Municipal d'Assistència Sanitària (CEIC-IMAS).

### **Antibodies**

HP-3B1(IgG2a) anti-CD94 monoclonal antibody (mAb) and anti CD94 F(ab')<sub>2</sub> fragments were prepared as previously described (39). Z199 (IgG2b) anti-NKG2A mAb was provided by Dr A. Moretta (University of Genova, Italy) and was conjugated to fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO). HP-3E4 anti-KIR2DL1/S1/S3 and HP-F1 anti-ILT2 were generated in our laboratory and have been previously described (40). 5.133 anti-KIR3DL2 was provided by Dr. Marco Colonna. Dx9 anti-KIR3DL1 mAb was provided by Dr. Lewis Lanier (UCSF, San Francisco, CA). CH-L anti-KIR2DL2/S2/L3 was provided by Dr. Silvano Ferrini (University of Genova, Italy). 3A1(IgG2b) anti-CD7 and 9E10 (IgG1) anti-myc hybridoma have been described (41,42), and the 20C2 (IgG1) anti-IL-12 hybridoma was obtained from ATCC (American Type Culture Collection). Anti-NKG2C (MAb1381, IgG2b) and anti-NKG2C-PE MAb were from R&D Systems (Minneapolis, MN). Anti-CD3-PerCP and anti-CD56-APC were from BD Biosciences Pharmingen (San Jose, CA). Indirect immunofluorescence analysis was carried out with a fluorescein isothiocyanate (FITC)-tagged F(ab')<sub>2</sub> rabbit anti-mouse Ig antibody (Dakopatts, Glostrup,

Denmark) or APC-labeled goat anti-mouse Ig (BD Biosciences Pharmingen, San Jose, CA, USA).

### **Immunofluorescence and flow cytometry analysis**

Immunofluorescence staining was performed according to the protocols detailed below, and samples were analyzed by flow cytometry (FACS) (BD LSR; Becton Dickinson). For immunophenotypic analysis, whole blood samples were incubated with anti-NKG2A-FITC; subsequently, samples were incubated with anti-NKG2C-PE (R&D Systems (Minneapolis, MN), anti-CD3PerCP, and anti-CD56-APC (BD Biosciences Pharmingen, San Jose, CA). After washing, erythrocytes were lysed with FACS lysis buffer (Becton Dickinson, San Jose, CA), and cells were resuspended in PBS. For indirect immunofluorescence staining of fresh PBMC, cells were isolated by centrifugation on Ficoll-Hypaque (Axis-Shield PoC AS, Oslo, Norway) and incubated with unlabeled antibodies (HP-3E4, 5.133, Dx9, CH-L and HP-F1) followed, after washing, by APC-labeled goat anti-mouse Ig, and NKG2A-FITC, NKG2C-PE and CD3-PerCP staining.

For indirect immunofluorescence staining of polyclonal NK cell populations and NK clones, samples were incubated with the individual unlabeled antibodies followed, after washing, by a fluorescein isothiocyanate (FITC)-tagged F(ab')<sub>2</sub> rabbit anti-mouse Ig antibody (Dakopatts, Glostrup, Denmark). Cells were treated with propidium iodide (Sigma, St. Louis, MO) in order to exclude dead cells during flow cytometry analysis; appropriate isotype-matched control mAbs were used to assess non-specific binding.

### **Cell cultures**

The RPMI-8866 B lymphoma cell line and the 721.221 (.221) HLA class I-deficient EBV-transformed B lymphoblastoid cell line (LCL) were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FCS, penicillin (100 U/ml) and streptomycin (10 µg/ml), referred to as complete

medium. The HLA-E+ 721.221-AEH (.221-AEH) cells, kindly provided by Dr. D. E. Geraghty (Fred Hutchinson Cancer Research Centre, Seattle, WA), were generated by stable transfection of .221 cells with a construct in which the leader sequence of the HLA-E\*0101 allele was replaced by that of HLA-A2 (43); .221-AEH cells were cultured in complete medium supplemented with 300 µg/ml hygromycin B (Calbiochem, San Diego, CA).

PBMC were isolated by centrifugation on Ficoll-Hypaque (Axis-Shield PoC AS, Oslo, Norway). To obtain polyclonal activated NK cell populations, PBMC ( $1.5 \times 10^6$  cells/ml) co-cultured in complete medium with irradiated (40Gy) RPMI-8866 or 221.AEH cells ( $0.5 \times 10^6$  cells/ml) in 24-well plates. After 8-10 days cells were harvested, washed and incubated with an anti-CD3 mAb followed by complement lysis with rabbit sera (Seralab, Sussex, United Kingdom) to remove T cells, as described (39). Purified CD3- CD56+ NK cell populations (>98%) were expanded in the presence of recombinant rIL-2 (400 U/ml). Polyclonal NK cells generated with 221.AEH cells were >92% NKG2C+ and are referred to as NKG2C+. Alternatively, NKG2C+ NK cell polyclonal populations were also generated by sorting of NKG2C+ cells from PBMC and stimulation at  $2 \times 10^5$  cells/ml with rIL-2 (1,000 U/ml), 2 µg/ml phytohemagglutinin (PHA), irradiated (40 Gy) allogeneic PBMC ( $4.5 \times 10^5$  cells/ml) and RPMI-8866 ( $9 \times 10^4$  cells/ml). NK cell polyclonal populations generated with RPMI-8866 contained NKG2A+ and NKG2C+ cells, and were subjected to a negative selection incubating with anti-NKG2C antibody followed by sheep anti-mouse IgG Dynabeads (DynaL Biotech ASA, Oslo, Norway), as recommended by the manufacturer. The resulting NKG2C- NK cell populations contained NKG2A+ cells as well as some NKG2C- NKG2A- cells and are referred to as NKG2A+ polyclonal NK cells. CD94/NKG2C+ and CD94/NKG2A+ NK cell clones were derived from fresh NKG2C+ or NKG2A+ PBL sorted by flow cytometry (FACSVantage SE, Becton Dickinson, San Jose, CA). As described (44), cells were cultured under limiting dilution conditions in 96-well plates with irradiated (40 Gy) allogeneic PBMC ( $1.5 \times 10^5$

## Chapter 5

cells/ml) and RPMI-8866 cells ( $0.3 \times 10^5$  cells/ml), in the presence of rIL-2 (1,000 U/ml) and 2  $\mu$ g/ml phytohemagglutinin (PHA) (Sigma), and were re-stimulated every 15 days with irradiated feeder cells and rIL-2. In some assays, polyclonal NK cells or NK clones were re-stimulated with irradiated (40 Gy) PBMC and RPMI-8866 cells, rIL-15 (30ng/ml), rIL-12 (20ng/ml) or rTGF $\beta$  (20ng/ml) (Peprotech, Rocky Hill, NJ) in the presence of rIL-2 (400U/ml).

### **Virus stock preparation**

Stocks of HCMV strain TB40E (45) (kindly provided by Christian Sinzger, Institute for Medical Virology, University of Tübingen, Germany) were prepared by infecting MRC-5 cells at low multiplicity of infection (MOI). Infected cell supernatants were recovered when maximum cytopathic effect was reached and cleared of cellular debris by centrifugation at  $1.750 \times g$  for 10 min. Virus was pelleted twice through a sorbitol cushion (20% D-sorbitol in TBS [25 mM Tris-HCl, pH 7.4, 137 mM NaCl]) by centrifugation 90 min at  $27.000 \times g$  at 15°C. Pelleted virus was resuspended in DMEM supplemented with 3% fetal calf serum and titrated by standard plaque assays on MRC-5 cells.

### **HCMV infection of dendritic cells**

After Ficoll-Hypaque separation of PBMC, monocytes were obtained by positive selection of CD14<sup>+</sup> cells using magnetic separation (Miltenyi, Bergisch Gladbach, Germany). To differentiate into dendritic cells CD14<sup>+</sup> cells were plated at  $1 \times 10^6$  cells/ml and cultured in the presence of IL-4 (25ng/ml) and GM-CSF (50ng/ml) (Peprotech, Rocky Hill, NJ). After 6 days, cells were CD14<sup>-</sup> CD1a<sup>+</sup> and CD83<sup>-</sup> as assessed by immunofluorescence staining. As previously described (46), monocyte-derived DC (moDC) were cultured overnight in 24-well plates ( $4 \times 10^5$  cells/well) with the TB40/E HCMV strain (MOI 100). After incubation, moDC were washed and incubated in 48-well plate ( $3 \times 10^4$  cells/well) with NK cells ( $3 \times 10^5$  cells/well) in a final volume of 400 $\mu$ l; cells were harvested

after 5 days for analysis. In some experiments, anti-IL-12 or anti-myc hybridoma supernatants were added (1/5 dilution) at the beginning of the co-culture. To test IL-12 production, supernatants of mock-treated or HCMV-infected moDC were harvested 48h post-infection and analysed by Human IL-12 (p70) ELISA test (Bender MedSystems San Bruno, CA).

### **Cytotoxicity assays**

As previously described (44), the cytolytic activity of NK clones against the murine FcγR<sup>+</sup> P815 mastocytoma cell line was analyzed in a 4-h <sup>51</sup>Cr-release reverse antibody-dependent cell-mediated cytotoxicity (rADCC) assay in the presence of different NKR-specific mAb (10 μg/ml) (effector/target= 1/1). As described (47), NK cell clones were as well tested in a 4 h <sup>51</sup>Cr-release assay against the HLA class I deficient 721.221 cell line and the HLA-E<sup>+</sup> 221-AEH transfectant, which displays surface HLA-E bound to an HLA class I leader sequence (43). All assays were set up in triplicate and specific lysis was calculated as described (47).

### **Statistical analysis**

Linear regression was used to assess the relationship between the distribution of the NKG2A<sup>+</sup> NKG2C<sup>+</sup> subset in NK and T cells. Analyses were performed with an SPSS (version 14.0; SPSS) statistical package. Results were considered to be significant at a *P* value (2-tailed) of .05

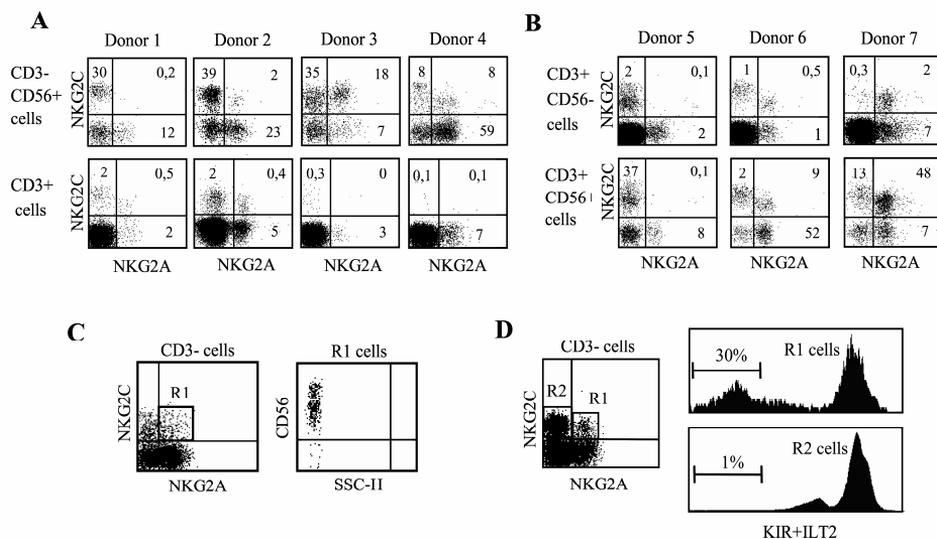
## RESULTS

### **NKG2A and NKG2C are co-expressed by subsets of peripheral blood NK and T cells.**

To study the distribution of CD94/NKG2A and CD94/NKG2C receptors, a multicolour flow cytometry analysis was carried out in peripheral blood samples from a population of adult individuals (n=195). Figure 1 presents representative examples of the different distribution patterns observed. The proportions of NK (CD3<sup>-</sup> CD56<sup>+</sup>) and T cells (CD3<sup>+</sup> CD56<sup>-</sup> and CD56<sup>+</sup>) stained by NKG2A and/or NKG2C specific mAbs are shown in Table I. Though most NK cells were either NKG2A<sup>+</sup>, NKG2C<sup>+</sup> or NKG2A<sup>-</sup> NKG2C<sup>-</sup>, variable numbers of double positive NKG2C<sup>+</sup> NKG2A<sup>+</sup> NK cells and T cells were detectable in different donors, indicating that both receptors may be expressed together at the cell surface in vivo. In contrast to a previous report (37), NKG2C<sup>+</sup> NKG2A<sup>+</sup> cells were found among both the CD56dim and CD56bright NK cell subsets (Figure 1C) and, predominantly, within the CD56<sup>+</sup> T cell subset (Figure 1B). No significant correlation between the proportions of double positive NKG2C<sup>+</sup> NKG2A<sup>+</sup> NK and T cells was substantiated.

Whether double positive NKG2A<sup>+</sup> NKG2C<sup>+</sup> cells bear additional inhibitory receptors for HLA class I molecules appeared a relevant question, considering that the NKG2C<sup>+</sup> population has been reported to contain higher proportions of KIR<sup>+</sup> and ILT2<sup>+</sup> cells than NKG2A<sup>+</sup> cells (22). To directly address this point, a multicolour analysis of NKG2C<sup>+</sup> NKG2A<sup>+</sup> cells was performed in PBMC from five donors, assessing the expression of KIRs (using a mixture of anti KIR mAbs) and ILT2 (LIR-1) receptors specific for HLA class I molecules on NK cells. Variable proportions of CD3<sup>-</sup> NKG2C<sup>+</sup> NKG2A<sup>-</sup> cells were stained by anti KIR (mean±SD= 48±15%) and anti ILT2 (mean±SD= 39±14%) mAbs; similarly a subset of CD3<sup>-</sup> NKG2A<sup>+</sup> NKG2C<sup>+</sup> NK cells displayed KIR (mean±SD= 26±4%) or ILT2 (mean±SD= 32±36%). Remarkably, a fraction of double positive cells that did not express KIR nor ILT2 was clearly identified

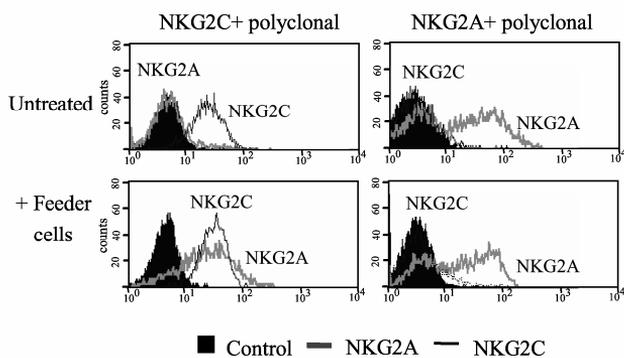
(Figure 1D), whereas most NKG2C+ NKG2A- cells were stained by the combination of ILT2 and KIR-specific mAbs (Figure 1D). It is of note that as homologous activating and inhibitory KIRs could not be discriminated by specific mAbs, co-expression of NKG2A with inhibitory KIRs could not be precisely defined. The detection of both KIR+ and KIR- NKG2A+ NKG2C+ cells was consistent with the observation that NKG2A and NKG2C co-expression is not restricted to the CD56bright population, previously reported to be predominantly KIR-negative.



**Figure 1. NKG2A and NKG2C are co-expressed in subsets of peripheral blood lymphocytes.** A multicolour flow cytometry analysis was carried out in samples from adult blood donors assessing the expression of NKG2A and NKG2C in NK cells (CD3-CD56+) and T lymphocytes (CD3+). Representative examples of different distribution patterns of NKG2A+ NKG2C+ cells observed are displayed comparing NK and T cell populations (panel A) or CD56+ and CD56- T cell subsets (panel B). Numbers correspond to the proportions of stained cells. The distribution of CD56bright and CD56dull subsets was assessed in NKG2C+ NKG2A+ populations (panel C). The expression of KIR and ILT2 by NKG2C+ NKG2A+ cells was also studied in five different individuals, and compared to the NKG2C+ NKG2A- subset; a representative distribution pattern is shown in panel D.

### Surface NKG2A expression is inducible upon CD94/NKG2C+ NK cell activation

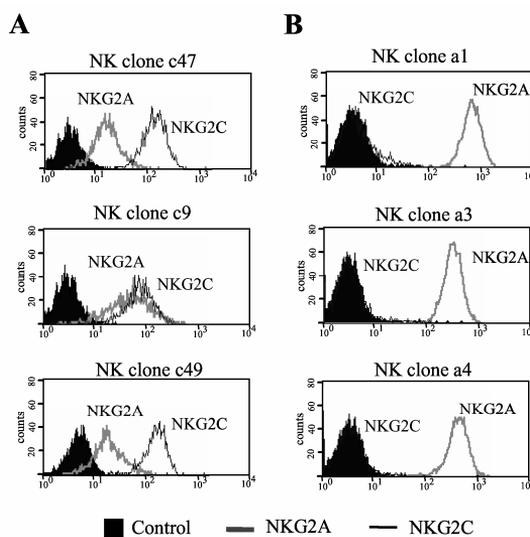
In previous studies we observed that NKG2C and NKG2A were occasionally found together at the surface of in vitro activated NK cells. We addressed whether co-expression of both NKG2 receptors resulted from the expansion of double positive cells or, alternatively, was induced upon cell activation. To this end, polyclonal NKG2C+ and NKG2A+ NK cell populations, the latter including also a subset of NKG2A- NKG2C- cells, were generated as described in Methods and their phenotype analysed upon in vitro stimulation. After a 5-day co-culture with irradiated allogeneic feeder cells (PBMC and RPMI-8866) and rIL-2, substantial proportions of NKG2A+ cells were detected among the NKG2C+ polyclonal population; by contrast, NKG2A+ cells remained NKG2C- (Figure 2).



**Figure 2. NKG2A expression is inducible on NKG2C+ polyclonal NK cells.**

NKG2C+ or NKG2A+ polyclonal NK cells were generated as described in material and methods and cultured with rIL-2 alone or with rIL-2 and irradiated feeder cells (allogeneic PBMC+RPMI-8866). After 5 days cells were stained with specific mAbs to analyse the expression of NKG2A and NKG2C on the cell surface. A representative experiment of three performed is shown.

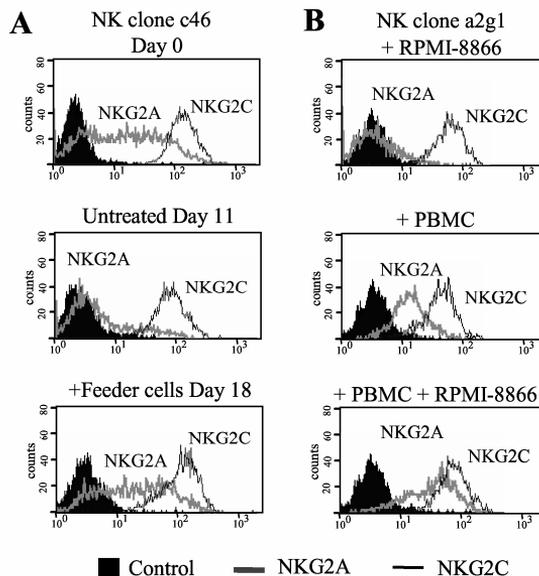
To more precisely assess whether NKG2A expression was inducible upon in vitro stimulation, ruling out the outgrowth of double positive NK cells, NKG2C+ cells were sorted and cloned under limiting dilution conditions in the presence of irradiated feeder cells and rIL-2. After expansion for at least one month, that required periodical re-stimulation, substantial proportions of NKG2C+ NK clones displayed variable levels of NKG2A (Figure 3A); under the same experimental conditions NKG2A+ clones remained NKG2C- (Figure 3B).



**Figure 3. Co-expression of NKG2A and NKG2C+ in NK cell clones.** NKG2C+ and NKG2A+ NK clones were generated by culturing under limiting dilution conditions NKG2C+ or NKG2A+ cells sorted from PBMC in the presence of irradiated feeder cells, rIL-2 and PHA. CD56+CD3- clones were analysed for NKG2C and NKG2A expression. Three clones derived from NKG2C+ (A) or NKG2A+ (B) cells are shown.

Sequential phenotypic analysis revealed that expression of NKG2A in NKG2C+ clones was unstable, progressively decreasing along in vitro culture in the presence of rIL-2 and becoming detectable again upon re-stimulation with irradiated feeder cells (Figure 4A).

To evaluate the relative contribution of feeder cells in the induction of NKG2A expression, NKG2C<sup>+</sup> clones were re-stimulated with irradiated PBMC or/and RPMI-8866 cells. Our results indicated that only incubation with PBMC was sufficient to induce NKG2A expression, whereas RPMI-8866 cells enhanced the effect (Figure 4B).

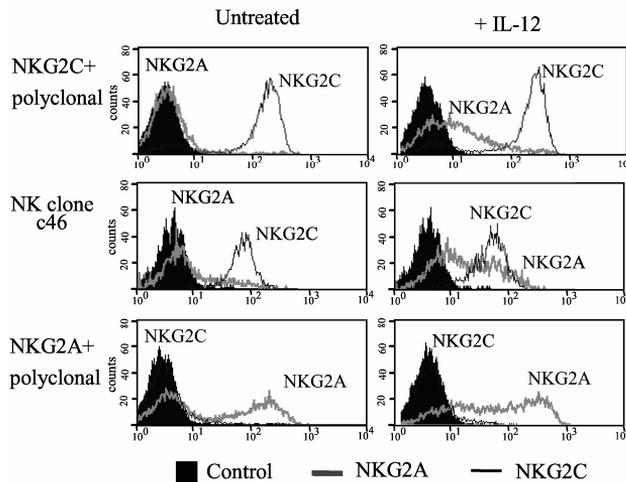


**Figure 4. NKG2A expression on NKG2C<sup>+</sup> clones is transient and can be induced after stimulation with feeder cells.** A) NKG2A<sup>+</sup>NKG2C<sup>+</sup> NK clones (day 0) cultured with rIL-2 alone were analysed for the expression of both surface receptors after >10 days (day 11). Subsequently, clones were re-stimulated with feeder cells (PBMC+RPMI-8866) and expression of NKG2A and NKG2C was assessed (day 18). B) NKG2C<sup>+</sup> clones stimulated in parallel with either RPMI-8866, allogeneic PBMC or both were analysed for NKG2A expression after 5 days. Results shown are representative of experiments performed with three different clones.

### IL-12 induces NKG2A expression on NKG2C<sup>+</sup> NK cells

Previous reports described that NKG2A expression was inducible in T cells by IL-12 stimulation (28) and upon TcR-dependent activation in the presence of IL-15 or TGF $\beta$  (26,27). Moreover, increased proportions of NKG2A<sup>+</sup>

lymphocytes were observed in NK cell populations cultured in the presence of IL-12 (48). To evaluate whether any of these cytokines accounted for the inducible expression of NKG2A in NKG2C+ cells, polyclonal NKG2C+ NK cell populations and NKG2C+ clones, cultured in rIL-2 containing medium, were treated with rIL-15, rTGF $\beta$  or rIL-12. As shown in figure 5, NKG2A was up-regulated in both NKG2C+ polyclonal populations and clones upon incubation with rIL-12, whereas no effect of the other cytokines was substantiated (data not shown). NKG2A expression appeared also induced in the NKG2A- NKG2C- subset, whereas none of these stimuli promoted NKG2C expression in NKG2A+ NK cells (Figure 5).



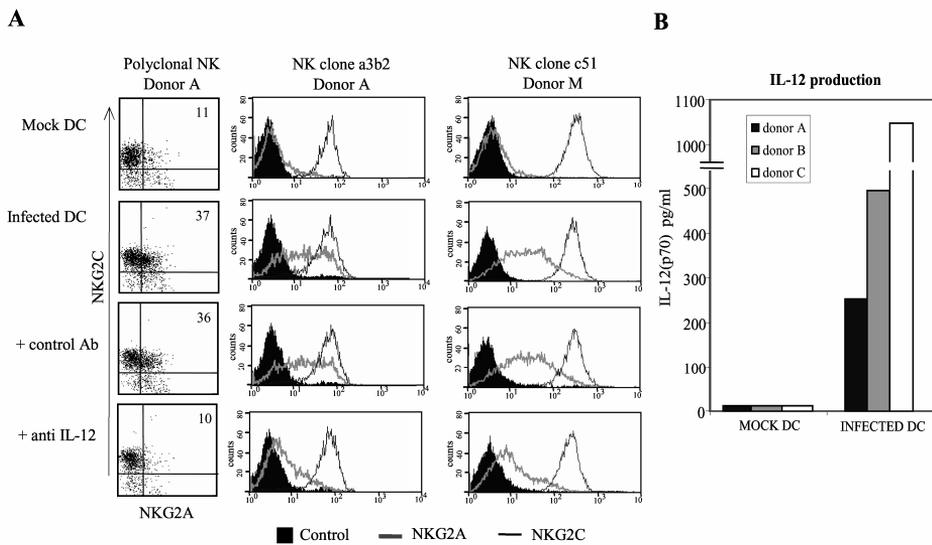
**Figure 5. IL-12 induces NKG2A expression on the surface of NKG2C+ NK cells.** NKG2C+ or NKG2A+ polyclonal NK cells and NKG2C+ NK clones (c46) were treated with 20ng/ml of rIL-12 and analysed for NKG2A expression after 5 days in the presence of rIL-2. Results are representative of three different experiments performed with NK polyclonal populations and 8 different NKG2C+ NK clones tested.

As the generation of polyclonal NK cell populations and clones involved pre-activation and expansion in the presence of rIL-2, these experiments did not discern whether rIL-12 stimulation was sufficient for inducing NKG2A

expression or, alternatively, it complemented other activating signals. To approach this issue, NKG2A<sup>-</sup> populations were obtained by negative selection from fresh PBMC and stimulated either with rIL-12 or with rIL-2 and rIL-12, analysing their phenotype 5 days later. Under these conditions, rIL-12 appeared sufficient to induce NKG2A expression (data not shown).

### **NKG2A expression is inducible in NKG2C<sup>+</sup> NK cells stimulated with HCMV-infected autologous dendritic cells**

To address whether NKG2A expression may be inducible in the context of the NK-DC crosstalk during the innate immune response to infections, we used HCMV-infected dendritic cells as an experimental model. To this end, monocyte-derived immature dendritic cells (moDC) were infected as described with the TB40E HCMV strain (46). The proportions of infected moDC identified by detection of the IE1 antigen ranged between 25 and 95 %, and cells remained CD83<sup>-</sup> after infection. Mock-treated and HCMV-infected moDC were co-cultured either with autologous polyclonal NKG2C<sup>+</sup> NK populations or clones. Under these conditions, NKG2A became detectable on the surface of NKG2C<sup>+</sup> cells stimulated with infected but not mock-treated moDC (Figure 6A). The effect could be markedly inhibited by an anti-IL-12 antibody, further supporting a central role of the cytokine. To directly check the presence of IL-12 in the system, mock-treated and virus-infected moDC were plated alone at the same concentrations employed for NK cell co-cultures, and supernatants were harvested after 48h. Consistent with their ability to induce NKG2A expression, IL-12 was detectable by ELISA only in the supernatants of infected moDC (Figure 6B). These data support that endogenous IL-12 secretion may up-regulate the expression of the NKG2A inhibitory receptor in the NKG2C<sup>+</sup> population during the innate immune response to HCMV infection.

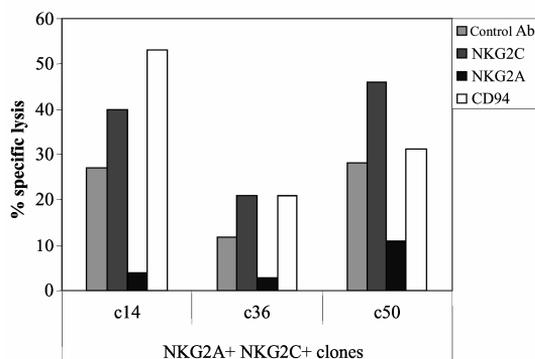


**Figure 6. IL-12 dependent induction of NKG2A on NKG2C+ NK cells co-cultured with HCMV-infected moDC.** A) NKG2C+ NK clones and polyclonal NK cells were cultured with autologous (donor A) or allogeneic (donor M) mock-treated or HCMV-infected immature moDC. In parallel, samples were incubated in the presence of either an anti-IL-12 mAb or an isotype-matched anti-myc mAb (control). After 5 days, NK cells were analysed for surface expression of NKG2A and NKG2C. Results are representative of four experiments performed combining moDC from three different donors and NK clones from two different donors. B) Mock-treated or HCMV-infected DC were incubated alone as described above. Supernatants were harvested after 48h and IL-12 was measured by ELISA. The proportions of infected cells expressing the IE1 antigen varied between 25% and 95% in different experiments. Data correspond to samples from three different donors.

### Engagement of the CD94/NKG2A receptor inhibits the cytolytic activity of CD94/NKG2C+ NK clones

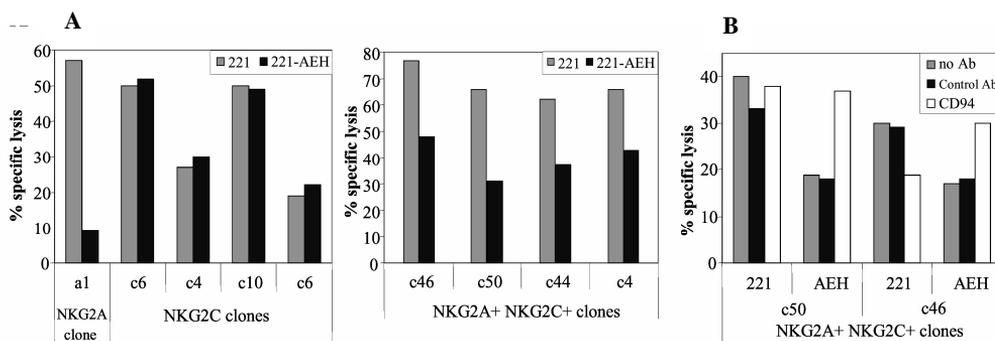
The implications resulting from co-expression at the clonal level of inhibitory and activating receptors specific for the same ligand depend on their signalling capacity. Our results suggested that the inducible expression of NKG2A in NKG2C+ NK clones might constitute a negative feedback regulatory

mechanism. To assess the inhibitory function of CD94/NKG2A in double positive NKG2C+ NKG2A+ NK clones, cells were first tested in redirected lysis assays against the Fc $\gamma$ R+ P815 mastocytoma cell line in the presence of mAbs specific for NKG2A, NKG2C or CD94. In agreement with previous results (33), stimulation with an anti NKG2C mAb triggered cytotoxicity in every case; conversely, selective engagement of NKG2A inhibited the lysis of P815 cells, proving that the receptor was functional (Figure 7). By testing in parallel a panel of NK clones, a clear correlation between surface expression levels of NKG2A and inhibition of cytotoxicity in redirected lysis assays was noticed (data not shown). Co-ligation of both receptors with a mAb specific for the common CD94 subunit activated target cell lysis in some clones (Fig. 7, c14 and c36), indicating that the function of NKG2C prevailed. By contrast, no effect of the anti CD94 mAb was detected in other samples (Fig. 7, c50), supporting that the antagonistic signalling pathways may effectively counteract each other.



**Figure 7. The CD94/NKG2A inhibitory receptor co-expressed in CD94/NKG2C+ NK clones is functional.** NK clones were tested in redirected cytotoxicity assays against the P815 cell line in the presence of mAbs specific for NKG2A, NKG2C and CD94 or control anti-CD7 mAb (E/T=2:1). Three representative examples of the different patterns of response observed are shown.

To further examine the inhibitory role of NKG2A in NKG2C+ cells, double positive NK clones were tested in cytotoxicity assays against the .221-AEH cell line that displays surface HLA-E in the absence of classical HLA class I molecules, and was generated by stable transfection of the 721.221 cell line with a construct in which the leader sequence of the HLA-E\*0101 allele was replaced by that of HLA-A2 (43). NKG2A+ NKG2C- and NKG2C+ NKG2A- clones were also comparatively studied in these assays. Cytotoxicity mediated by double positive NK clones, but not by NKG2C+ NKG2A- cells, against .221-AEH cells appeared lower than that detected against wild-type .221 cells (Figure 8A), but was restored in the presence of anti CD94 mAb F(ab')2 (Figure 8B). These data support that the inducible CD94/NKG2A inhibitory receptor may regulate the response of CD94/NKG2C+ NK cells by engaging HLA-E bound to leader sequence peptides from other HLA class I molecules (11-13); it is of note that CD94/NKG2A has a higher affinity for HLA-E than CD94/NKG2C (14-16).



**Figure 8. CD94/NKG2A inhibits the cytolytic activity of NKG2C+NKG2A+ NK cell clones against HLA-E+ target cells.** (A) NKG2A+, NKG2C+ and double positive NKG2A+ NKG2C+ NK clones were tested in cytotoxicity assays against the HLA class I deficient 721.221 cell line (.221) and its HLA-E+ .221-AEH transfectant. (B) Cytotoxicity of NKG2A+ NKG2C+ NK clones against .221 and .221-AEH cells was tested in the presence of anti CD94 F(ab')2 or anti-CD7 (control) mAbs.

## DISCUSSION

The inhibitory CD94/NKG2A and activating CD94/NKG2C receptors are constitutively displayed by discrete NK cell subsets (1,22). In the present report we provide evidence supporting that, under the influence of IL-12 stimulation, CD94/NKG2A is also transiently inducible in NK cells bearing the homologous CD94/NKG2C activating receptor. Moreover, our results indicate that CD94/NKG2A is functional, regulating the response of CD94/NKG2C+ cells against targets bearing HLA-E, the natural ligand shared by both lectin-like receptors. This situation is reminiscent of the activation-dependent expression of CTLA-4 on T lymphocytes which counteracts CD28-mediated co-stimulation (38). By analogy, it is conceivable that acquisition of the NKG2A inhibitory receptor may play a physiological role, providing a reversible regulatory feedback mechanism to control the activation of NKG2C+ cells. The higher affinity of CD94/NKG2A for HLA-E likely favours its competition with CD94/NKG2C for ligand engagement, as described for the CD28/CTLA-4 pair. This would enhance the efficiency of the regulatory mechanism, compensating the lower surface levels of the inhibitory receptor transiently expressed by activated CD94/NKG2C+ cells. In NKG2C+ NK clones the surface levels of the activating receptor appeared rather stable, whereas NKG2A expression decreased after stimulation, predictably reaching a threshold avidity for HLA-E peptide complexes unable to counteract the triggering signals. A clear correlation between the expression levels of NKG2A and the inhibition of cytotoxicity in redirected lysis assays was noticed when a panel of NK clones were tested in parallel.

CD94/NKG2A expression was previously shown to be inducible in T cells stimulated with IL-12 (28) or upon TCR-dependent activation under the influence of IL-15 or TGF $\beta$  (26,27). Our results indicate that incubation with rIL-12 alone promoted NKG2A expression by NKG2C+ polyclonal NK cell populations and clones, whereas IL-15 and TGF $\beta$  had no detectable effect. It is

of note that stimulation of resting purified NK cells with rIL-12 alone induced NKG2A expression, thus suggesting that secretion of the cytokine during an inflammatory response is sufficient to promote this regulatory mechanism controlling the activation of CD94/NKG2C+ cells.

Information about the mechanisms that regulate transcription of the different NKG2 genes is limited. The NKG2A promoter has been partially characterized and shown to be under the control of GATA3 (49). Further studies are required to define how constitutive and inducible NKG2A expression are differentially regulated, and whether IL-12R signalling via STAT-4 plays a direct role in the process. On the other hand, the possibility that NKG2C expression may be inducible in NKG2A+ cells activated in response to other stimuli cannot be excluded.

It is currently accepted that individual NK cells acquire along differentiation various combinations of inhibitory receptors specific for MHC class I molecules, including NKG2A. The NKR repertoire of an individual will be ultimately conditioned by the variability of the inherited KIR haplotypes (25). NKG2A becomes detectable during NK cell differentiation at earlier stages than KIR molecules and is constitutively expressed by an NK cell subset (1,25,29) As compared to NKG2C+ cells, the NKG2A+ subset tends to include lower proportions of KIR+ and ILT2+ (LIR-1, CD85j) cells and higher expression levels of NCR (NKp30 and NKp46) (22,23). The inducible expression pattern of NKG2A in NKG2C+ cells described in this study reveals that the NKR repertoire may be transiently altered during NK cell activation depending on the influence of IL-12. From a practical standpoint, this should be carefully taken into account when performing functional studies on CD94/NKG2C+ cells.

Phenotypic studies of PBMC indicated that a subset of NKG2C+ NKG2A+ NK cells are KIR- ILT2-, thus supporting that NKG2A expression may play a central inhibitory role in this NKG2C+ population. On the other hand, the

function of the inducible NKG2A molecule may be overlapping with that of other inhibitory receptors for class I molecules (i.e. KIRs and ILT2) expressed by NKG2C<sup>+</sup> cells. In this regard, our previous functional studies (40,47) indicated that CD94/NKG2A and ILT2 may contribute in a complementary way to repress NK cell function, whereas in clones co-expressing CD94/NKG2A and KIR the inhibitory function of the latter may be dominant. Thus, it can be predicted that the inducible expression of NKG2A may have an additive effect with other receptors in some NKG2C<sup>+</sup> NK cells, contributing to establish the inhibitory threshold, while being redundant in cells that are effectively repressed by appropriate inhibitory KIR-HLA class I interactions.

NK cells have been reported to interact with dendritic cells, regulating their survival and function (50-52). The KIR- NKG2A<sup>+</sup> cell subset was shown to kill autologous immature DC, whereas up-regulation of HLA-E expression protected mature DC by engaging the CD94/NKG2A inhibitory receptor (53). It is of note that IL-12 has been reported to be pivotal in regulating the NK-DC crosstalk (54). Our results support that IL-12 secretion by cells of the myelomonocytic lineage may play a dual role activating NK cell functions and concomitantly inducing the expression of the NKG2A inhibitory receptor on NKG2C<sup>+</sup> cells, thus contributing to prevent their potential autoreactivity. This regulatory mechanism may be particularly relevant in the context of the NK-DC crosstalk established during the innate immune response to some infections (55). In this regard, increased numbers of circulating CD94/NKG2C<sup>+</sup> NK cells were previously associated to a positive serology for HCMV (22,23). Moreover, an expansion of NKG2C<sup>+</sup> populations was observed upon co-culture with HCMV-infected fibroblasts (31), suggesting that this subset may be involved in the defence against the viral infection. In the present study we provide data supporting that endogenous IL-12 secretion in HCMV-infected moDC cultures induced NKG2A expression in NKG2C<sup>+</sup> cells and thus might modulate their response against infected cells. Paradoxically, this effect might provide an advantage for the virus, that down-regulates classical HLA class I expression

while maintaining surface HLA-E (56); in that scenario, the induced CD94/NKG2A expression could reinforce the immune evasion mechanism.

IL-12 was detectable by ELISA in supernatants of HCMV-infected moDC cultures and, moreover, expression of NKG2A by NKG2C+ NK clones was blocked by an anti IL-12 mAb. Whether IL-12 is secreted by the HCMV-infected moDC or by the fraction of cells that remain uninfected is being studied. The discrepancy with a previous report showing that IL-12 was undetectable by intracellular staining in HCMV-infected DC cultures (57) may be explained by technical differences (i.e. cytokine detection assays).

It has been recently reported that decidual and peripheral blood CD56bright cells may co-express NKG2A and NKG2C (37). In the present study, we observed that minor subsets of peripheral blood NK cells, including CD56 bright and CD56dim, as well as T lymphocytes, mainly CD56+, displayed both NKG2A and NKG2C at the cell surface; the proportions of these subsets widely varied in different donors. Whether they correspond to NK cells which have transiently acquired NKG2A in vivo and/or they represent a distinct subset stably expressing both receptors is uncertain.

**Acknowledgements:** We are grateful to Oscar Fornas for excellent advice in flow cytometry analysis, Gemma Heredia for technical assistance and Aura Muntasell for critical reading of the manuscript and helpful discussion.

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## Chapter 5

(2) Source of support: This work was supported by grants SAF2007-61814 to ML-B (Ministerio de Ciencia e Innovación), MRTN-CT-2005-019248 (Marie Curie Training Network, EU), Red Heracles (Instituto de Salud Carlos III) and SAF2005-05633 to A.A. (Ministerio de Ciencia y Tecnología). AS-B is supported by a fellowship from DURSI (Generalitat de Catalunya), NR is supported by a fellowship from Instituto de Salud Carlos III and GM is supported by a Marie Curie Training Network (EU).

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(4) Abbreviations: HCMV, Human Cytomegalovirus. NKR, Natural Killer cell Receptors

## Chapter 6

### Expansion of NKG2A+ NK cells upon stimulation of PBMC with autologous Epstein-Barr virus infected B cells

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*Preliminary results*



## INTRODUCTION

Recent work from our laboratory has suggested a role for NKG2C+ NK cells in the immune response to HCMV infection. Higher proportions of NKG2C+ NK cells were found in seropositive individuals, and NKG2C+ NK cells were shown to expand upon HCMV-infection of fibroblasts *in vitro* <sup>168,245</sup>. On the other hand, our studies showed that NKG2A is induced on NKG2C+ NK cells co-cultured with HCMV-infected dendritic cells, and that NKG2A is able to regulate NKG2C+ NK cell function upon binding of HLA-E, supporting that it may act as a relevant regulatory feedback mechanism <sup>258</sup>. These results suggest that both CD94/NKG2A and CD94/NKG2C receptors may influence the response of NK cells to Human Cytomegalovirus infection *in vivo*. Yet, little is known about the role of NKG2A and NKG2C receptors in response to other herpes viral infections.

Epstein-Barr virus belongs to the family of gamma-herpes viruses. As other herpes viruses it establishes a life-long latent infection, and the seroprevalence in the adult population reaches 90%. The virus is commonly transmitted orally through saliva, it infects and replicates in epithelial cells, and spreads to mucosal B cells. In latently infected individuals, circulating B cells are the main viral reservoir and in contrast to HCMV, EBV integrates in the host genome of infected cells. It has been implicated in the development of B, NK and T cell lymphomas as well as of epithelial tumors and nasopharyngeal carcinoma. Furthermore, EBV infection has been associated to the development of some autoimmune diseases (i.e. multiple sclerosis and rheumatoid arthritis)<sup>259</sup>. The virus elicits specific CD4+ and CD8+ T cell responses, and T cell clones specific for both latent and lytic cycle antigens can be detected in peripheral blood of seropositive individuals. As observed for HCMV, the virus has developed several strategies to down-modulate class I and II molecules, and avoid T cell recognition <sup>260</sup>. However, as compared to HCMV, much less is known about the role of NK cells in response to Epstein-Barr virus, and no evasion strategies have

## Chapter 6

been identified so far. Studies on infectious mononucleosis patients reported high frequencies of CD56bright cells in peripheral blood <sup>261</sup>, although the mechanisms leading to such increase are unclear. Interestingly, CD56bright cells were also shown to limit B cell transformation in vitro through IFN $\gamma$  secretion <sup>30</sup>. On the other hand, lymphoblastoid cell lines (LCL) generated in vitro by infecting B cells from peripheral blood are considered relatively resistant to NK cell lysis. Lysis was observed upon masking of MHC-I by specific mAb, and in this setting, NKp46 was shown to participate in triggering cytotoxicity <sup>150</sup>. Only decidual NK cells (CD56bright) have been described to mediate variable LCL killing after prolonged exposure to the virus, although the mechanism for such effect was not addressed <sup>262</sup>. Studies on the lytic cycle of EBV have reported an increased expression of ULBP1 and Nectin-2 on the Burkitt lymphoma-derived cell line AKATA during active viral replication, leading to increased NK cell mediated killing by respective engagement of NKG2D and DNAM <sup>120</sup>.

So far, there are no reports addressing the role of CD94/NKG2 receptors in the response to EBV infection. In order to approach this question, we conducted a pilot study in which the response of NK cells against autologous LCL was studied in vitro.

## MATERIALS AND METHODS

### Subjects

Heparinized blood samples were obtained from 6 healthy adult individuals. Written informed consent was obtained from every donor, and the study protocol was approved by the Comitè de Ètica e Investigacion Clínica-Institut Municipal d'Assistència Sanitària (CEIC-IMAS).

## Cell cultures

Cell cultures were conducted in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FCS, penicillin (100 µg/ml) and streptomycin (10 µg/ml), referred to as complete medium. EBV infectious particles were obtained by culturing the EBV+ B95.2 marmoset cell line in complete medium for one week and recovering, filtering and freezing the supernatant (EBV stock). LCLs were generated from 6 different donors by incubating  $4 \times 10^6$  PBMC with 0.5 ml of EBV stock overnight in the presence of 1mg/ml Cyclosporin A (CsA), washing and culturing in 24 well plates in complete medium with 1mg/ml of CsA. LCLs growth was detected after 3-4 weeks in culture and samples were tested for CD19 expression by flow cytometry.

PBMC were isolated by centrifugation on Ficoll-Hypaque (Axis-Shield PoC AS, Oslo, Norway) and  $2 \times 10^6$  cells were cultured with autologous LCL cells (1:1) ratio in the presence of IL-2 (100U/ml) in 24-well plates. Cells were cultured for 10 days in the presence of IL-2 (100U/ml), were fed and split when needed, and their phenotype was subsequently analysed by FACS. In some experiments cells were labelled with CFSE before setting the culture. As described, PBMC were resuspended in RPMI-1640 ( $10^7$ /ml) and incubated for 10 min at 37°C with the intracellular fluorescent dye 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) (2 µM). After two washes, CFSE-labeled PBL were cultured with autologous LCL as described above, and proliferation was analysed by FACS.

## Antibodies

Monoclonal antibodies (mAbs) specific for NKG2A, KIR, ILT2, NCR and NKG2D have been previously detailed <sup>168</sup>. 9E10 (IgG1) anti-myc hybridoma has been described <sup>258</sup>, and the 20C2 (IgG1) anti-IL-12 hybridoma was obtained from ATCC (American Type Culture Collection). Anti-NKG2C (MAb1381, IgG2b) and anti-NKG2C-PE MAb were from R&D Systems (Minneapolis, MN). Anti-CD3-PerCP and anti-CD56-APC were from BD Biosciences Pharmingen (San

## Chapter 6

Jose, CA). Indirect immunofluorescence analysis was carried out with a fluorescein isothiocyanate (PE)-tagged F(ab')<sub>2</sub> rabbit anti-mouse Ig antibody (Dakopatts, Glostrup, Denmark).

For immunofluorescence staining, cells were pretreated with human aggregated Ig (10 µg/ml) to block FcR, and subsequently labeled with the different mAb and analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA), as described <sup>255</sup>.

### **Functional assays**

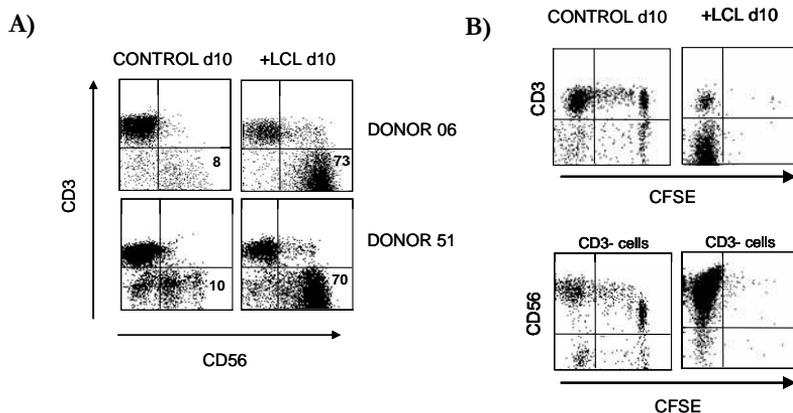
For degranulation assays, PBMC cultured with LCL for 10 days were harvested, washed, and incubated with autologous LCL (ratio 2:1) in 96 well plates with monensin (5µg/ml) and FITC-labeled anti-CD107 mAb (3ul/well). Cells were incubated for 5 hours and then stained with CD3PerCP and CD56PE for FACS analyses.

## RESULTS AND DISCUSSION

### **Increased proportions of NKG2A+ NK cells in response to EBV-infected autologous cell lines**

To analyse the possible role of CD94/NKG2A and CD94/NKG2C receptors in the response to EBV infection, we first studied the overall response of the NK cell compartment to EBV-infected cells *in vitro*. To this end, EBV-infected lymphoblastoid cell lines (LCL) from 4 different adult healthy individuals were cocultured with autologous PBMC in the presence of IL-2, and proportions and phenotype of NK cells were analysed after 10 days.

Under these conditions, the proportions of NK cells increased considerably, becoming the major lymphocyte subset detectable by the end of the culture (Figure 1A). To confirm that increased NK cell numbers were the result of cell division and not of increased selective mortality of other lymphocytes, experiments with CFSE labeled cells were performed. In cocultures with LCL, a higher cell division rate of the NK cells was observed as compared to the control, thus confirming that this lymphocyte subset vigorously proliferates upon stimulation with EBV+ LCLs (figure 1B). This response is reminiscent of the well-known ability of NK cells to expand when PBMC are cocultured with the Burkitt lymphoma-derived EBV+ RPMI-8866 cell line, a method conventionally used to expand NK cells *in vitro* (data not shown).

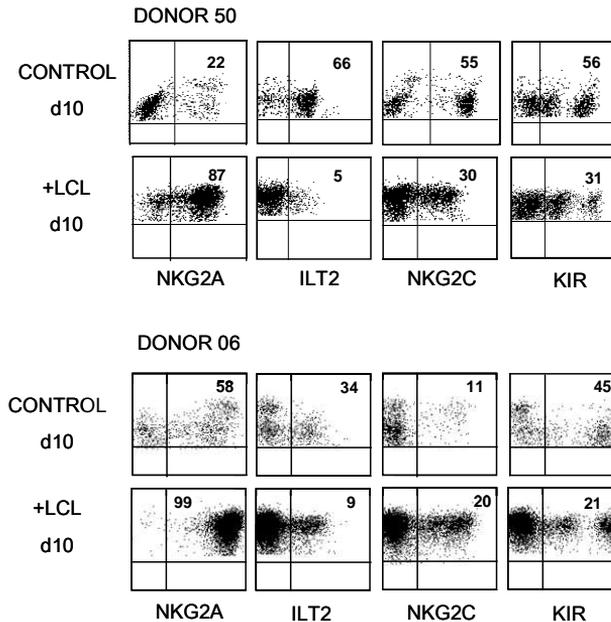


**Figure 1: NK cells proliferate in response to EBV+lymphoblastoid cell lines.**

A) PBMCs from four different donors were cocultured with or without autologous LCL (1:1) in the presence of IL-2 for 10 days and the proportion of NK cells was assessed. Representative examples of two different donors are shown. B) PBMC were stained with CFSE prior to culture with autologous LCL. After 10 days cells were stained with CD3 and CD56 and deletion of CFSE staining was assessed.

The phenotype of NK cells was also studied at day 10 of culture. To this end, cells were stained with mAbs against NKG2D, ILT2, KIR, NKG2A, NKG2C, NKp30, NKp46, 2B4 and DNAM-1. Remarkably, as compared to control samples, most NK cells from cocultures with LCLs were NKG2A+, and higher proportions of NKG2C+ cells were observed in 3 out of 4 donors tested. Moreover, the proportions of ILT2+ and KIR+ NK cells were decreased in every donor (n=4). Expression of NKG2D, NKp30, NKp46, 2B4 and DNAM-1 remained rather stable over the culture (data not shown). These results indicate that besides a variable proliferation of NKG2C+ NK cells, there is a shift in the usage of inhibitory receptors by NK cells stimulated with LCL, that preferentially express NKG2A while ILT2+ and KIR+ cells tend to decrease (Figure 1). The difficulty to discriminate between activating and inhibitory KIR with the panel of available mAb did not allow a precise definition of the KIRs expressed after the

culture. Studies are in progress to overcome this limitation following a recently described approach <sup>263</sup>

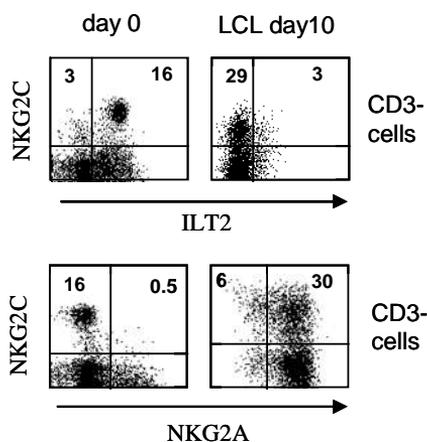


**Figure 2: NKR expression on NK cells stimulated with autologous LCL.** PBMC from adult blood donors (n=4) were cultured with autologous LCL in the presence of IL-2 and the phenotype of NK cells was studied after 10 days. Data from two representative donors is shown.

### Increased proportions of NKG2A+NKG2C+ NK cells

The % of NKG2A+ and NKG2C+ NK cells detected at day 10 of culture indirectly indicated the presence of double positive cells. Peripheral blood NKG2C+ NK cells have been described to contain high proportions of ILT2, and only low proportions of NKG2C+ NK cells have been detected to coexpress NKG2A <sup>258</sup>. Thus, the phenotype of NKG2C+ NK cells was studied at day 10 of culture. In contrast to the control, expanded NKG2C+ NK cells were shown to be mainly ILT2- but coexpressed NKG2A (figure 3). These results indicate that, as described during HCMV infection of dendritic cells in

in vitro <sup>258</sup>, an NKG2A+NKG2C+ double positive subpopulation of NK cells emerges upon coculture of PBMC with EBV-infected LCL. Moreover, in this case, the double positive population includes low proportions of ILT2+ cells. The increased proportions of NKG2A+NKG2C+ cells could be the result of an induced NKG2A expression on the cell surface of NKG2C+ cells or a consequence of the proliferation of the preexisting NKG2A+NKG2C+ NK cell subset along the culture.

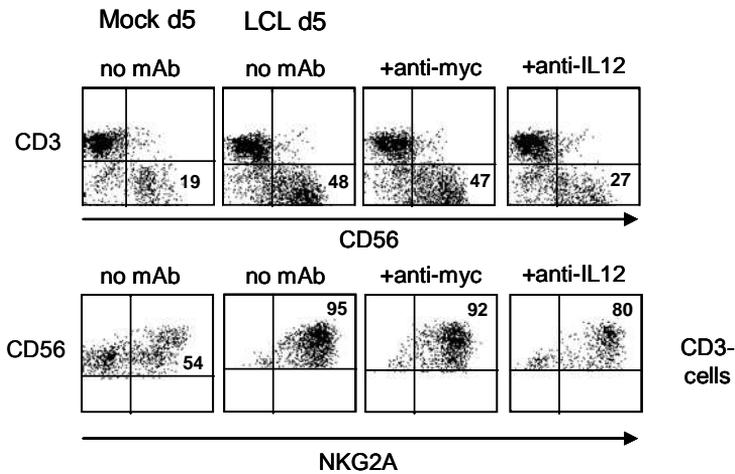


**Figure 3: Emergence of an ILT2-NKG2A+NKG2C+ cell subset after coculture of PBMC with autologous LCL** NKG2A and ILT2 expression was studied on NKG2C+NK cells from two donors at day 0 and day 10 of culture of PBMC with autologous LCL. A representative experiment is shown.

### Role of IL-12 in the expansion of NK cells

LCLs have been described to spontaneously secrete IL-12 <sup>264</sup>. This cytokine is an important factor involved in the activation of NK cells, and we described that it induces NKG2A expression on NKG2C+NK cells <sup>258</sup>. IL-12 could influence NK cell proliferation and NKG2A+ NK cell numbers in cocultures with EBV+ LCL. To indirectly address this question, the effect of an anti-IL12 mAb was tested. The proportions of NK cells at day 10 were lower in cultures where IL-12 was neutralized, suggesting that the cytokine contributes to NK cell proliferation.

However, the % of NKG2A+ cells were only slightly diminished, indicating that other factors may contribute to promote the preferential expansion of the NKG2A+ subset.

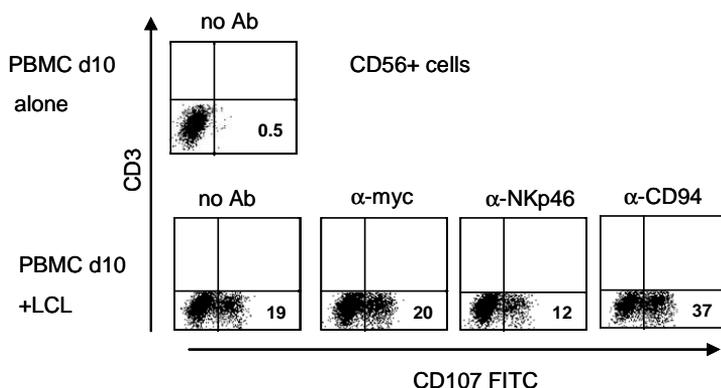


**Figure 4: IL-12 contributes to NK cell proliferation upon culture with LCL.** PBMC were cocultured with autologous LCL in the presence of anti-IL12mAb and the proportion of NKG2A+ NK cells was assessed after 5 days.

#### EBV-stimulated NK cells can kill LCLs in vitro

LCLs have been described to be resistant to NK cell lysis<sup>150</sup>. To test whether the NK cell receptor shift could influence NK cell killing of LCLs, CD107a expression was measured upon coculture of LCL with PBMCs from day 10 of culture. As detected by CD107 staining, a subset of NK cells was shown to degranulate in response to LCL, indicating that these cells are capable of mediating LCL killing. In this setting, blockade of the Nkp46 receptor partially interfered with cytotoxicity, consistent with data previously reported in a system where MHC-I was blocked to allow killing. To test whether NKG2A expression could repress LCL lysis, degranulation was measured in the presence of an anti-CD94 (Fab')<sub>2</sub> mAb. The results indicated that cytotoxicity was increased upon

CD94 masking, suggesting that CD94/NKG2A expression accounts for partial inhibition of LCL killing.

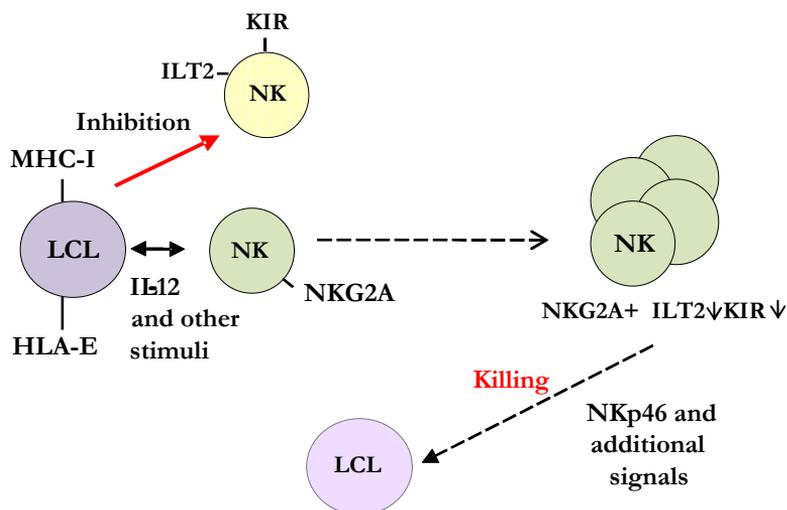


**Figure 5: NK cells stimulated with LCL lyse autologous LCL targets.** After 10 days of culture with LCL, PBMC from two different donors were recovered and assayed for CD107 degranulation against autologous LCL in the presence or absence of the indicated mAb. Cells were stained for CD56 and CD3 to assess CD107 expression on NK cells. A representative experiment is shown.

Altogether these results support that NK cells actively proliferate upon encounter with autologous LCLs *in vitro*, similarly to the effect observed upon culture with EBV+ transformed B cell lines (i.e. RPMI-8866, 721.221). Yet, in contrast to cultures with RPMI-8866 cells (data not shown), a shift in the NK cell receptor usage upon culture with autologous LCL is observed, as recovered cells show higher proportions of NKG2A+ NK cells and reduced % of ILT2+ and KIR+ cells as compared to controls. Importantly, our results indicate that a fraction of NK cells recovered from day 10 of culture was able to degranulate upon interaction with autologous LCL, whereas in previous reports, killing of LCL by NK cells was only observed after blocking of MHC-I. Addition of anti-NKp46 mAb partially inhibited degranulation, suggesting that ligands for the

receptor are expressed on LCL as previously described <sup>150</sup>. The possibility that other receptors may be involved in the response is not excluded.

Though preliminary, these results set up a suitable experimental ground to explore and get a more precise insight on the mechanisms and receptors involved in the regulation of the NK cell response to EBV+LCL. We propose a model in which preferential expansion of ILT2-NKG2A+NK cells would represent an advantage in the response to the pathogen (Figure 6). The expansion of ILT2+KIR+ cells may be hampered due to engagement of the inhibitory receptors by MHC-I on the LCL surface. By contrast, the interaction of CD94/NKG2A with HLA-E does not appear to inhibit proliferation of this subset, though it partially controls cytotoxicity



**Figure 6: Hypothesis on the mechanism of response of NK cells to EBV+ LCL**

Upon contact with LCL, NKG2A+ NK cells proliferate while ILT2+ KIR+ NK cells might be inhibited due to MHC-I mediated inhibition. NK cells including low proportions of ILT2+ and KIR+ cells are able to kill autologous LCL through engagement of NKp46 and the help of additional signals.

## Chapter 6

Our future work will try to elucidate which receptors and cytokines are involved in the response of NK cells to EBV+ LCL, and whether CD94/NKG2C may play an active role in the defense against the pathogen.

**PART IV**

**DISCUSSION AND CONCLUSIONS**



Chapter 7  
Discussion

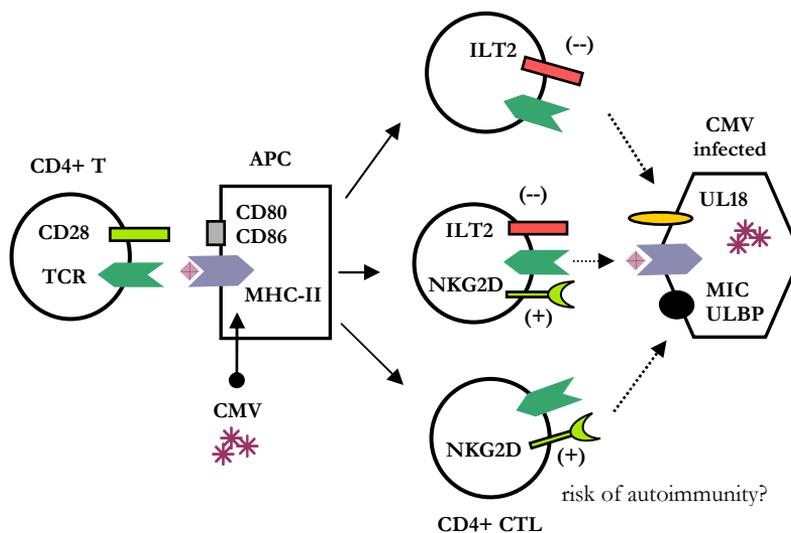
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The results presented in the first part of this work indicate that NK cell receptors can be expressed by CD4+T cells specific for Human Cytomegalovirus. Expression of NKR on peripheral blood CD8+T cells has been well documented<sup>163</sup>. However, expression of NKR on peripheral blood CD4+T cells from healthy subjects is usually very low or undetectable, and reports on NKR+CD4+T cells have been restricted to some pathological conditions such as RA, Crohn's disease and cancer patients<sup>171-173</sup>. We found increased proportions of NKG2D+, ILT2+ and KIR+ CD4+T cells upon HCMV stimulation of PBMC from healthy seropositive individuals. NKG2D was detected on CD28- or CD28low CD4+T cells in combination with intracellular perforin and granzyme. Furthermore, we proved that NKG2D-delivered signals complemented suboptimal TCR stimulation of CD4+T cells, activating proliferation and cytokine secretion. Analysis of Vb13.1+CD4+T-Large Granular Lymphocytosis patients allowed us to study NKR expression on HCMV-specific CD4+T cell clones *in vivo*, and results obtained resembled those observed *in vitro*. We found increased proportions of NKR+CD4+T cells in peripheral blood of 8 out of 9 CD4+T-LGL patients studied, and expression of NKR on Vb13.1+ cells indirectly supported that these molecules may be expressed by CD4+T cells specific for the gB HCMV antigen.

Previous studies on CD8+T cells showed that NKG2D costimulates the TCR mediated cytotoxic response to HCMV-infected fibroblasts<sup>153</sup>. The loss of CD28 and acquisition of NKG2D observed on HCMV-specific CD4+T cells may favour the response of this subset to HCMV-infected targets expressing NKG2D-ligands in peripheral tissues. NKG2D ligands have been shown to be expressed upon HCMV-infection of fibroblasts *in vitro*<sup>67</sup>, and the virus has developed several mechanisms to subvert NKG2D-ligand expression, underlining the importance of NKG2D in the response to the infection<sup>185</sup>. Our results suggest that NKG2D may participate in the response of CD4+T cells to MHC-II+ HCMV-infected targets. It is of note that myelomonocytic cells have been described to harbour the virus in latently infected individuals<sup>265</sup>, and thus

NKG2D+CD4+T cells may have a role in the recognition of MHC-II+ monocytes and dendritic cells undergoing viral reactivation. Expression of NKG2D ligands on HCMV-infected myeloid cells is currently being studied in our laboratory (G.Magri and N.Romo). On the other hand, IFNs have been shown to induce MHC-II expression on a number of cell types (i.e. fibroblasts, endothelial and epithelial cells)<sup>266</sup>, and it is thus conceivable that NKG2D may costimulate the response of CD4+T cells to HCMV-infected cells that transiently acquire MHC-II expression under the influence of antiviral cytokines (Figure 7).



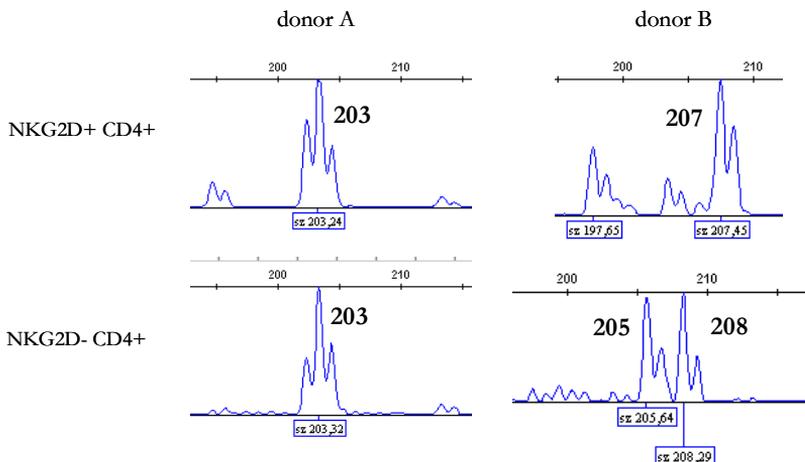
**Figure 7. Hypothesis on the generation and function of NKR+CD4+T cells**  
 HCMV-specific CD28+CD4+T cell clones expand upon antigenic stimulation by professional APCs. CD4+T memory cells lose CD28 expression, but may acquire different combination of NK cell receptors. NKR expression on CD4+T cells may influence their response to HCMV-infected targets expressing NKR ligands (i.e UL18 and MIC/ULBP). On the other hand, CD4+T cells expressing NKG2D in the absence of other inhibitory receptors may give rise to a subset of cells with an increased autoimmune potential. *Sáez-Borderías et al 2006*

In this regard, experiments were done to assess the role of NKG2D in the response of CD4+T cells to HCMV-infected cells. CD4+T cells expanded in vitro with HCMV secreted IFN $\gamma$  in response to IFN $\gamma$ -treated MHC-II+ autologous fibroblasts (data not shown). This response could be blocked by an anti-CD3 or anti-CD4 mAb supporting specific TCR mediated recognition of the infected cells (data not shown). Yet, evidence for a contribution of the NKG2D receptor to the response could not be obtained, since addition of a blocking anti-NKG2D mAb had no detectable effect. It is conceivable that the costimulatory function of NKG2D may operate only transiently under suboptimal TCR stimulation during early stages of viral replication, and preceding viral downregulation of NKG2D-L. On the other hand, attempts to generate and expand NKG2D+CD4+T cell clones were unsuccessful, probably because this subset has already undergone extensive proliferation. The difficulty to work with autologous fibroblasts and to generate NKG2D+CD4+T cell clones did not allow more precise experiments to evaluate of the role of NKG2D in the response to MHC-II+ targets. Recently the conditions to infect moDC have been set up in our laboratory (G. Magri, unpublished) allowing to explore this issue.

Our results showed that NKG2D+CD4+ T cells express intracellular perforin and granzyme, and redirected killing assays confirmed that HCMV-stimulated CD4+T cells were able to kill the p815 cell line when engaged by an anti-CD3 mAb (data not shown), proving that these cells were cytotoxic and suggesting they may be able to lyse HCMV-infected targets, in addition to their ability to secrete proinflammatory and antiviral cytokines.

The antigen specificity of NKG2D+CD4+T cells expanding upon HCMV stimulation in vitro remains to be studied. TCR rearrangement analysis showed that CD4+T cells expanded upon HCMV stimulation were oligoclonal (Figure 8). In some donor, the analysis of sorted NKG2D+ and NKG2D- CD4+T cells subsets revealed that they shared the same TCR rearrangement pattern, indicating

that they were essentially derived from the same clone (Fig 8, donor A). By contrast, in one case, NKG2D<sup>+</sup> and NKG2D<sup>-</sup> CD4<sup>+</sup> subsets (Fig 8, donor B) displayed a different TCR rearrangement pattern.



**Figure 8: T cell clonality of the NKG2D<sup>+</sup> and NKG2D<sup>-</sup> CD4<sup>+</sup>T cell subsets:** PBMC stimulated with HCMV for 10 days were stained with mAb specific for CD4 and NKG2D and subsequently NKG2D<sup>+</sup> and NKG2D<sup>-</sup> subsets were sorted. T-cell clonality was evaluated as previously described by PCR amplification with specific oligonucleotides for the TCR $\gamma$ -chain gene <sup>267</sup>. Two different donors are shown representative of the different TCR rearrangement patterns observed

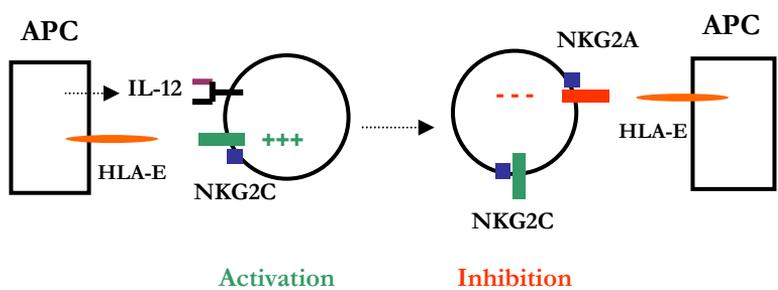
Some reports point to IE1, gB and pp65 as the immunodominant HCMV antigens <sup>194,195</sup>. However, a mutant virus lacking pp65 was able to elicit comparable CD4<sup>+</sup>T cell proliferation and expression of NKR in two donors tested (data not shown), suggesting that at least in some individuals NKG2D<sup>+</sup>CD4<sup>+</sup>T cells expanded in vitro may have a different specificity. In this regard, study of the LGL patients bearing Vb13.1<sup>+</sup> CD4<sup>+</sup>T cell expansions indirectly confirmed that gB-specific CD4<sup>+</sup>T cell clones may express NKR in vivo.

The expression of NKR observed on T lymphocytes may influence their response to HCMV but also eventually to self-antigens (Figure 7). Expression of NKG2D on HCMV-specific CD4+T cells in the absence of other inhibitory receptors, as detected in one of the donors tested, might give rise to a subset of NKG2D+CD4+T cells with autoimmune potential, since our results proved that NKG2D engagement lowers down the threshold for activation on these cells. Several studies have reported increased proportions of CD28-CD4+T cells in peripheral blood of RA patients, and suggested that this population could be implicated in the development of the disease <sup>268-271</sup>. More recently, Groh et al. also described increased proportions of NKG2D+CD4+T cells in peripheral blood of RA patients, and showed that these cells lacked CD28 expression <sup>171</sup>. Accordingly, our results suggested that HCMV should be considered as a candidate for driving the increase of NKG2D+ CD28- CD4+T cells observed in RA patients, consistent with studies reporting an association between HCMV serology and the expansion of CD4+CD28- cells <sup>272</sup>. A recent report confirmed that CD4+CD28- T cells found peripheral blood and synovial fluid of RA patients respond to HCMV antigens <sup>273</sup>.

Increased risk of autoimmunity might result from expression of NKG2D in the absence of other inhibitory receptors, as detected in one donor. Expression of the individual NKR may be differentially regulated, since we observed a variable expression pattern of NKR on CD4+T cells from HCMV-stimulated cultures depending on the donor, as well as on HCMV-specific CD4+T cells from LGL patients. Mechanisms regulating individual NKR acquisition by T cells are unclear and further studies are required to more precisely understand how and when NKR are expressed. Our findings are in line with previous reports describing NKR expression on memory T cells (i.e. ILT2 and KIR). Prolonged antigenic stimulation alone may promote NKR expression on T cells, and in this regard NKG2A expression was reported to be induced on CD4+T cells after CD3 stimulation <sup>183</sup>. On the other hand, cytokines secreted in the context of infection might also influence expression of NK cell receptors on T cells. IL-15

has been shown to induce NKG2D expression on CD4+T cells <sup>171</sup>, and the inhibitory C-type lectin-like receptor NKG2A was reported to be induced on CD8+T cells by TGFβ, IL-15 and IL-12 together with TCR stimulation <sup>164 165 166</sup>.

The expression of some NK cell receptors on NK cells has also been shown to be modulated by the effect of soluble factors that may be secreted during infections. NKG2D is constitutively expressed by virtually all NK cells, but levels of NKG2D expression were shown to be increased upon IL-12 treatment <sup>114</sup>, and downmodulated by the action of IL-21 or TGFβ <sup>118 274</sup>. In this regard, our results indicate that secretion of IL-12 in the context of infection may also influence the expression of the NKG2A receptor on NK cells. We detected variable proportions of NK and T cells coexpressing NKG2A and NKG2C receptors *in vivo*, and demonstrated that NKG2A can be induced on NKG2C+NK cells upon *in vitro* stimulation with allogeneic PBMC, rIL-12 or HCMV-infected DC. Our results suggest that acquisition of NKG2A by NKG2C+NK cells may constitute a regulatory feedback mechanism, since NKG2A inhibits cytotoxic activity of NKG2C+clones against 221 cells expressing HLA-E (Figure 9).



**Figure 9: Hypothesis on the induction and function of CD94/NKG2A on CD94/NKG2C+ NK cells:** Secretion of IL-12 by APC may induce NKG2A expression on NKG2C+NK cells, constituting a regulatory feedback mechanism to control NK cell activation and terminate the response.

On the other hand, in the context of HCMV infection, NKG2A induction could have a counterproductive effect. HCMV downmodulates MHC-I expression to avoid T cell recognition. This renders infected cells susceptible to NK cell attack, but the leader peptide of the viral UL40 protein stabilizes expression of HLA-E on the surface, engaging NKG2A and inhibiting the response of NKG2A<sup>+</sup> cells as a viral escape mechanism <sup>246,247</sup>. Under the influence of IL-12, UL40-mediated stabilization of HLA-E may also inhibit the response of NKG2C<sup>+</sup> cells that have transiently acquired NKG2A expression. Previous studies support that NKG2C<sup>+</sup> cells may be involved in the response to HCMV-infected fibroblasts <sup>245</sup>. It would be interesting to study whether the response of NKG2C<sup>+</sup> cells to HCMV-infected dendritic cells is regulated by the inducible expression of NKG2A.

The inducible expression of NKG2A on NKG2C<sup>+</sup> cells may also be relevant in the context of the response to other pathogens. IL-12 has been described to be secreted by phagocytic cells and B lymphocytes <sup>275</sup>. In this regard, we detected the emergence of an NKG2A<sup>+</sup>NKG2C<sup>+</sup> NK cell subpopulation upon coculture of PBMC with EBV-infected LCL *in vitro*, that have been described to secrete high levels of IL-12 <sup>264</sup>. The presence of an anti-IL12 mAb reduced the expansion of NK cells but had a minor effect on the dominant proportions of NKG2A<sup>+</sup> cells, supporting the involvement of other stimuli. Interestingly, NKG2A expression was able to inhibit degranulation of a subset of NK cells against autologous LCL. Studies are in progress to address the role of NKG2C in the response to EBV infection and to assess whether NKG2A inhibits the response of NKG2C<sup>+</sup> cells to autologous LCLs.

Altogether these results support that expression of NK cell receptors can be modified during the course of infections, either by persistent antigenic stimulation and/or under the influence of cytokines. Acquisition of NKR provides additional mechanisms to control immune cell activation. In the case of CD4<sup>+</sup>T cells, NKG2D engagement enhances TCR delivered signals, whereas

## Chapter 7

NKG2A induction on NKG2C+NK cells provides a potential regulatory negative feedback mechanism. Conversely, as mentioned above, NKR expression may have also negative effects, favoring self-antigen recognition or viral immune subversion mechanisms.

Chapter 8  
Conclusions

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1. Oligoclonal HCMV-specific CD4<sup>+</sup> T cell populations expanded in vitro from PBMC of seropositive individuals may express different NK cell receptors including NKG2D, ILT2, KIR and CD94.
2. The distribution of NKG2D and inhibitory NKR (i.e. ILT2, KIR) in HCMV-specific CD4<sup>+</sup> T lymphocytes is not coordinated, suggesting that their expression is differentially regulated.
3. NKG2D is expressed by HCMV-specific CD4<sup>+</sup> CTL and functions as a prototypic co-stimulatory receptor, enhancing proliferation and cytokine production in response to suboptimal TCR-dependent stimulation.
4. PBMC samples from CD4<sup>+</sup> T Large Granular Lymphocytosis (LGL) patients display increased proportions of NKR<sup>+</sup> CD4<sup>+</sup> T cells.
5. NKR are detectable in both Vb13.1<sup>+</sup> CD4<sup>+</sup> T-LGL, reported to be specific for the gB HCMV antigen, and Vb13.1<sup>-</sup> CD4<sup>+</sup> T-LGL.
6. Minor subsets of peripheral blood NK and T cells coexpress surface CD94/NKG2A and CD94/NKG2C receptors
7. CD94/NKG2A is transiently induced on CD94/NKG2C<sup>+</sup> clones after stimulation with allogeneic PBMC or rIL-12
8. CD94/NKG2A is inducible on CD94/NKG2C<sup>+</sup> clones cocultured with HCMV-infected dendritic cells, and this effect can be partially prevented by an anti-IL-12 mAb

## Chapter 8

9. CD94/NKG2A is functional in CD94/NKG2C+ clones, inhibiting redirected lysis of the P815 cell line and cytotoxicity against the HLA-E+ 721.221-AEH target cell line.
  
10. Altogether, our results support that the expression of lectin-like NKR may be modified during the immune response to infections, either by persistent antigenic stimulation and/or under the influence of cytokines, contributing to regulate NK and CD4+ T cell functions.





## ANNEX I

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## ANEX II

### Abbreviations

ADCC	Antibody dependent cellular cytotoxicity
CTL	Cytotoxic Lymphocytes
CD	Crohn disease
DAP10	10kDa DNAX adaptor protein
DAP12	12kDa DNAX adaptor protein
DC	Dendritic cells
EBV	Epstein-Barr virus
FcR	Receptor for the constant region of immunoglobulins
GM-CSF	Granulocyte-macrophage colony stimulating factor
HCMV	Human Cytomegalovirus
HIV	Human Immunodeficiency Virus
HLA	Histocompatibility leukocyte antigen
iDC	Immature dendritic cells
ILT	Ig-like transcripts
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KIR	Killer Ig-like receptor
KLR	Killer cell lectin-like receptor
LCL	Lymphoblastoid cell line
LGL	Large Granular Lymphocytosis
mAb	Monoclonal antibody
MCMV	Murine Cytomegalovirus
MIC	MHC class I chain related
moDC	Monocyte-derived dendritic cells
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptors

NK	Natural killer
NKG2	Natural killer group 2
NKR	Natural Killer cell receptors
PBMC	Peripheral blood mononuclear cells
PTK	Protein Tyrosine Kinase
RA	Rheumatoid arthritis
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TNF	Tumor necrosis factor
ULBP	UL-16 binding protein

