Innate response to human cytomegalovirus and the role of infections in the pathogenesis of atherosclerosis

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THESIS ABSTRACT

We comparatively analyzed the natural killer (NK) cell response against HCMV-infected pro-inflammatory (M1) and anti-inflammatory (M2) MΦ derived from autologous monocytes. M1 MΦ were more resistant to infection, secreting TNF-α, IL-6, IL-12 and type I IFN. By contrast, in HCMV-infected M2 MΦ the production of proinflammatory cytokines, type I IFN and IL-10 was limited, and IL-12 undetectable. NK cell degranulation was triggered by interaction with HCMV-infected M1 and M2 MΦ and was partially inhibited by specific anti-NKp46, anti-DNAM-1 and anti-2B4 mAbs, thus supporting a dominant role of these activating receptors. By contrast, only HCMV-infected M1 MΦ efficiently promoted NK cell-mediated IFN-γ secretion, an effect partially related to IL-12 production. These observations reveal differences in the NK cell response triggered by distinct HCMV-infected monocyte-derived cell types, which may be relevant in the pathogenesis of this viral infection.

HCMV infection has been proposed to contribute to the development of atherosclerosis, a chronic inflammatory process in which MΦ play a key role. The contribution of HCMV to vascular disease may depend on features of the immune response not reflected by the detection of specific antibodies. Persistent HCMV infection in healthy blood donors has been associated with changes in the distribution of NK cell receptors (NKR). The putative relationship among HCMV infection, NKR distribution, subclinical atherosclerosis and coronary heart disease was assessed. An association of overt and subclinical atherosclerotic disease with LILRB1+ NK and T cells was observed, likely reflecting a relationship between the immune challenge by infections and cardiovascular disease risk, without attributing a dominant role for HCMV.

RESUM DE LA TESI

Hem analitzat la resposta de la cèl·lula NK als macròfags proinflamatoris (M1) i antiinflamatoris (M2) derivats de monòcits autòlegs infectats pel citomegalovirus humà (HCMV). Els macròfags M1 son més reistents a la infecció i secreten TNF-α, IL-6, IL-12 i IFN de tipus I. Per altra banda, en els macròfags M2 infectats per HCMV la producció de citoquines proinflamatories, IFN de tipus I i IL-10 es limitada i la IL-12 indetectable. La cèl·lula NK degranula al interaccionar amb els macròfags M1 i M2 infectats. Aquesta degranulació s'inhibeix parcialment al bloquejar amb anticossos específics anti-NKp46, anti-DNAM-1 i anti-2B4, això indica que aquests receptors tenen un rol important en el procés. En canvi, només els macròfags M1 infectats amb HCMV promouen de manera eficient la producció d'IFN-γ per part de la cèl·lula NK, degut parcialment a la producció de IL-12. Aquestes observacions posen de manifest diferències en la resposta de la cèl·lula NK a diferents tipus de macròfags infectats per HCMV que pot ser relevant en la patogènesis d'aquesta infecció viral.

S'ha proposat que la infecció per HCMV contribueix al desenvolupament de l'aterosclerosis, un procés inflamatori crònic en el que els macròfags tenen un paper clau. La contribució del HCMV a la malaltia cardiovascular pot dependre de la resposta immune. La infecció per HCMV en donants de sang sans s'ha associat a canvis en la distribució dels receptors de les cèl·lules NK. S'ha evaluat la possible relació entre la infecció per HCMV, la distribució dels receptors de les cèl·lules NK i l'infart agut de miocardi. S'ha observat una associació de l'infart agut de miocardi i l'aterosclerosi subclínica tant amb les cèl·lules NK LILRB1+ com amb les cèl·lules T LILRB1+. Això possiblement reflexa la relació entre la pressió que les infeccions exerceixen en el sistema immunitari i el risc cardiovascular sense atribuir un paper principal al HCMV.

PREFACE

HCMV is a β herpesvirus that establishes an asymptomatic life-long latent infection in immunocompetent hosts, but may cause severe congenital disease and important disorders in immunocompromised patients. Hematopoietic cells of the myeloid lineage constitute a key reservoir for the virus, which undergoes occasional reactivation in healthy subjects, allowing its replication, dissemination to different cell types and transmission to new hosts. Controlling the persistent infection represents a permanent challenge, and a potentially important burden for the immune system, as the virus has reciprocally developed different immune evasion strategies. These events take place recurrently along the individual´s life and, though they are generally subclinical, their effects may be potentially relevant in immunosenescence, as well as in the pathogenesis of some chronic inflammatory disorders, such as atherosclerosis. Understanding the host-pathogen relationship may provide a deeper insight in this regard.

CONTENTS

PART I

INTRODUCTION AND AIMS

Chapter 1

Introduction

1. Natural Killer cell biology

Natural killer (NK) cells are large granular lymphocytes of the innate immune system that comprise around 15% of peripheral blood lymphocytes and are phenotypically defined by their expression of CD56 and lack of expression of CD3. NK cells were originally identified on a functional basis because of their ability to lyse certain tumours in the absence of previous stimulation [1]. Under normal conditions, NK cells are mostly confined to peripheral blood, spleen and bone marrow, but can migrate to inflamed tissues in response to different chemoattractants.

NK cells are involved in the innate immune response against viruses, parasites, bacteria and tumoral cells and also link the innate and adaptive immune responses. Such responses are mediated through two major effector functions, the direct cytolysis of infected or transformed cells and the production of proinflammatory cytokines and chemokines [2].

NK cells display rapid effector functions because they constitutively express transcripts for interferon-γ (IFN-γ) and contain cytolytic granules (granzymes and perforin) [3]. Moreover, NK cell functions are modulated by cytokines secreted by other immune cells such as type-I interferons, interleukin-15 (IL-15), IL-12 or IL-18. An important NK cell function is antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells express CD16 an activating Fc receptor for IgG (FcγR) and can lyse target cells coated with specific IgG [4].

NK cells are regulated by the balance of activating and inhibitory signals transmitted by membrane receptors that recognize ligands on the cell surface of potential target cells. An NK cell can simultaneously express different combinations of activating and inhibitory receptors, thus having complementary pathways to control NK cell activation (Table 1).

Table 1. Activating and inhibitory NK cell receptors

Activating receptors on NK cells		Inhibitory receptors on NK cells	
Receptor	**Ligand**	**Receptor**	**Ligand**
CD94/NKG2C	HLA-E	LILRB1	HLA class I
NKG2D	ULBP, MICA/B	CD94/NKG2A	HLA-E
NKp30	BAT3, pp65	KIR2DL/3DL	HLA class I
NKp44	Hemaglutinins	CD66a (CEACAM1)	CEACAM1, CEACAM6, CEA
NKp46	Hemaglutinins	LAIR	collagens
NKp80	AICL	IRP60	?
DNAM-1	Nectin-2, PVR		
CD16	IgG		
2B4	CD48		
KIR2DS/3DS	HLA class I		
KIR2DL4	HLA-G?		
CD69	?		

Initially, NK cells were described as non-MHC restricted in their recognition process because of their ability to kill target cells that lacked MHC class I or expressed allogenic MHC class I molecules. The "missing self hypothesis" was formulated by K.Kärre and colleagues in 1984 and stated that NK cells detect information that is missing in the target cell and present in the host [5]. NK cells express inhibitory receptors for MHC class I molecules and are activated when they encounter a cell that lacks MHC class I or over expresses ligands for activating receptors. When both activating ligands and MHC class I molecules are expressed the outcome is determined by the balance of signals (Figure 1) [4].

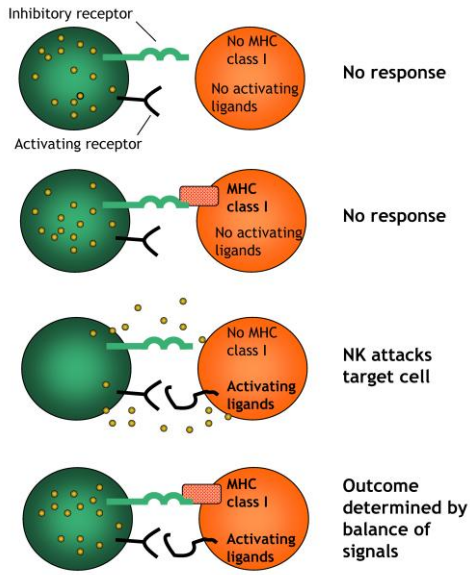

Figure 1. Regulation of NK cell function by activating and inhibitory signals. Adapted from Lanier, L. L., Annu Rev Immunol, 2005 [4].

Activating receptors are linked to different adapter molecules through a charged aminoacid in their transmembrane region. DAP12, FcεRIγ-chain or CD3ζ adaptors contain in their cytoplasmic tail immunoreceptor tyrosine-based activation motifs (ITAM). When the receptor is engaged, ITAMs are phosphorylated by tyrosine kinases leading to an activating signal cascade. DAP10 is a different adapter molecule that contains an YxxM motifs coupling the receptor to PI3K activation [6].

Inhibitory receptors contain immunoreceptor tyrosine-based inhibition motifs (ITIM) in the cytoplasmic tail. Upon receptor engagement, ITIMS are phosphorylated and tyrosine phosphatases containing SH2 domains are recruited dephosphorylating proteins coupled to activating receptors and dampening the signalling cascade [7].

2. Macrophage biology

Macrophages (MΦ) were initially recognized by Elie Metchnikoff as phagocytic cells responsible for pathogen elimination and homeostatic functions [8]. MΦ clear senescent blood cells, remove cellular debris generated during tissue remodelling and eliminate apoptotic cells. Those processes occur independently of other immune cells through scavenger receptors, phosphatidyl serine receptors, thrombospondin receptors, integrins and complement receptors [9]. By contrast, debris generated during necrosis lead to the production of cytokines and pro-inflammatory mediators. MΦ identificate danger signals through Toll-like receptors (TLRs) and other intracellular pattern recognition receptors [10].

MΦ are heterogeneous and display an important degree of plasticity, acquiring different morphology and functional properties depending on the tissue and the immunological environment. The circulating precursors of MΦ are monocytes. It is unclear whether different monocyte subsets give rise to different types of MΦ or if MΦ are randomly derived from the monocyte pool [11]. Moreover, after their differentiation in a given tissue microenvironment, is unclear whether they are still flexible to change their phenotype [12].

Inflammation results in resident MΦ activation and in an increase in the production of cytokines and chemokines. Mirroring the Th1/Th2 paradigm, MΦ can be functionally polarized and termed M1 and M2 [13, 14]. M1 or classically activated MΦ are generated *in vitro* from monocytes stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF) and are characterized by their ability to secrete high levels of pro-inflammatory cytokines (i.e. TNF-α, IL-1β, IL-12, IL-6, IL-23, IL-18) and chemokines (i.e. CCL15, CCL20, CXCL8-11, CXCL13). M1 MΦ have enhanced antimicrobial and tumoricidal capacity, efficiently kill intracellular pathogens by endocytosis, production of superoxide anions, oxygen and nitrogen

radicals and support Th1 responses. On the other hand, M2 or alternatively activated macrophages are obtained *in vitro* with macrophage colony-stimulating factor (M-CSF) and secrete the anti-inflammatory cytokine IL-10, express scavenger and mannose receptors, contribute to Th2 responses, enhance phagocytosis, eliminate parasites and promote tissue repair (Figure 2) [15, 16]. Polarization of MΦ function should be viewed as a useful, simplified, conceptual framework describing different degrees of activation [17].

M1 and M2-like MΦ subsets have been identified throughout the body with different functional potential. For example, alveolar MΦ are shown to be immunosuppressive and display a poor antigen-presenting capacity [18]; similarly some tumour-associated MΦ may also share characteristics with the M2 MΦ population, promoting cancer progression and metastasis [19, 20]. On the other hand, M1 MΦ are key mediators in several autoimmune diseases, including rheumatoid arthritis [21] and inflammatory bowel disease [22].

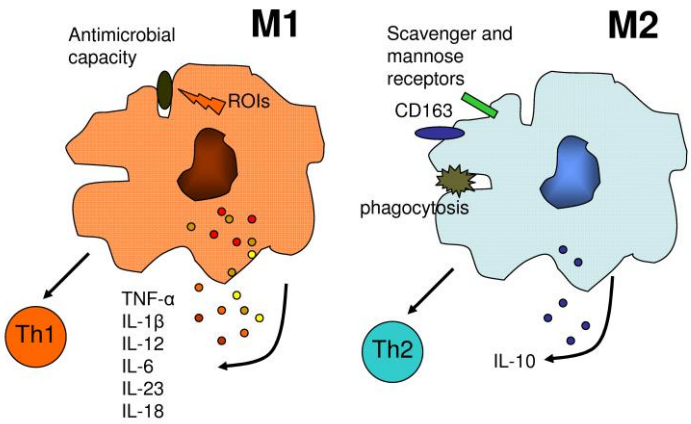

Figure 2. M1 and M2-polarized MΦ.

2.1. M1 and M2 MΦ in bacterial and viral infections

MΦ play a central role as effector cells in immunity to intracellular pathogens but they also provide a habitat for their survival. During *Salmonella enterica*-infection of MΦ it has been shown that IFN-γ promotes intracellular killing of the bacteria and that response is defective in MΦ from patients with IFN-γ receptor deficiency [23, 24]. *Coxiella burnetii* is an intracellular bacterium that causes the Q fever, an infective endocarditis. Patients with valvulopathy exhibited increased levels of circulating apoptotic leukocytes and have the highest risk to develop infective endocarditis. The binding of apoptotic cells to monocytes and macrophages, the hosts of *C. burnetii*, may be responsible for the immune impairment observed in Q fever endocarditis. Apoptotic lymphocytes increased *C. burnetii* replication in monocyte and monocyte-derived MΦ inducing an M2 program. In co-culture with apoptotic cells monocytes produced IL-10, IL-6 and increased CD14 while MΦ released TGF-β1. Neutralization of IL-10 and TGF-β1 prevented the replication of *C. burnetii* indicating that those cytokines are involved in bacterial replication. IFN-γ re-directed MΦ to an M1 phenotype and prevented replication of the bacteria. *C. burnetti* stimulated an M2 activation program that may account for its persistence in MΦ [25, 26].

Another study compared M1 and M2 monocyte-derived MΦ treated with *Mycobacterium tuberculosis* lysates. IL-23 was secreted by M1 MΦ in response to mycobacteria but exogenous IFN-γ addition was needed to produce IL-12. In contrast, M2 MΦ failed to produce IL-12 and IL-23 [27]. M1 MΦ also secreted IL-1β, IL-6, TNF-α, as well as IL-8, MCP-1, MIP-1β and RANTES, corroborating their pro-inflammatory function. M2 MΦ maintained the IL-10 secretion and produced no or relatively low IL-1β, IL-6 or TNF-α, but secreted high levels of IL-8 and MIP-1β [27]. Binding, uptake and intracellular growth of *Mycobacterium* was supported by both MΦ subsets but more efficiently by M2 than M1 MΦ [28]. M1 MΦ stimulated with

mycobacteria efficiently supported a Th1 response whereas M2 did not. Accordingly, only M2 MΦ down-modulated MHC class II, CD86 and CD40 [28]. MΦ are also HIV-1 targets, contribute to cell-to-cell spread of the virus and are HIV-1 reservoirs. HIV-1 replication was transiently inhibited in M1 and M2 polarized MΦ but was of shorter duration in M1 MΦ [29]. That could be explained because during MΦ polarization a down-modulation of CD4 occurred. However, only M1 polarization inhibited early events of HIV-1 replication. HIV-1-infected M1 MΦ also secreted higher amounts of CCL3, CCL4, and CCL5, potent inhibitors of viral entry. In contrast, changes in cytokine production were modest, only IL-6 and IL-1 were increased in M1 infected MΦ and IL-10 in M2 MΦ [29]. These results suggest that MΦ heterogeneity may be an important determinant of immunity and disease outcome in intracellular bacterial and viral infection.

2.2. NK cell-MΦ crosstalk

Information on the crosstalk between NK cells and MΦ is quite limited. It has been described that autologous human MΦ activate NK cell proliferation, IFN-γ secretion and enhanced NK cell cytotoxicity against target cell lines (K562 and 721.221). Crosstalk between activated NK cells and MΦ inducing IFN-γ secretion was recently shown to involve DNAM-1 and 2B4 [30, 31], while NKp46 and DNAM-1 contributed to the lysis of MΦ [30]. NKp30, although important in DC-mediated NK cell activation was not involved in NK cell activation by MΦ.

NK cells killed LPS-activated MΦ through the engagement of the NKG2D receptor [31]. Recently it has been described that engagement of TLR-4 by LPS up-regulated MICA and not MICB. TLR-4 ligation decreased expression of microRNAs (miR-17-5, miR-20a, and miR-93) that target MICA, suggesting an important role for MICA and NKG2D in the regulation of MΦ-NK cell crosstalk [32].

Little is known about the NK cell response to MΦ infected by intracellular pathogens. A study performed with *Mycobacterium tuberculosis* shows that the expression of NKp30, NKp46, NKG2D activating receptors was enhanced in NK cells after co-culture with infected monocytes but DNAM-1 and 2B4 expression remained unaltered. Antibodies against NKp46 and NKG2D blocked lysis of infected monocytes, but anti-NKp30 and 2B4 mAb had no effect. *M. tuberculosis*-infected monocytes up-regulated the NKG2D ligand ULBP1 in a TLR2-dependent process. The same results were further confirmed in *M. tuberculosis*-infected alveolar MΦ [33].

A recent report studied the interaction between NK cells and *Salmonella enterica*-infected MΦ. NK cells produced IFN-γ and degranulated upon stimulation with infected MΦ but none of the studied receptors (NKG2D, NKp46, NKp30, NKp44, NKp80 and 2B4) appeared involved in the process. IL-12 and IL-18, were implicated in the control of bacterial replication promoting NK cell-mediated IFN-γ production and infected cell killing [34].

3. Human Cytomegalovirus

Human cytomegalovirus (HCMV) is a member of the *herpesviridae* family that has a lineal double-stranded DNA of 235 kbp containing more than 160 open reading frames (ORFs). The HCMV genome is composed of unique long (UL) and unique short (US) domains, flanked by terminal repeated sequences (TRL and TRS) in one end, and internal repeats (IRL and IRS) in the opposite. After infection of cultured fibroblasts the viral genes are sequentially expressed in a highly organized cascade of immediate early (IE), early (E) and late (L) transcription. The HCMV virion is composed of an icosahedral capsid containing the DNA genome and an outer layer of proteins called tegument, enveloped by a cellular lipid layer containing viral glycoproteins [35].

The prevalence of HCMV infection in adult populations worldwide ranges from ≈50 to 100%, and the virus is acquired often through contact with secretions during childhood [36]. HCMV infection is usually asymptomatic in healthy individuals, but the virus is not cleared and persists in a life long latent state with occasional replication and shedding to assure the transmission to new hosts. Primary infection or reactivation in immunocompromised hosts can lead to pneumonia, hepatitis or graft failure in transplant recipients, and retinitis in HIV infected patients. Moreover, HCMV is the leading viral cause of congenital disorders, such as mental retardation, hearing loss or chorioretinitis [37].

Cytomegaloviruses commit a large percentage of their genome to the modulation of host cell behaviour and immune response to infection [38]. HCMV encodes proteins interfering at different levels with host defence and inflammation (i.e. MHC function, leukocyte activation, susceptibility to apoptosis, cytokine and antibody-mediated defence).

3.1. Immune responses to HCMV infection

3.1.1 T cells

An effective response against HCMV requires the participation of both adaptive and innate immune responses [39]. The CMV-specific CD4+ T-cell response precedes CMV-specific CD8+ T-cell responses and displays an effector-memory phenotype [40]. The magnitude of the CMV-specific CD8+ T cell response has been shown to be exceptionally strong and may progressively increase with age. In normal adult blood donors the percentage of T cells specific for HCMV is estimated to range from 0.1% to 5% [41] and most of this response is specific for pp65, gB and IE-1 HCMV proteins [42].

Several HCMV proteins have been described to down-modulate MHC class I and MHC class II expression via post-translational mechanisms resulting in

an impairment of T cell responses [43]. US3 is an IE protein that physically interacts with MHC class I heavy chains and retains MHC class I proteins in the endoplasmic reticulum (ER) [44], moreover US3 can bind to tapasin and interfere with peptide loading and MHC complex assembly, resulting in a delay in maturation [38, 45]. US2 and US11 are E proteins that translocate the MHC class I heavy chain from the ER to the cytosol and target it for degradation [46]. US6 is a late gene that binds to TAP interfering with the peptide transport to the ER [43, 47]. US2 and US3 also down-modulate MHC class II molecules, impairing the translocation of gene products or preventing antigen presentation by disruption of the invariant chain interaction, respectively [48].

3.1.2. NK cells

As shown in experimental animal models, NK cells are also important players in the response to HCMV. A patient lacking NK cells was reported to suffer several herpesviruses infections [49]. Recently studies in a patient lacking T cells recovering from a primary HCMV infection suggested that human NK cells contribute to control HCMV viremia [50].

Viral infection results in NK cell proliferation and recruitment to targeted tissues. Type I interferons (IFNs) are secreted by infected cells and directly upregulate NK cell cytotoxic functions. Type I IFNs also facilitate the production of IL-15, an NK cell growth factor that also enhances cytotoxicity and cytokine production. Moreover, HCMV-induced activation of myeloid cells results in the production of IL-12 and IL-18, which are potent inducers of IFN-γ production by NK cells. Reciprocally, NK cell secreted IFN-γ activates MΦ and dendritic cells (DC) at the site of infection [51].

Down-modulation of MHC class I molecules to subvert T cell recognition theoretically renders infected cells susceptible to NK cell lysis by releasing the engagement of the MHC class I inhibitory receptors [52, 53]. Yet, other

studies suggest that infected cells become resistant to NK cell lysis [54] supporting that HCMV possess mechanisms to evade the NK cell recognition, despite the loss of MHC class I on the surface of the infected cell (Figure 3) [55].

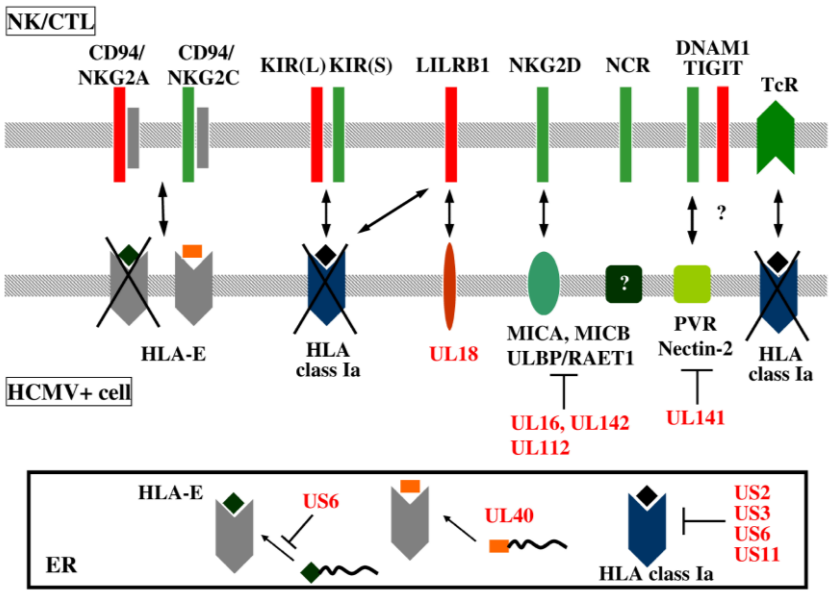

Figure 3. HCMV immunevasion mechanisms. Adapted from Lopez-Botet, M. et al., Tissue Antigens, 2004 [37].

3.1.3. Natural Killer cell receptors in the response to cytomegalovirus infection

LILRB1/ILT2

The human ILT gene (LIR, LILRB, CD85) family is located on human chromosome 19, encoding for molecules preferentially expressed by the myeloid lineage. Of the 13 *ILT* genes, *ILT2, ILT3 ILT4, ILT5* and *LIR8* encode for inhibitory receptors, but only two, ILT2 and ILT4 bind MHC class I molecules whereas other ligands remain unknown (Figure 4). All the other ILT receptors contain a charged transmembrane residue (Arg) and may

have an activating function by coupling to the ITAM-bearing FcεRIγ adaptor. LILRB1 (CD85j/ILT2/LIR-1) is a type I transmembrane protein of the immunoglobulin superfamily that is expressed by B cells, monocytes, and subsets of NK and T cells. LILRB1 expression has been associated to late differentiation stages of T lymphocytes specific for different microbial pathogens [56].

The protein consists of four Ig-like extracellular domains and a cytoplasmic tail containing four ITIMs conferring inhibitory function to the receptor [57]. LILRB1 recognizes a broad spectrum of classical and non-classical MHC class I molecules, by binding with low affinity to a conserved region in the α3 domain. *In vitro* engagement of LILRB1 represses cell activation through other receptors. Monocytes display a higher cell surface expression of LILRB1 than NK and T cells. It has been recently described that LILRB1 expression is driven by different promoters in monocytes and lymphocytes. The lymphocyte promoter maps 13kb upstream of the monocyte promoter, resulting in the inclusion of an extra exon that represses LILRB1 protein translation, resulting in lower expression levels in NK and T cells [58].

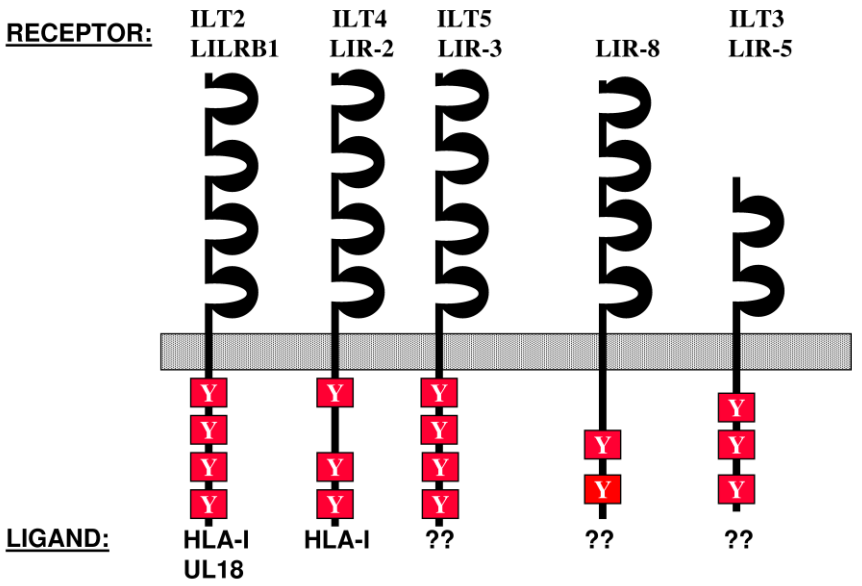

Figure 4. Inhibitory ILT receptors.

LILRB1 binds to the UL18 HCMV glycoprotein, a homolog of MHC class I [59] with >1000-fold higher affinity, illustrating how a viral protein may maintain an inhibitory receptor engaged while MHC class I molecules are down-modulated [60, 61]. Yet, the role of UL18 during HCMV infection is still unclear. The expression of UL18 in transfected cells or infected fibroblast resulted in a enhanced NK-mediated killing [62], by contrast, in another report, the resistance of HCMV-infected MΦ to lysis was described to be independent of UL18 expression [63]. It has been described that CD8+ T cells lyse UL18 expressing cells, whereas cells infected with the UL18 deletion mutant are resistant. Lysis was independent on TCR specificity and blocked by anti-LILRB1 and anti-UL18 mAbs [64].
Previous studies in our laboratory showed an increased expression of LILRB1 in peripheral blood T cells from healthy adult HCMV seropositive blood donors and children [65, 66].

KIR

The killer-cell immunoglobulin-like receptor (KIR) family has evolved in primates to generate diverse receptors with unique structures that enable them to recognize MHC-class I molecules with locus and allele-specificity. KIR proteins have been classified into 13 groups (KIR3DL1-2, KIR3DS1, KIR2DL1-5, and KIR2DS1-5) according to the number of Ig-like extracellular domains, the length of the cytoplasmic tail and the sequence similarity. The number of Ig-like domains is described as 2D for 2 domains or 3D for 3 domains; the length of the cytoplasmic tail is expressed as L for long or S for short. The inhibitory KIR have long cytoplasmic tails containing pairs of immune receptor tyrosine-based inhibition motifs (ITIMs), whereas KIR with short cytoplasmic tails are activating receptors associated with the DAP12 adaptor molecules via a positively charged lysine residue in their transmembrane domain [67]. KIR genes are clustered in the leukocyte receptor complex on chromosome 19q13.4. Different *KIR*

haplotypes including variable numbers of genes have been identified. Some inhibitory KIRs discriminate subsets of classical HLA class I allotypes (HLA-B, HLA-C or HLA-B) that share homology in the α1 domain. Some triggering KIRs interact as well with HLA class I molecules but with a lower affinity than the inhibitory ones, whereas the ligands for others remains unknown. Inhibitory KIRs monitor the surface expression of HLA-C and subsets of HLA-A and HLA-B molecules maintaining tolerance [68]. The clonal distribution of inhibitory KIR allows different NK-cell subsets to discriminate variable alterations of MHC class I expression [68-71].

Although HCMV has developed several mechanisms for altering MHC class I expression, thus far no specific KIR has been shown to be important in the immune response to CMV [72]. An individual whose NK cells all expressed the inhibitory receptor KIR2DL1 developed recurrent infections including HCMV [73]. A patient suffering of a primary T cell immunodeficiency who recovered from HCMV disease displayed an oligoclonal expansion of NK cells recognized by a mAb specific for KIR2DL2/2DS2/2DL3+ [50].

CD94/NKG2 receptors

CD94 and NKG2 are lectin-like membrane glycoproteins encoded at the NK gene complex (NKC) in human chromosome 12. CD94/NKG2 heterodimers are selectively expressed by NK cells and some cytotoxic T cells. CD94 binds to NKG2 glycoproteins through disulfide bridges and is required to stabilize their surface expression, although it may also form homodimers.

The CD94/NKG2A heterodimer constitutes an inhibitory receptor because of the ITIM-bearing NKG2A subunit. By contrast, CD94/NKG2C forms a triggering receptor coupled to the DAP12 adaptor molecule. The function of other putative activating molecules encoded by the *NKG2E* gene remains unknown (Figure 5) [74].

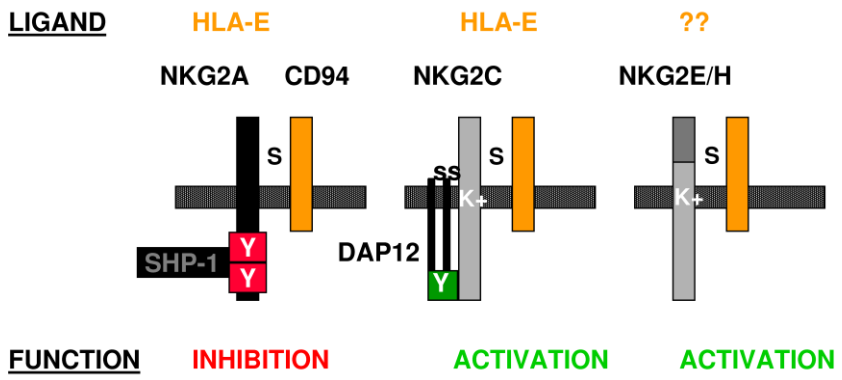

Figure 5. CD94/NKG2 receptors.

CD94/NKG2A and CD94/NKG2C recognize the non-classical HLA class I molecule HLA-E. HLA-E binds a restricted set of nonamer peptides derived from the leader sequences of MHC class I molecules. Binding of HLA-E to CD94/NKG2A is considered a mechanism evolved to survey the normal synthesis of HLA class I molecules, preventing self-reactivity of NK cells. The inhibitory receptor has higher affinity for the ligand than the activating homologue [75]. The function of the activating receptor is unknown. It has been suggested that HLA-E bound to a high affinity peptide could trigger CD94/NKG2C signalling and it might activate NK cytotoxicity in pathological conditions when the down regulation of surface HLA class I molecules would impair inhibitory signals [76, 77]. In the majority of NK cells the expression of NKG2A and NKG2C is mutually exclusive, but we have recently reported that a minor subset of peripheral blood NK and T cells co-express both CD94/NKG2A and CD94/NKG2C in healthy adult donors. NKG2A is transiently displayed by CD94/NKG2C+ NK cells under the influence of IL-12, providing a potential negative regulatory feedback mechanism [78].

The surface expression of HLA-E, was described to be stabilized by a nonamer derived from the leader sequence of the HCMV glycoprotein UL40

[79, 80]. In order to be preserved in the infected cell the UL40 peptide presentation is independent of TAP and, thus refractory to the US6 action [79]. Previous studies in our laboratory showed that US2 and US11 preserved HLA-E and selectively down-modulated HLA class I molecules [43]. Preservation of the HLA-E expression protected infected cells from CD94/NKG2A+ cell lysis [81]. However, in contrast with previous reports in fibroblasts, HCMV-infected monocyte-derived dendritic cells (moDC) down-modulate HLA-E, becoming susceptible to CD94/NKG2A+ NK cell lysis [82].

It has been described in our laboratory that a positive serology for cytomegalovirus, but not for other herpesviruses (i.e. EBV, HSV) is associated with a higher percentage of NKG2C+ NK and T cells in peripheral blood from adult donors. It is of note that NKG2C+ NK cells expressed lower levels or NKp30 and NKp46 and higher levels of ILT2 and KIR than NKG2A+ cells [65]. An expansion of CD94/NKG2C+ cells was also described in HIV positive individuals and were shown to be also related to HCMV infection [83-85]. Recent studies demonstrate that a positive serology for HCMV and an active excretion of the virus are also associated with increased numbers of NKG2C+ NK and T cells in healthy children [66].

These results where further supported by studies *in vitro* co-culturing PBLs from seropositive donors with HCMV-infected fibroblasts (AD169, Towne strains). Under these experimental conditions an expansion of NKG2C+ NK cells was observed and was prevented with an anti-CD94 mAb [86]. The mechanism underlying this response remain unknown [87].

NKG2D

NKG2D is another C-type lectin-like molecule that forms homodimers. The *NKG2D* gene is located within the NK complex region in human chromosome 12. NKG2D is expressed by all human NK cells as well as TCRγδ(+) and TCRαβ(+)CD8(+) T lymphocytes and a subset of cytotoxic CD4+ T cells [88]. NKG2D associates with the adaptor molecule DAP-10, which contains an YxxM motif that links it to phosphoinositide 3-kinase (PI3-K) signalling pathway. NKG2D-ligands are class I MHC-related molecules including MICA, MICB and the ULBP family of proteins (ULBP1-6) [89, 90]. NKG2D ligands are absent or expressed at low levels in normal cells but can be upregulated during different cell conditions, including infections and cellular transformation. Stimulation of NK cells through NKG2D triggers cell-mediated cytotoxicity and cytokine and chemokine secretion.

Several proteins encoded by HCMV evolved to prevent NKG2D ligands expression in the infected cell. UL16 impairs the expression of MICB, ULBP1 and ULBP2 [91, 92], the HCMV gene product UL142 prevents cell surface expression of MICA and ULBP3 [93-95]. Recently a microRNA (miRNA) encoded by HCMV (UL112) that downregulates MICB expression has been described [96, 97].

Previous studies in our laboratory showed that *in vitro* stimulation of PBMCs from seropositive individuals with HCMV promoted variable expansion of CD4+ NKG2D+ T lymphocytes potentially having a role in the response against infected MHC class II+ cells displaying NKG2D ligands [88].

A recent work showed a transient reduction of NKG2D expression on NK cells when infecting PBMCs with HCMV. This effect is cytokine-mediated (by IL-12 and type I interferons) and may represent a feedback mechanism to control NK cell reactivity against normal cells expressing the NKG2D ligands in response to the viral infections [98].

Natural Cytotoxicity Receptors

Natural cytotoxicity receptors (NCRs) comprise NKp46, NKp30, NKp44 and NKp80 molecules. NCRs are expressed by all NK cells but NKp44 is expressed only upon NK cell activation.

NCRs are type I transmembrane glycoproteins belonging to the Ig superfamily. NKp46 is characterized by two C-type Ig-like domains and NKp30 and NKp44 have a single extracellular domain and their cytoplasmic tail associates with adaptor proteins that contain ITAM motifs. NCRs are activating receptors that have been involved in the recognition of certain tumor cells [99].

The cellular NCR ligands remain ill-defined. On the other hand NKp44 and NKp46 bind to hemagglutinins (HA) from influenza virus and trigger NK cell-mediated lysis of the infected cell [100]. The HCMV tegument protein pp65 has been described as a ligand for the NK cell activating receptor NKp30. This interaction produces an NK cell inhibition mediated by the dissociation between NKp30 and its adaptor [101]. Recently, it has been shown in our laboratory that moDC express the NKp30 and NKp46 ligands and both are down-modulated upon HCMV infection. Yet, only NKp46 was involved in the NK cell response to infected moDC [82].

NKp80 is expressed by virtually all NK cells and by a minor subset of CD56+ T cells [102]. NKp80 binds to AICL, a myeloid-specific activating receptor. AICL-NKp80 interaction promoted NK cell-mediated cytolysis of malignant myeloid cells and the release of proinflammatory cytokines from both cell types [103].

DNAM-1

DNAX accessory molecule-1 (DNAM-1, CD226) is expressed by all NK cells, T cells and monocytes. Encoded in human chromosome 18, DNAM-1 is characterized by two Ig-like domains in the extracellular portion and a cytoplasmic tail containing three tyrosine residues. Cross-linking of DNAM-

1 results in Fyn-mediated tyrosine phosphorylation and triggering of cytotoxicity in NK cells [104]. Poliovirus receptor (PVR/CD155) and Nectin-2 (CD112), members of the Nectin family, have been described as cell surface ligands for DNAM-1 [105]. These proteins are highly expressed in tumour cell lines of epithelial, haematopoietic or neuronal origin. Remarkably, DNAM-1 cooperated with NKp30 in the NK-mediated killing of both immature and mature DCs and the degree of contribution of DNAM-1 appeared to correlate with the surface densities of its specific ligands PVR and Nectin-2 [106].

The HCMV protein UL141 has been shown to down-regulate surface expression of DNAM-ligands PVR and Nectin-2 in human fetal foreskin fibroblast (HFF) [107, 108]. Recently, we have also shown that PVR and Nectin-2 are down-modulated in moDC infected with the TB40/E HCMV strain, further supporting a role of the activating receptor DNAM-1 in the response to HCMV [82].

SLAM-related receptors: 2B4

SLAM-related receptors are a group of six distinct molecules named SLAM (CD150), 2B4 (CD244), Ly-9 (CD229), CD84, NK-T- and B-cell antigen (NTB-A) and CD2-like receptor-activating cytotoxic cells (CRACC). All are members of the Ig-superfamily and form a gene cluster in chromosome 1. Except 2B4, all members of the family are implicated in homotypic self-interactions. 2B4 ligand is CD48 a member of the CD2 family broadly expressed in haematopoietic and endothelial cells [109, 110]. Engagement of 2B4 in mature human NK cells promotes NK cell activation. In contrast, triggering of 2B4 in immature NK cells inhibit cell activation, potentially preventing reactivity against self during NK cell maturation [111, 112]. This dual function could be explained by the fact that mature NK cells express the 2B4 adaptor proteins SAP (that activates NK cells) and EAT-2 (that inhibit NK cell activity) but immature NK cells do not express SAP. The

2B4 promoter is also influenced by the NK cell activation state. IL-2 stimulation leads to an up-regulation of SAP expression having functional consequences for the stimulation of NK cell cytotoxicity by 2B4. In resting NK cells, 2B4 acts only as a co-receptor but in IL-2 activated NK cells triggering via 2B4 alone is sufficient to induce NK cell cytotoxicity [111, 113].

In vivo, mouse cytomegalovirus (MCMV) infection up-regulated CD150 expression on NK cells [114]. However, little is known about the role of SLAM receptors during CMV infection.

3.2. HCMV infection of MΦ

Myeloid cells were described as HCMV reservoirs and dissemination vectors in the blood circulation. HCMV establishes a latent state in myeloid cells and can be reactivated upon allogenic stimulation. Some reports have proposed that IFN-γ could be critical for viral reactivation [115, 116]. IFN-γ and TNF-α induce the formation of HCMV permissive MΦ that are refractory to the antiviral activities of these cytokines [117]. HCMV infection of monocytes induces their differentiation towards an M1 MΦ phenotype sharing some characteristics with M2 MΦ, these MΦ are permissive to viral replication potentially promoting viral spread [117-121].

On the other hand, little is known about the functional consequences of HCMV infection of MΦ. Various HCMV strains have been tested for their capacity to infect monocyte-derived MΦ *in vitro*. The endotheliotropic strains VHL/E and TB40/E efficiently infected MΦ supporting a lytic replicative cycle while other strains (i.e. AD169) infected <0.1% of cells [122, 123].

MΦ infection is associated with a down-modulation of MHC class I and class II molecules [122, 124] and also CD80, CD86, and CD14 are negatively regulated. HCMV infection produces a paralysis of the MΦ motility by

down-regulating chemokine receptors, reorganizing the cytoskeleton, and releasing macrophage migration inhibitory factor (MIF) [123].
HCMV infection also reduces matrix metalloproteinase 9 activity, potentially contributing to atherogenesis [125]. However, it has been described that HCMV infection inhibits the development of the foam cell phenotype in the MΦ-derived cell line THP-1 suggesting that the virus does not promote the lipid uptake in the infected cell [126] but, other reports describes that alters the lipid metabolism increasing oxLDL uptake and scavenger receptor expression in macrophages and vascular smooth muscle cells [127, 128].

3.3. NK cell response to HCMV-infected myelomonocitic cells

To our knowledge the NK cell receptors involved in the lysis of HCMV-infected MΦ remains unknown; yet, we have recently described that NK cells efficiently respond against HCMV-infected immature moDC (HCMV TB40/E strain). Infected moDC, that down-modulate MHC class I molecules, trigger NK cell cytotoxicity and DNAM-1 and NKp46 receptors play a central role in this process. NKG2D ligands are not detected in the infected cell likely due to the effect of immune evasion mechanisms explaining why this receptor is not apparently involved in killing [82].
Opposite to what has been described for moDC, HCMV-infected MΦs were reported to be less susceptible to NK cell lysis. Experiments with deletion mutants showed that this process was independent of down-regulation of MHC class I molecules and expression of the UL18 HCMV class I homologue [63].

4. Atherosclerosis

Atherosclerosis is a major underlying cause of cardiovascular disease (CVD). CVD refers to a group of disorders that includes stroke, coronary artery

disease, myocardial infarction, congestive heart failure, angina, and aneurysms. CVD is the main cause of death in Western societies, causing 1.5 million deaths per year in the European Union. One out of eight male dies before the age of 65 resulting of CVD [129] which is rapidly increasing in developing countries due to an accumulation of risk factors such as obesity and diabetes [130].

Atherosclerosis is an inflammatory process that may begin in childhood life and is initiated when cholesterol-containing low-density lipoproteins accumulate in the intima layer of blood vessels and activate the endothelium giving rise to fatty streak lesions [131]. Classical risk factors for the development of atherosclerosis are smoking, high blood pressure, increased blood cholesterol levels, obesity and diabetes.

These risk factors do not completely explain the disease and other markers have been proposed. Proinflammatory cytokines (i.e. IL-6) drive the hepatic production of acute-phase response proteins such as the C-reactive protein (CRP) [132], that contributes to microbial defense and inflammation. Increased CRP levels were reported to be associated with CAD and CRP high sensitivity tests have been developed to predict future cardiovascular events; yet it has been shown that CRP is a relatively modest predictor of coronary heart disease and subclinical atherosclerosis [133].

Carotid Intima-Media Thickness (CIMT, IMT) is the measurement of the distance between the anatomical boundaries lumen-intima and media-adventitia interfaces in an ultrasound image (Figure 6). Measurements at different points of the right and left carotid can be performed; the common carotid (CCA) (the arterial far wall 1 cm proximal to the bulb), the carotid bulb (the arterial far wall between the carotid dilatation and the carotid flow divider) and the internal carotid (the arterial far wall 1 cm distal of the carotid flow divider). A plaque is a focal structure of at least 1.5mm or 50% higher of the surrounding IMT value [134]. The CIMT is a measurement of

subclinical atherosclerosis and several prospective studies have demonstrated an association between CIMT and CVD [135, 136].

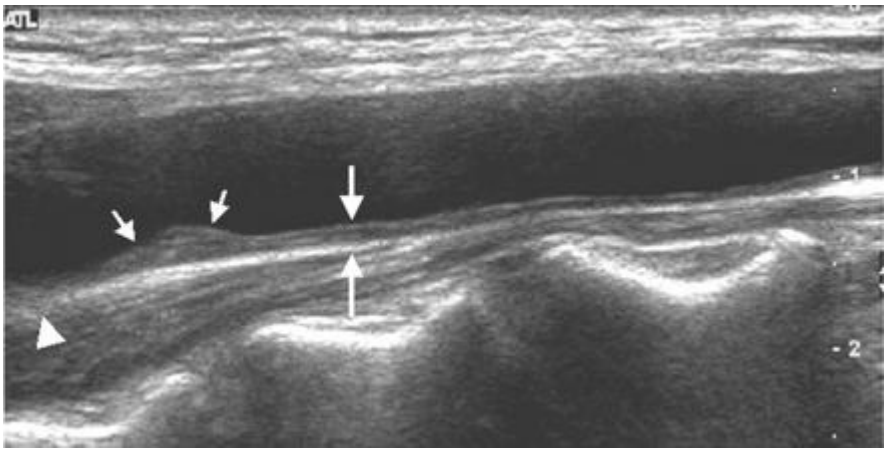

Figure 6. Longitudinal scan of carotid ultrasonogram. Measurement of CCA-IMT (*large arrows*) at the far wall of the common carotid artery is shown. The carotid plaque (*small arrows*) in the distal common carotid artery is seen. The *arrowhead* represents carotid bifurcation (Lee, E. J. et al., Am J Neuroradiol, 2007) [137].

Widely used models of atherosclerosis are the apolipoprotein E deficient mice (ApoE$^{-/-}$) and LDL-receptor deficient mice (LDLR$^{-/-}$). As ApoE is critical for elimination of cholesterol-containing lipoproteins in mice, ApoE$^{-/-}$ mice have increased cholesterol and develop accelerated atherosclerosis. LDLR$^{-/-}$ mice responds to fat feeding by cholesterolemia and blood vessel lesion formation [138].

4.1. The atherosclerotic process

The structure of the normal blood wall comprises three layers, from outside to inside: the adventitia, media and intima (Figure 7). The adventitia layer comprises connective tissue, capillaries, fibroblast and fat cells that are separated from the outer media by an elastic lamina. The lamina media contains vascular smooth muscle cells (SMCs) within an interstitial matrix

containing type I collagen, fibronectin, as well as dermatan and chondroitin sulphate proteoglycans. Separating the media and the intima there is an internal elastic lamina composed by elastin. The inner layer, intima comprises a monolayer of connected endothelial cells lying on a basal membrane composed by type IV collagen, laminin and heparan sulphate proteoglycans [139].

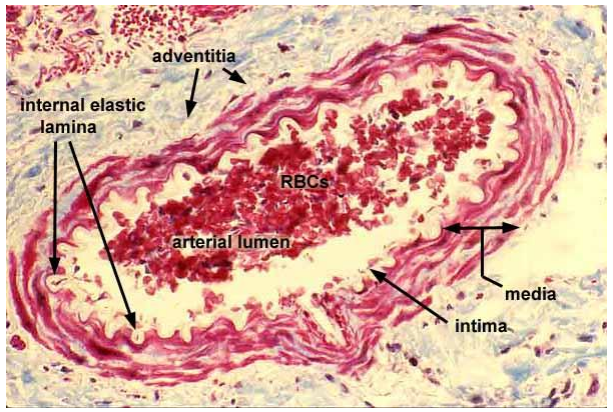

Figure 7. Structure of a blood vessel (Mescher, A. , Junqueira's Basic Histology, 2010) [140].

At the beginning of the atherosclerosis, lipids and immune cells accumulate in the artery, giving rise to fatty streak lesions. The majority of cells are MΦ loaded of lipids (foam cells) and T cells, but also DC, NK cells, B cells and mast cells are detected to a lesser extent (138-140). Morphological and functional studies of the earliest stages of atherosclerosis in human and animal models indicate that the initiating step is subendothelial accumulation of apolipoprotein B-containing lipoproteins (ApoB-LPs) (141). ApoB-LPs are produced by the liver as very low-density lipoproteins, which are converted in the circulation in low-density lipoproteins (LDL). Intestinal ApoB-LPs are secreted as chylomicrons, which are converted by lipolysis into atherogenic particles called remnant lipoproteins. Subsequently, LPs infiltrate the intima (142) where they are retained in the extracellular matrix and modified by oxidation, releasing phospholipids that activate endothelial cells leading to recruitment of blood monocytes (143). Activated endothelial

cells secrete chemokines that promote directional migration of monocytes. Monocytes roll on endothelial cells through the interaction of monocyte P-selectin glycoprotein ligand-1 (PSGL-1) with endothelial selectins (143), then becoming adhered to endothelial cells through the interaction of monocyte integrins VLA-4 (very late antigen-4) and LFA-1 (lymphocyte fuction-associated antigen-1) and their respective endothelial cell ligands, VCAM-1 (vascular cell adhesion molecule-1) and ICAM-1 (intercellular adhesion molecule-1) (143, 144). Firm adhesion of monocytes is followed by their entry into the subendothelial space (diapedesis) (145). Driven by M-CSF and probably other factors monocytes differentiate into MΦ (143, 146). MΦ clear oxidized lipoproteins via scavenger receptors, the type A scavenger receptor (SRA) and CD36 generating foam cells (147). These early lesions have been detected in children and can disappear with time or progress to atherosclerotic lesions (148).

Atherosclerotic lesions (atheromas or atherosclerotic plaques) are asymmetrical thickenings of the intima layer. The lesions are composed of a lipid core surrounded by MΦ, foam cells, mast cells, lymphocytes, and connective tissue. In an advanced stage also NK and NKT cells are present representing ~0.1% and 2%, respectively of the total lymphocyte population (149, 150). A smooth muscle fibrous cap covers the plaque. The progressive reduction of the lumen size can lead to symptoms like effort angina. On the other hand, fibrous plaques can destabilize due to the action of cytokines, proteases and prothrombotic factors, which promote collagen degradation and a thinning of the cap. When the cap is disrupted exposing prothrombogenic material, platelets and coagulation factors form a thrombus [141] which leads to ischemic stroke or myocardial infarction.

4.2. Inflammation and atherosclerosis

Immune cells dominate the atherosclerotic lesions accelerating their progression end eliciting the acute coronary syndromes [142]. The main components in atherosclerotic lesions are T cells and MΦ. However, other immune cell types (i.e. B and NK cells) have also been described to play a role in the inflammatory process [143-146].

TCR αβ+ CD4+ and CD8+ T cells [147] that may interact with MΦ and DC [148, 149] are present in atherosclerotic lesions, but CD4+ cells predominate in number [150, 151] (Figure 8) and play a pro-atherogenic role, as shown using different animal models. For instance, CD4 deficient C57Bl/6 mice on an atherogenic diet were protected against fatty streak formation [152]. A subset of infiltrating CD4+ T cells are reactive against antigens on the atherosclerotic plaque such as oxLDL and heat shock proteins (HSP) [153, 154]. In fact, ≈10% of human CD4+ T cell clones derived from plaques are specific for oxLDL as an MHC class II-restricted antigen [153, 155-157]. Activation of naïve T cells requires ligation of the TCR by antigen/MHC and ligation of costimulatory molecule CD28 on T cells by CD80 or CD86 on the APC. Other costimulatory molecules important for T cell activation are CD40-ligand on T cells and CD40 on APC and Ox40 on T cells and Ox40-ligand on a wide array of cells. Abrogation of either of those interactions reduced lesion formation [155-157].

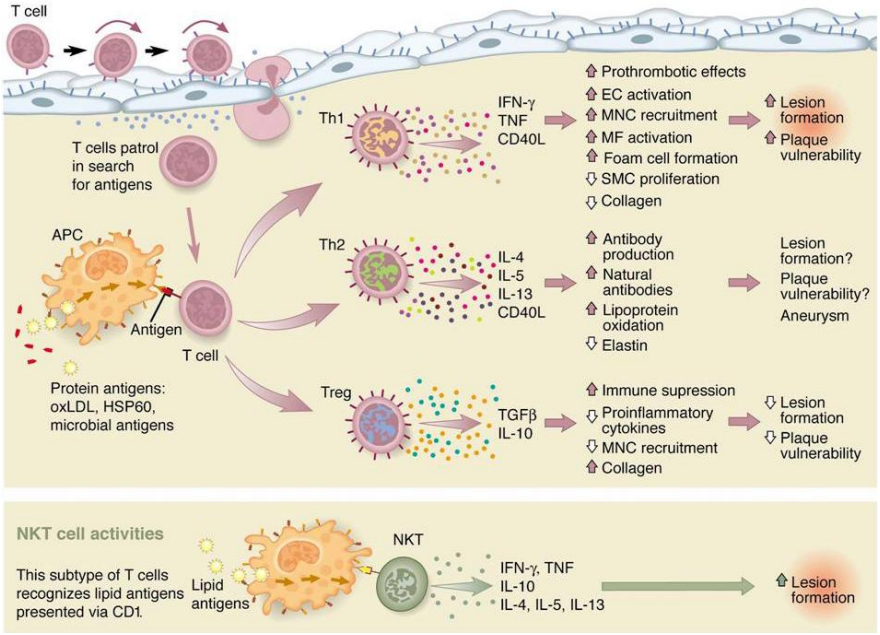

Figure 8. Recruitment and activation of T cells in atheromas (Hansson, G.K. et al., Annu Rev Pathol, 2006) [141].

Animal and human studies show a predominant Th1 pattern in atherosclerosis. The Th1-stimulating cytokines IL-12 and IL-18 have been detected in lesions [158] and a proatherogenic role of these cytokines has been observed [159, 160]. Th1 cells also produce TNF-α and lymphotoxin (LT, TNF-β), two proinflammatory cytokines with proatherogenic effects [161].

Th2 cells produce IL-5 and IL-4, promoting B cell activation and the production of antibodies, that may recognize oxLDL and inhibit cholesterol uptake [162]. Though IL-4 was also predicted to be protective against the disease, studies in IL-4-deficient mice supported a proatherogenic role [159, 163]; this effect may be due to the ability of IL-4 to upregulate CD36 and cause matrix degradation [163].

It has been proposed that CD8+ cells may contribute to the atherosclerotic process upon antigen stimulation during viral infections [164]. ApoE$^{-/-}$ CD8$^{-/-}$ mice show no changes in the development of atherosclerosis compared

with ApoE$^{-/-}$ [165]. However, activation of antigen-specific CD8+ T cells by a foreign antigen expressed in SMCs in ApoE$^{-/-}$ mice, leads to an accelerated atherosclerosis [166]. It has been proposed that CD8+ cells may contribute to the atherosclerotic process upon antigen stimulation, including viral infections [164].

Regulatory T cells (Treg) recognize self antigens maintaining self-tolerance and preventing autoimmunity [167]. Treg inhibit activated lymphocytes by producing the anti-inflammatory cytokines IL-10 and TGF-β [168-170]. Recent reports suggest that Treg are present in lesions of ApoE$^{-/-}$ mice [171]. IL-10 deficiency in C57BL6 mice fed with an atherogenic diet promotes early atherosclerotic lesion formation with increased activated T cells [168]. Recently it has been shown that absence of IL-10 promotes a switch towards the Th1 immune response [169]. An anti-atherogenic effect of TGF-β has been demonstrated in ApoE$^{-/-}$ mice using neutralizing antibodies and TGF-β-deficient animals [172, 173]. TGF-β has an anti-inflammatory effect and also stabilizes the atherosclerotic plaque. Altogether these results suggest that Treg cells may have an important role in the control of lesion development and progression [170].

NKT cells are T cells expressing TCR specific for lipids displayed on the antigen presenting cell (APC) bound to CD1d. CD1d has been detected in human atherosclerotic lesions suggesting a role in the development of atherosclerosis [174], which is slower in CD1d$^{-/-}$ mice [175-177]. In ApoE$^{-/-}$ deficient mice injection of the CD1d-ligand α-GalCer exacerbate atherosclerosis [175]. Moreover, NKT cell activation leads to secretion of IFN-γ and other pro-inflammatory cytokines, resulting in an aggravation of atherosclerosis [177].

NK cells have been detected in immunohistochemical samples from all stages of atherosclerotic lesions [178]. However, they role in lesion development remains unclear. The hints that NK cells may be implicated in atherosclerosis stem from studies in an animal model deficient of NK cells

that maintain normal B and T lymphocytes levels [143]. NK cell deficiency in LDLR$^{-/-}$ mice results in a significant reduction in the development of early stage lesions suggesting that NK cells might be pro-atherogenic [144, 145]. Th1 cells, CD8+ T cells, NKT cells and NK cells might contribute to the pathogenesis of atherosclerosis through IFN-γ secretion. IFN-γ is produced locally in the atherosclerotic lesions [179] and promotes MΦ and endothelial activation. In IFN-γ$^{-/-}$ mice lesion development was reduced [180] indicating a proatherogenic role of that cytokine.

As mentioned before, MΦ differentiation and activation is essential for the development of atherosclerosis (Figure 9). MΦ uptake lipids via scavenger receptors and transform into foam cells up-regulating TLRs expression [181]. TLRs bind to bacterial toxins, DNA motifs and HSP60 promoting MΦ activation [182-184]. This also affects other cell types expressing TLRs like DCs, mast cells and endothelial cells [183]. Most MΦ and endothelial cells in atherosclerotic lesions express TLR4 and TLR2 [185]. ApoE/TLR4 or ApoE/MyD88 (an adapter protein involved in TLR signalling) deficient mice had a significant reduction in lesion size and in the numbers of infiltrating MΦ in lesions [186]. MΦ may also activate T cells via antigen presentation (oxLDL, HSP60 and microbial antigens) and respond to cytokines produced by T cells. MΦ activation leads to release of nitric oxide, eicosanoids and reactive oxygen species, and may also contribute to plaque destabilization secreting proteolytic enzymes [187].

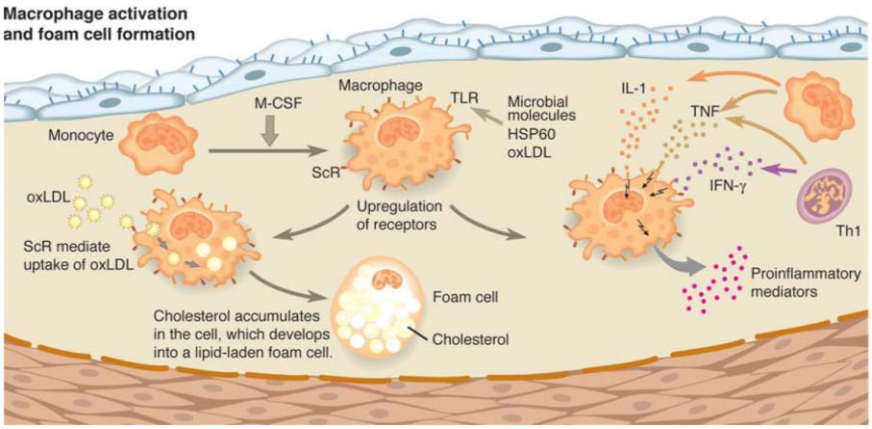

Figure 9. Activation of MΦ in atheroma (Hansson, G.K. et al., Annu Rev Pathol, 2006) [141].

Mast cells are effectors cells of allergic reactions that are activated when IgE bound to their Fc receptors interacts with antigens, in addition mast cells can also be activated by PAMPs. Activated mast cells secrete proteases, histamine, lipid mediators and cytokines that may contribute to the pathogenesis of atherosclerosis destroying matrix components and promoting plaque rupture [188].

Not all immune responses are detrimental during atherosclerosis development and B cells may have a protective role [189]. In ApoE$^{-/-}$ mice, after splenectomy leading to a reduction of B cells atherosclerotic lesions were aggravated, and transfer of spleen B cells reduced atherosclerosis development [146]; moreover, injection of immunoglobulins reduced ApoE$^{-/-}$ mice lesions [190].

4.3. Infections and atherosclerosis

The causes of vascular endothelial injury appear related to conventional risk factors, such as oxidized LDL, cigarette smoke, and stress caused by mechanical forces associated with hypertension, but may also include infections. Several viruses and bacteria, which cause chronic infections, have

been implicated in the pathogenesis of atherosclerosis; however the mechanisms whereby microbial pathogens might contribute to atherogenesis remain uncertain. Bacteria (i.e. *Chlamydia pneumoniae, Mycoplasma pneumoniae* and *Helicobacter pylori*) as well as herpes viruses (i.e. CMV, Epstein-Barr virus and Herpes-simplex virus type I) have been associated to vascular disease, mainly based on seroepidemiological studies, not systematically confirmed [191].

The "infectious burden" hypothesis proposes that the risk of vascular disease results from a cumulative effect exerted by different infections [192]. A recent study calculated a weighted index for infectious burden (IB) taking into account the serologies for HCMV, *C. pneumoniae, H. pylory*, HSV1 and HSV2 [193] and found a correlation between IB and carotid plaque thickness [194]. Further studies are needed to explore the putative association of infectious burden with stroke and AMI, and markers that reflect the impact of infections on the atherosclerotic process are warranted.

4.3.1. *Chlamydia pneumoniae*

C. pneumoniae has been detected in atherosclerotic lesions, but infection in mice does not cause the disease. There are contradictory studies about the role of *C. pneumoniae* accelerating the development of fatty streak lesions in hypercholesterolemic mice [195, 196]. Similarly, epidemiological studies of anti-*Chlamidia* antibody titres gave conflicting results [192, 197] and clinical trials have failed to show any beneficial effect of antibiotic therapy on myocardial infarction recurrence or long-term complications in patients with chronic coronary disease [198]. A possible link between infection and inflammation in atherogenesis is molecular mimicry between microbial antigens and human epitopes present in atherosclerotic plaques. Antibodies against bacterial HSP65 can recognize human HSP60 and induce cytotoxic damage in endothelial cells [147]. Moreover, anti-HSP60 antibodies can be detected in the majority of patients with CAD [199].

4.3.2. HCMV

HCMV infection is believed to be involved in the development of atherosclerosis based on the association of vascular disease with the detection of anti-HCMV antibodies, not systematically confirmed [192, 200]. Additional studies demonstrated that only high anti-HCMV antibody levels appear associated to atherosclerosis [201]. Moreover, HCMV has been associated to vascular lesions in chronic graft rejection and coronary re-stenosis post-angioplasty [202, 203], and viral DNA has been detected in atherosclerotic lesions [204].

In ApoE$^{-/-}$ mice, murine CMV infection accelerates the development of vascular lesions [205, 206], an effect also induced with inactivated virus [207], thus supporting its indirect contribution to the inflammatory process. Moreover mouse CMV infection alone results in an increase of blood pressure due to the secretion of pro-inflammatory cytokines (IL-6, TNF-α), renin and angiotensin II causing vasoconstriction and hypertension [208].

In vitro data also revealed CMV modulation of cellular gene products involved in the pathogenesis of atherosclerosis. HCMV-infected smooth muscle cells, MΦ and endothelial cells secrete inflammatory cytokines (i.e. IL-12, IL-18, TNF-α and IFN-γ) potentially contributing to the disease progression [209].

HCMV-infection can contribute by multiple ways to the pathogenesis of atherosclerosis. HCMV infects endothelial cells and stimulates renin expression indicating that HCMV hypertension could be the mechanism to aggravate atherosclerosis [208]. HCMV infection also alters the lipid metabolism increasing oxLDL uptake and scavenger receptor expression in vascular smooth muscle cells [128] although it inhibits the conversion of THP-1 derived MΦ into foam cells [126]. HCMV infection of MΦ has been shown to alter the metalloproteinase-9 activity in infected cells potentially contributing to plaque destabilization [125, 210].

It has also been proposed that immune responses to particular HCMV proteins might result in autoimmunity through a mechanism of molecular mimicry. Patients with CAD have antibodies against an epitope of the HSP60 molecule [199] that shares homology with the HCMV proteins UL122 and US28, suggesting that anti-HSP60 antibodies might derive from an anti-HCMV response [211].

On the other hand, CMV acts as a ligand for TLR2, expressed in monocytes and MΦ and this interaction is independent of viral replication suggesting that the increase in lesion size observed with the UV-inactivated virus in hypercholesterolemic mice is mediated by TLR ligation followed by pro-inflammatory cytokine secretion [207, 212].

Chapter 2

Aims

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

- Comparatively analyze the NK cell response to autologous HCMV-infected pro-inflammatory and anti-inflammatory macrophages.

- Address whether the impact of HCMV infection on the NKR distribution might reflect its putative role in the pathogenesis of atherosclerosis.

PART II

RESULTS

Chapter 3

Natural Killer cell-mediated response to human cytomegalovirus-infected macrophages is modulated by their functional polarization

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Natural Killer cell-mediated response to human cytomegalovirus-infected macrophages is modulated by their functional polarization

Running Title: NK cell response to HCMV-infected MΦ

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ABSTRACT

Macrophages (MΦ) comprise a heterogeneous population of cells that contribute to host defence and maintenance of immune homeostasis. MΦ may be infected by human cytomegalovirus (HCMV), which has evolved different strategies to subvert the immune response. In the present study, we comparatively analyzed the natural killer (NK) cell response against HCMV (TB40E)-infected pro-inflammatory (M1) and anti-inflammatory (M2) MΦ derived from autologous monocytes cultured in the presence of GM-CSF and M-CSF, respectively. M1 MΦ were more resistant to infection and secreted IL-6, TNF-α, IFN-α and IL-12; by contrast, in HCMV-infected M2 MΦ, proinflammatory cytokines, IL-10 and IFN-α production were limited, being IL-12 undetectable. NK cell degranulation was triggered by interaction with HCMV-infected M1 and M2 MΦ at 48 h post-infection. The response was partially inhibited by specific anti-NKp46, anti-DNAM-1 and anti-2B4 mAbs, thus supporting a dominant role of these activating receptors. By contrast, only HCMV-infected M1 MΦ efficiently promoted NK cell-mediated IFN-γ secretion, an effect partially related to IL-12 production. These observations reveal differences in the NK cell response triggered by distinct HCMV-infected monocyte-derived cell types, which may be relevant in the immunopathology of this viral infection.

INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus that establishes a persistent infection in 50–80% of adult populations [1]. HCMV infection may cause severe congenital disorders, as well as important complications in immunodeficient and immunosuppressed patients; moreover, it has been associated with immunosenescence and chronic inflammatory disorders (i.e. atherosclerosis) in immunocompetent hosts [2, 3]. Among the wide variety of cell types that may be infected by HCMV, myeloid cells are believed to be responsible for its systemic spread, and HCMV viremia has been associated to the detection of viral DNA in monocytes and macrophages [4-6].

Macrophages (MΦ) comprise a heterogeneous cell population that participates in immune defense and homeostasis. Both innate and adaptive immune responses may drive macrophages to develop different functional programs [7]. By analogy with $CD4^+$ T helper (Th) cells, these polarized differentiation pathways have been designated M1 and M2. Among a number of phenotypic and functional differences, M1 MΦ (pro-inflammatory) produce IL-1β, IL-6, IL-12 and tumor necrosis factor-α (TNF-α) whereas M2 MΦ (anti-inflammatory) secrete predominantly IL-10 [8-10]. Yet, there is evidence that macrophages may exhibit a remarkable plasticity, allowing them to adapt their functional programs at different levels along the M1-M2 polarization spectrum [7, 11, 12]. Functionally specialized MΦ subsets are found in different tissues and alveolar MΦ were reported to be immunosuppressive, displaying a poor antigen-presenting capacity [13]; similar M2 MΦ-like populations have been found in tumour infiltrates [14, 15]. On the other hand, M1 MΦ are key players in the pathogenesis of some autoimmune disorders, such as rheumatoid arthritis and inflammatory bowel disease [16, 17].

In vitro studies have shown that MΦ are susceptible to lytic infection by low-passage clinical isolates and some endothelial-cell-propagated HCMV strains [18]. Down-regulation of MHC class I and class II molecules, and chemokine receptors have been reported in HCMV-infected MΦ [18, 19]. This viral infection has also been shown to reduce matrix metalloproteinase 9 activity, potentially contributing to atherogenesis [20]. Moreover, HCMV infection of monocytes was observed to promote their differentiation towards a mixed M1-M2 MΦ phenotype [21, 22].

An effective defense against HCMV requires the participation of both innate and adaptive immune responses, involving natural killer (NK) and T cells, together with the production of specific antibodies [23]. Down-modulation of MHC class I molecules in HCMV-infected cells impairs the activation of specific T lymphocytes, rendering them potentially vulnerable to NK cells [24]. NK cell recognition of infected targets involves MHC-specific inhibitory NK cell receptors (NKR) and different activating molecules that control cytotoxicity and cytokine production. HCMV has developed a variety of immune evasion strategies to escape NK cell surveillance, keeping inhibitory receptors engaged [25] or interfering with the expression of ligands for activating receptors [26-28]. Most studies on the NK cell-mediated response to HCMV infection have been carried out in fibroblasts and thus cannot be directly extrapolated to precisely understand the specificities of the cross-talk between NK cells and HCMV-infected myeloid cells. We recently characterized the NK cell response to HCMV-infected autologous monocyte derived dendritic cells (moDC) [29]. Our data indicated that NKp46 and DNAM-1 play a dominant role triggering cytotoxicity and IFN-γ production against infected moDC; the effectiveness of the response was dependent on the time-course of the NK-infected moDC interaction, which influenced the expression of ligands for activating receptors.

In the present report, we extended these studies comparatively analyzing the NK cell response to autologous HCMV-infected M1 and M2 MΦ. Our results reveal differences in the NK cell response triggered by HCMV-infection of different monocyte-derived cell types, which may be relevant for understanding the immunopathology of this viral infection.

MATERIAL AND METHODS

Subjects

Heparinized blood samples were obtained from healthy adult individuals. Written informed consent was obtained and the study protocol was approved by the institutional Ethics Committee (CEIC-IMAS). Standard clinical diagnostic tests were used to analyze serum samples for circulating IgG antibodies against HCMV (Abbott Laboratories, Abbott Park, IL).

Virus stock preparation

TB40/E HCMV stocks (kindly provided by Dr. 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 x 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 for 90 min at 27.000 x g at 15ºC. Pelleted virus was resuspended in DMEM supplemented with 3% fetal calf serum and titrated by standard plaque assays on MRC-5 cells. Virus was inactivated by ultraviolet (UV) light using a UV crosslinker (Biorad GS genelinker UV chamber). A fraction of viral stocks was passed through 0.1 μm filter to eliminate viral particles.

Reagents

Ultra pure Escherichia coli LPS was purchased from InvivoGen (San Diego, CA). Recombinant human IL-12 (rhIL-12) and IFN-α (rhIFN-α) were purchased from PeproTech (London, United Kingdom) and Roche (Nutley, NJ) respectively.

Antibodies, immunofluorescence and flow cytometry analysis

Flow cytometry analysis was performed using monoclonal antibodies specific for the following surface molecules: CD14-Phycoerythrin (PE), CD3-Fluorescein isothiocyanate (FITC), CD56-PE (BD Biosciences Pharmingen, San Jose, CA), CD69-PE and CD25-PE (Immunotools, Friesoythe, Germany), CD163-PE (eBioscience, San Diego, CA). HP-1F7 anti-HLA class I was generated in our laboratory. 2B4.69 (anti-2B4) and 99A anti-CD48 were kindly provided by Dr. P. Engel (Universitat de Barcelona) and Dr. R. Villella (Hospital Clínic, Barcelona) respectively. L95 (IgG1, anti-PVR) and L14 (IgG2a, anti-Nectin-2) were kindly provided by Prof. A. Moretta (University of Genova, Italy) and were previously described [30]. Control IgG2a-PE and PE-conjugated $F(ab')_2$ rabbit anti-mouse Ig were purchased from Becton Dickinson (Mountain View, CA) and DAKO (Glostrup, Denmark), respectively.

Cells were pretreated with human aggregated IgG (10μg/ml) to block Fc receptor, and subsequently labeled with specific antibodies. In the indirect immunostaining, samples were incubated with the unlabeled Abs followed, after washing, by PE-conjugated $F(ab')_2$ rabbit anti-mouse Ig. Flow cytometry analysis was performed with a FACSCalibur and a LSRI instrument; data were processed with Cell Quest Pro (Becton Dickinson, CA) and FlowJo (Tree Star, Inc., OR) softwares. Cellular viability was assessed using the Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol.

For blocking experiments, supernatants of the following monoclonal antibodies were used at saturating concentrations, as described [29]: KL247 (IgM, anti-NKp46), F252 (IgM, anti-NKp30), F5 (IgM, anti-DNAM-1), L95 (IgG1, anti-PVR), L14 (IgG2a, anti-Nectin-2), CO54 (IgM, anti-2B4) and CO202 (IgM, anti-CD48) were kindly provided by Prof. A. Moretta (University of Genova, Italy). BAT221 (IgG1, anti-NKG2D) was kindly provided by Dr. D. Pende. Human IL-12 neutralizing mAb (clone 20C2,

IgG1) was obtained from the American Type Culture Collection. Blocking antibody against IFNAR chain 2 (IFNAR) (clone MMHAR-2, IgG2a) was obtained from Calbiochem (Darmstadt, Germany). Anti-myc mAb (9E10, IgG1) was used as a negative control.

NKp30-Fc and NKp46-Fc recombinant fusion proteins were expressed as previously described [29]. To perform staining, cells were pretreated with rabbit serum (50µl) to block Fc receptors. Subsequently, cells were incubated with NKp30-Fc or NKp46-Fc (3 µg) for 45 min at 4°C followed by PE-conjugated anti–human Ig (Jackson ImmunoResearch Laboratories). Human IgG1 (2 µg) was used as a negative control and 4',6-Diamidino-2-pheny lindole (DAPI) (Sigma, St. Louis, MO) was added to exclude dead cells from the analysis.

Macrophage isolation and differentiation

PBMC were separated from fresh blood by Ficoll-Paque PLUS centrifugation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and extensively washed with PBS for platelet removal. Monocytes were isolated from total PBMC by negative selection using the EasySep™ human monocytes enrichment kit without CD16 depletion (StemCell Technologies, WA), following the manufacturer guidelines. Purity of cell preparation was assessed by flow cytometry using CD14 as a monocyte marker. About 95% cells were $CD14^+$. MΦ were obtained as described previously [31]. Briefly, highly pure M1 and M2 MΦ were obtained after 6 days of culturing in medium (RPMI-1640/glutamax source medium, Invitrogen Life Technologies, Paisley, UK) with 10% (v/v) heat-inactivated low endotoxin fetal bovine serum (Greiner Bio-One GmbH, Frickenhausen, Germany) supplemented with 100ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF, PeproTech, London, United Kingdom) or with 10ng/ml recombinant macrophage colony-stimulating factor (rhM-CSF, Immunotools, Germany) respectively.

Macrophage infection

MΦ were treated overnight with medium (mock), TB40/E (moi 10-100) or the same concentration of UV-inactivated TB40/E (UV-TB40/E), washed twice and used in different assays. Cells at 48h after infection were harvested and cytospin glass slides were prepared by centrifugation ($7x10^4$cells/100µl) for 3 minutes at 500 rpm, using a Cytospin4 Cytocentrifuge (ThermoShandon). Slides were fixed in ice cold absolute methanol and dried at room temperature. After permeabilization with triton, fixed cells were incubated with mouse anti-CMV IE-1/IE-2 monoclonal antibody (clone mab810, Chemicon, Temecula, CA) for 60 min followed by Alexa 448-conjugated $F(ab')_2$ goat anti-mouse Ig (Invitrogen, Carlsbad, CA) and counterstained with DAPI. The percentage of IE1/IE2 positive cells was calculated using Leica DM6000B fluorescence microscope, and cell images were analyzed with the Leica FW4000 Fluorescence Workstation software (Leica, Bensheim, Germany). Based on the percentage of IE-1/IE-2^+ cells, the infection rate of MΦ varied from 10% to 90% in different experiments. When indicated, for each viral preparation the moi was adjusted to achieve comparable infection rates in both MΦ populations (moi M1: 50-75, moi M2: 5-15).

When indicated, rhIFNα or an anti-IFNAR mAbs were added at the time of the infection. Anti-IL-15 mAb (clone 34559) from R&D Systems (Minneapolis, MN) was included as a control. Cells were harvested at 48h post-infection and the percentage of infected cells was assessed by fluorescence microscopy.

NK cell purification and co-culture with macrophages

PBMC were stimulated overnight with 40 U/ml rIL-2 (Proleukin; Chiron, Emeryville, CA) and NK cell enrichment was performed by negative selection using EasySep™ Human NK Cell Enrichment kit (StemCell

Technologies, WA) according to manufacturer instructions, obtaining >98% $CD3^-$ $CD56^+$ populations.

NK cells were resuspended in complete medium and co-cultured for 48 hours with autologous M1 and M2 MΦ (uninfected, UV-TB40/E or TB40/E infected MΦ) in 96 well flat-bottom plates at a MΦ/NK ratio 1:4. All experiments were performed in triplicate.

Degranulation assays

NK cell degranulation was assessed by flow cytometry using surface mobilization of CD107a. Monensin (5ng/ml, Sigma, St. Louis, MO) and FITC-anti-CD107a mAb (BD Biosiciences Pharmingen, San Jose, CA) were added to the NK cell/macrophage coculture for 5h. NK cells were harvested and stained with an anti-CD56-PE mAb. $CD107a^+$ $CD56^+$ cells revealed the degranulated NK cells. HLA class I-defective erythroleukemia K562 cell line was used as a positive control for degranulation. Some experiments were performed in the presence of a panel of blocking NK cell receptor-specific mAbs. When indicated, data were normalized referring for each experiment the numbers of CD107a+ cells to the response of NK cells to HCMV infected MΦ in the absence of mAbs (100%).

Cytokine detection

Cytokine production was assessed in mock, UV-TB40/E or TB40/E HCMV-infected cells 18h post-treatment. IFN-α and IFN-γ were detected in cell supernatants using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Bender MedSystems, San Bruno CA). IL-6, IL-10 and TNF-α were also detected by ELISA (Immunotools, Germany). IL-12 production was analyzed by Human IL-12 (p70) ELISA kit (eBioscience, San Diego, CA).

Statistical analysis

Statistical analysis was performed by the Mann-Whitney U test, using the SPSS 15.0 software (SPSS, Chicago, IL). Results were considered significant at the 2-sided P level of .05.

RESULTS

M1 and M2 MΦ infection by the TB40/E HCMV strain

Monocytes were isolated from total PBMC by negative selection and M1 and M2 MΦ were obtained by culturing monocytes in medium supplemented with rhGM-CSF or rhM-CSF, respectively as detailed in Material and Methods. After 6 days, M1 MΦ were $CD14^{+low}$ $CD163^{-}$ and M2 MΦ were $CD14^{+bright}$ $CD163^{+}$ as assessed by immunofluorescence staining (Figure 1A).

Subsequently, MΦ were incubated either with medium (mock), UV-inactivated TB40/E (UV-TB40/E) or infected with TB40/E HCMV at different moi (1-75); cytospin slides were stained with an anti-IE1/IE2 mAb at 48h after infection. Nuclear IE1 staining was undetectable in mock treated cultures and rare $IE1^{+}$ cells were occasionally observed in UV-TB40/E-treated cultures. M1 MΦ appeared to be more resistant to HCMV infection than M2 MΦ (Figure 1B).

As compared to mock and UV-TB/40E-treated MΦ, the expression of HLA class I molecules decreased in TB40/E HCMV-infected cells, as already reported in previous studies [18, 19]. In some experiments, the moi was adjusted to achieve comparable infection rates in both MΦ populations, which showed similar downregulation of HLA class I molecules (Figure 1C). The viability at 48h after infection, assessed by Annexin V/ propidium iodide staining was of 87-95% of live cells and was comparable to that of UV-TB40/E-treated MΦ (data not shown).

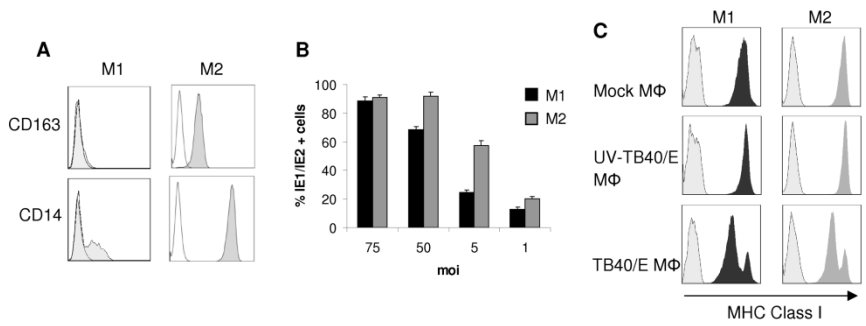

Figure 1. M1 and M2 MΦ phenotype upon HCMV infection. (A) M1 and M2 MΦ phenotype was assessed by immunofluorescence staining. **(B)** MΦ were infected at different moi and at 48h post-infection cells were stained with anti-IE1/IE2 viral antigen mAb and counterstained with DAPI. **(C)** At 48h post-infection, cells were stained with an anti-MHC class I mAb and analyzed by flow cytometry (open histograms, isotype control; shaded histograms specific staining). A representative experiment out of four performed is shown (% of IE1/IE2$^+$ cells in M1 and M2 MΦ was 70 and 65%, respectively).

Cytokine production by HCMV-infected M1 and M2 MΦ

To assess cytokine production in response to HCMV infection, MΦ were mock-, UV-TB40/E and TB40/E HCMV-treated for 18 hours. Cell culture supernatants were harvested and cytokine production was measured by ELISA. Upon LPS treatment, M1 and M2 MΦ secreted IL-6 and IL-10 respectively, confirming their pro- and anti-inflammatory phenotypes (Figure 2A). TB40/E HCMV-infected M1 MΦ produced IL-12 and high concentrations of IL-6; by contrast, only limited concentrations of IL-6 and IL-10 were detected upon infection of M2 MΦ. Moreover, HCMV-infected M1 MΦ also produced higher concentrations of IFN-α and TNF-α than M2 MΦ (Figure 2A).

The possibility that IFN-α secretion by M1 MΦ accounted for their relative resistance to HCMV infection was addressed. Incubation of M1 MΦ with an anti-IFNAR mAb at the time of the infection rendered them more permissive to HCMV infection. On the other hand, upon incubation with IFN-α M2 MΦ became more resistant, thus supporting a role of IFN-α in

the different susceptibility of M1 and M2 MΦ to HCMV infection (Figure 2B).

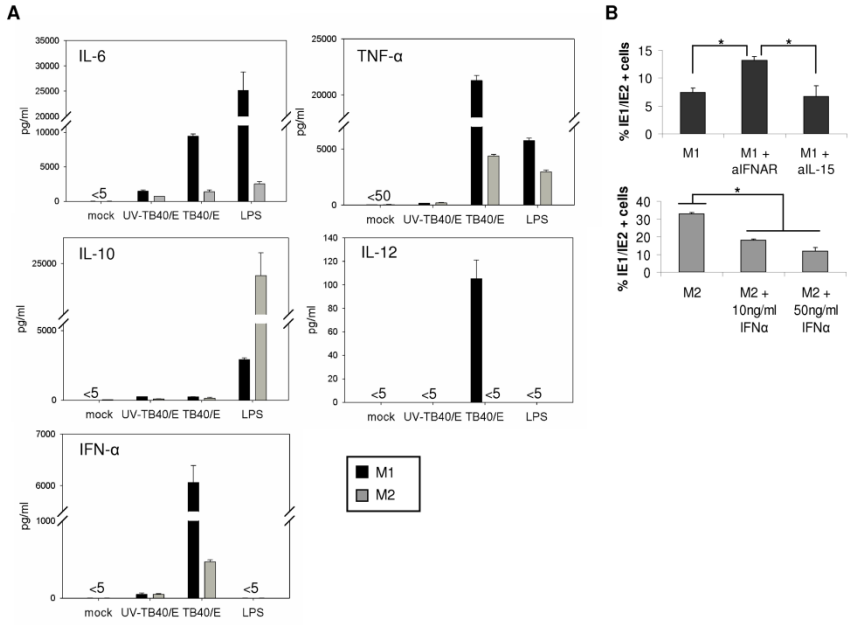

Figure 2. Cytokine production by HCMV-infected M1 and M2 MΦ. (A) IL-6, TNF-α, IL-10, IFN-α and IL-12 were measured by ELISA in the supernatants of mock, LPS (100ng/ml), UV-TB40/E or TB40/E HCMV-treated for 18h M1 and M2 MΦ. A representative experiment out of five performed is shown (% of IE1/IE2$^+$ cells in HCMV-infected M1 and M2 MΦ was 30%). **(B)** M1 and M2 MΦ were TB40/E HCMV-infected in the presence of an anti-IFNAR, an anti-IL-15 mAb, or different concentrations of exogenous IFN-α, respectively. At 48h post-infection, cells were indirectly stained with anti-IE1/IE2 viral antigen mAb. Statistical analysis was performed by the Mann-Whitney U test. Data correspond to mean ± SEM (*$p<0.05$).

NK cells comparably degranulate in response to HCMV-infected M1 and M2 MΦ

To determine the ability of HCMV-infected M1 and M2 MΦ to trigger NK cell cytotoxicity, we analyzed NK cell degranulation by assessing the surface expression of CD107a (LAMP-1). NK cells were purified by negative selection from PBMC cultured overnight with IL-2. CD107a surface expression was analyzed by flow cytometry in purified NK cell populations

incubated with mock, UV-TB40/E and TB40/E HCMV-treated autologous M1 and M2 MΦ for 5h (MΦ/NK ratio=1:4). The moi was adjusted to achieve comparable infection rates in both MΦ populations (moi M1= 50, moi M2= 10) (Figure 1C). The K562 erythroleukemia cell line was included as a positive control (Figure 3A). A significant increase in the percentage of $CD107a^+$ $CD56^+$ NK cells was specifically detected in response to TB40/E HCMV-infected MΦ, but not upon incubation with mock or UV-TB40/E-treated MΦ. HCMV-infected M1 and M2 MΦ comparably triggered NK cell cytotoxicity (Figure 3B).

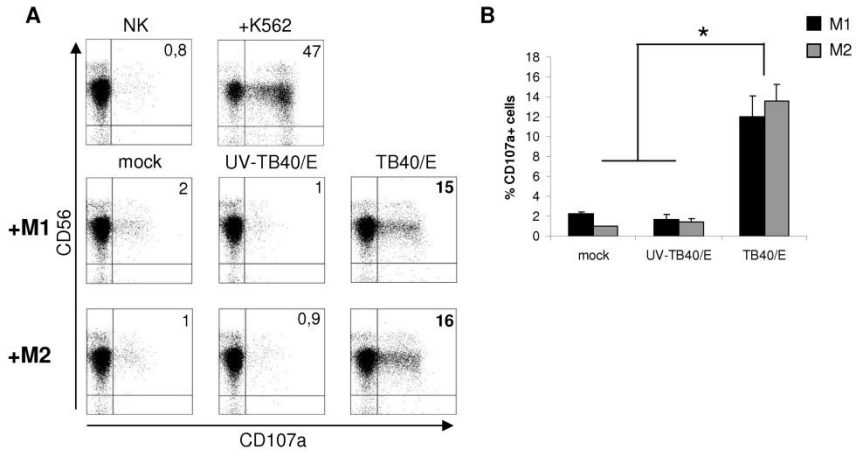

Figure 3. NK cell degranulation against HCMV-infected autologous M1 and M2 MΦ. M1 and M2 MΦ were untreated (mock), incubated with UV-TB40/E or TB40/E HCMV. Moi was adjusted to achieve comparable infection rates in both populations. **(A)** After 48h, cells were co-cultured with autologous purified NK cells as explained in methods, and the percentages of $CD107a^+$ NK cells were assessed by flow cytometry. The target cell line K562 was added as a positive control (% of $IE1/IE2^+$ cells in M1 and M2 MΦ; 45%). **(B)** Graphs represent the proportions of $CD107a^+$ NK cells (% of $IE1/IE2^+$ cells in M1 and M2 MΦ was 30-45%). Statistical analysis was performed by the Mann-Whitney U test. Data correspond to mean ± SEM (*$p<0.05$).

To investigate the nature of activating receptors involved in this process, degranulation assays were carried out in the presence of blocking mAbs specific for different activating receptors. As shown in Figure 4, anti-NKp46 (NCR1), -DNAM-1 and -2B4 mAbs partially prevented CD107a expression,

supporting that these receptors participate in the NK cell response against HCMV-infected M1 and M2 MΦ. To further confirm the role of 2B4 and DNAM-1 receptors, the effect of mAbs specific for their ligands was tested.

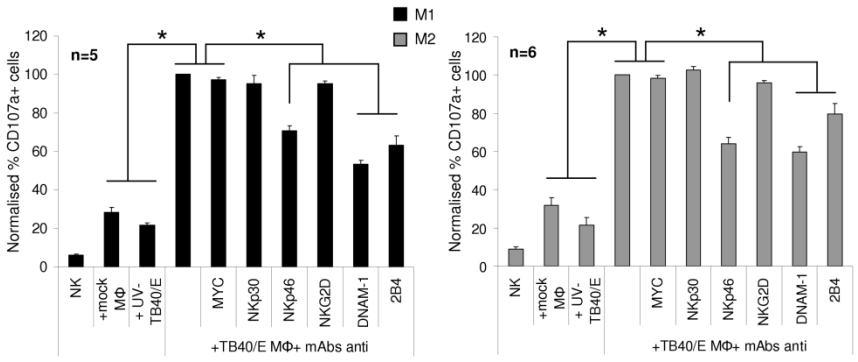

Figure 4. NK cell degranulation against HCMV-infected M1 and M2 macrophages is partially blocked by anti-NKp46, -DNAM-1 and -2B4 mAbs. Autologous NK cells were incubated with different NKR-specific blocking mAbs and co-cultured with TB40/E HCMV-infected M1 and M2 MΦ. Data were normalized referring for each experiment the numbers of $CD107a^+$ cells to the response of NK cells to HCMV infected MΦ in the absence of mAbs (100%). Statistical analysis was performed by the Mann-Whitney U test. Data correspond to mean ± SEM (*$p<0.05$).

Anti-CD48 and -2B4 mAbs comparably inhibited the response to HCMV-infected MΦ (Figure 5B). Though DNAM-1 ligand mAbs (PVR and Nectin-2) individually exerted a limited antagonistic effect on NK cell degranulation, they did inhibit the response similarly to anti-DNAM-1 mAb (Figure 5A) when used in combination. It is of note that no effect was observed with the anti-NKG2D mAb as previously described by moDC [29].

PVR and Nectin-2 were previously reported to be down-regulated in moDC infected by the TB40/E HCMV strain, an effect known to be mediated by the UL141 immunoevasin [28, 32]. At 48 h post infection, the surface levels of PVR and Nectin-2 were also partially reduced in TB40/E-infected M1 and M2 MΦ as compared to UV-TB40/E treated MΦ (Figure 5C). Yet, their contribution to the NK cell response against infected cells was perceived at

that stage of infection, as shown above. Remarkably, CD48 was also down-regulated in HCMV-infected cells, thus suggesting the existence of a putative viral immune evasion strategy targeting the 2B4 pathway. It is of note that the involvement of 2B4 was not perceived in our previous studies on moDC, that do not express CD48 [33].

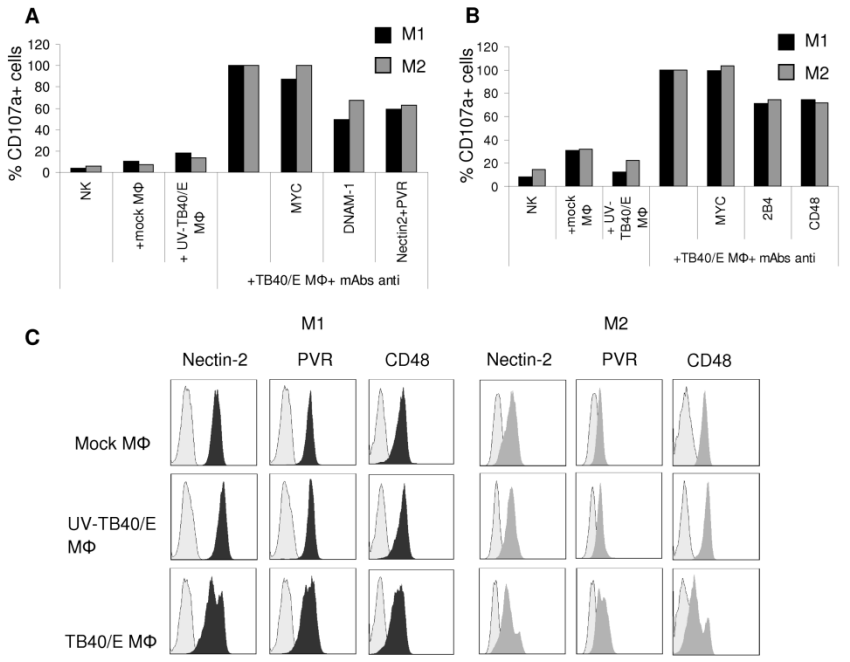

Figure 5. HCMV infection of M1 and M2 MΦ down-regulates the expression of CD48 (2B4 ligand), Nectin-2 and PVR (DNAM-1 ligands). (A) Autologous NK cells were incubated with TB40/E-infected M1 and M2 MΦ in the presence of anti-DNAM-1 or a combination of anti-PVR and anti-Nectin-2 mAbs. CD107a expression by NK cells was analyzed. **(B)** Autologous NK cells were incubated with TB40/E-infected M1 and M2 MΦ, and CD107a expression was analyzed in the presence of anti-2B4 or anti-CD48 mAbs at 48h of co-culture. Results of a representative experiment out of three is shown. (% of IE1/IE2+ cells in M1 and M2 MΦ: 60%). **(C)** At the same time point, expression of Nectin-2, PVR and CD48 was assessed in mock, UV-TB40/E treated, or TB40/E HCMV-infected M1 and M2 MΦ (open histograms, isotype control; shaded histograms, specific staining). A representative experiment out of three is shown (% of IE1/IE2+ cells in M1 and M2 MΦ: 70%).

As reported for moDC [29], NKp30 and NKp46 ligands analyzed using the corresponding NCR-Fc (natural cytotoxicity receptor-Fc) recombinant

proteins, were also constitutively expressed in M1 and M2 MΦ, and their expression at 48 hours post-infection was decreased (data not shown). Yet, only anti-NKp46 mAb inhibited degranulation indicating that this NCR plays a dominant role in the response to HCMV-infected macrophages.

Differential ability of HCMV-infected M1 and M2 MΦ to trigger NK cell-mediated IFN-γ production

NK cell populations were incubated alone or in the presence of mock, UV-TB40/E and TB40/E HCMV-treated autologous M1 and M2 MΦ (MΦ/NK ratio=1:4). The moi was adjusted to achieve comparable infection rates in both MΦ populations. IFN-γ secretion was measured by ELISA in culture supernatants at 24 and 48h of co-culture. In the presence of HCMV-infected M1 MΦ, NK cells secreted higher amounts of IFN-γ than in the presence of HCMV-infected M2 MΦ, displaying a minimal or undetectable response to mock or UV-TB40/E-treated MΦ (Figure 6A). HCMV-infected M1 MΦ also induced the expression of CD25 and CD69 NK cell activation markers more efficiently than HCMV-infected M2 MΦ (Figure 6B).

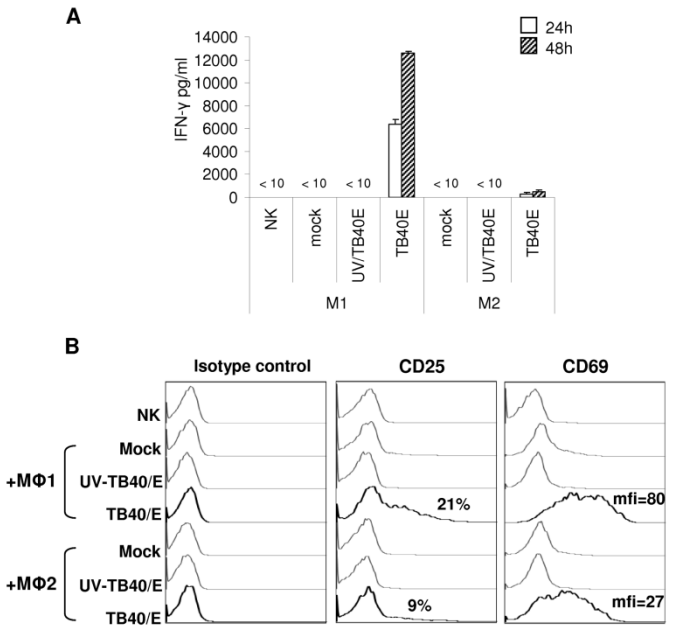

Figure 6. HCMV-infected M1 MΦ activate NK cell-mediated IFN-γ production and induce CD25 and CD69 expression more efficiently than M2 MΦ. Autologous NK cells were co-cultured with mock, UV-TB40/E and TB40/E HCMV infected M1 and M2 MΦ. **(A)** IFN-γ secretion was measured by ELISA in culture supernatants at 24 and 48h of co-culture. **(B)** Expression of CD25 and CD69 was assessed by flow cytometry in NK cells at 48h of co-culture. A representative experiment out of four is shown (% of IE1/IE2+ cells in M1 and M2 MΦ: 35%).

Several approaches were undertaken to elucidate the basis of the different NK cell-mediated IFN-γ production triggered by TB40/E-infected M1 and M2 MΦ. First, filtered supernatants from HCMV infected-M1 MΦ were observed to partially restore the ability of NK cells co-cultured with M2-infected MΦ to produce IFN-γ (Figure 7A), thus indirectly supporting the contribution of soluble factors. Considering the different ability of both populations to produce IL-12 (Figure 2A), NK cells and M2-infected MΦ were co-cultured in the presence of suboptimal concentrations of exogenous rhIL-12. These experiments revealed that the cytokine synergized with HCMV-infected-M2 MΦ to induce NK cell-mediated IFN-γ production (Figure 7B). On the other hand, when NK cells were co-cultured with

TB40/E HCMV-infected M1 MΦ in the presence of anti-IL-12 blocking mAb, a significant reduction of IFN-γ secretion was observed (Figure 7C). Altogether, these results support that the poor IL-12 production by HCMV-infected M2 MΦ determines, at least partially, their reduced ability to trigger NK cell-mediated IFN-γ secretion.

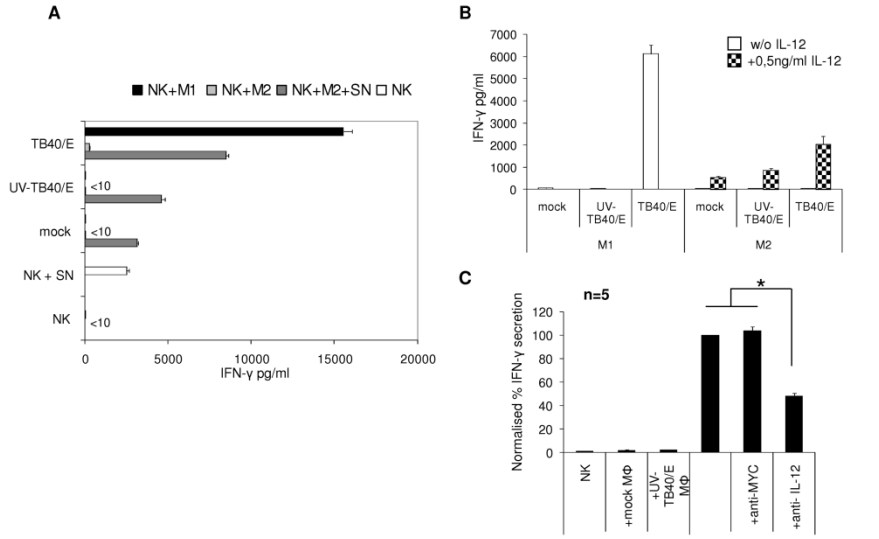

Figure 7. IL-12 is involved in the NK cell-mediated IFN-γ production in response to HCMV infected M1 MΦ. (A) NK cells were co-cultured with autologous TB40/E-infected M1 and M2 MΦ in the absence or presence of filtered supernatants from infected-M1 MΦ; IFN-γ secretion was measured at 48h by ELISA. Supernatants of infected-M1 MΦ partially restored the ability of NK cells cultured with M2-infected MΦ to produce IFN-γ. **(B)** Exogenous IL-12 (0.5 ng/ml) synergized with HCMV-infected-M2 MΦ to induce NK cell-mediated IFN-γ production. A representative experiment out of three performed is shown (% of IE1/IE2+ cells in M1 and M2 MΦ: 25%). **(C)** Autologous NK cells were co-cultured with TB40/E HCMV-infected M1 MΦ in the presence of anti-IL-12 mAb, and IFN-γ secretion was measured at 48h. In each experiment, data were normalized to the IFN-γ levels detected in supernatants of NK/ MΦ co-culture in the absence of mAbs (100%). Statistical analysis was performed by the Mann-Whitney U test. Data correspond to mean ± SEM of 5 different experiments (*$p<0.05$). (% of IE1/IE2+ cells in M1 and M2 MΦ: 30%).

DISCUSSION

Information on the immune response to HCMV is largely based on *in vitro* studies carried out with infected fibroblasts, despite the fact that the virus displays a wide tropism for different cell types. Among them, myeloid cells may play a key role as they are susceptible to HCMV infection and, moreover, they differentiate from hematopoietic precursors that constitute a viral reservoir, thus contributing to the systemic dissemination of the pathogen [4]. To more precisely understand the immunopathology of HCMV infection, we developed suitable experimental conditions to comparatively analyze the NK cell response to different autologous myelomonocytic cell types infected with the TB40/E HCMV strain [18]. We herein report our observations on the interaction between NK cells and monocyte-derived HCMV-infected pro-inflammatory MΦ (M1) or anti-inflammatory MΦ (M2), extending our recent studies on infected moDC [29]. The results reveal qualitative differences in the NK cell response upon infection of the distinct monocytic cell types, thus providing further insights on the immunopathology of the viral infection.

Remarkably, a different susceptibility of M1 and M2 macrophages to HCMV was observed, and higher moi were required to infect M1 MΦ; similar differences in resistance to infection of M1 and M2 MΦ were reported for other intracellular pathogens *(i.e. Mycobacterium)* [34]. Moreover, the cytokine secretion pattern in response to HCMV infection also differed, reflecting at least in part their functional polarization. HCMV-infected M1 MΦ produced IL-12, IL-6, TNF-α and IFN-α, whereas M2 MΦ secreted lower amounts of TNF-α and IFN-α, failed to release detectable levels of IL-12 and secreted poorly IL-10, despite that, consistent with their anti-inflammatory phenotype, they secreted IL-10 upon LPS treatment. Interestingly, M2 infected MΦ, albeit to a moderate extent, displayed a pro-inflammatory cytokine secretion pattern [35]. The relative resistance of M1 MΦ to

infection appeared related to their ability to secrete higher levels of type I IFN.

The NK cell cytolytic machinery was triggered by both HCMV-infected M1 and M2 MΦ involving NKp46, DNAM-1 and 2B4 receptors, as indirectly assessed by the antagonistic effects of the corresponding mAbs. NKp46 and DNAM-1 were previously reported to play a dominant role in the response to HCMV-infected moDC [29], reflecting the similarities in the pattern of response to the different myelomonocytic cell types. Interestingly, 2B4 was involved in the response to macrophages but not to moDC, which did not express its ligand (CD48) [33]; these results are consistent with the role of 2B4 as a triggering receptor in activated NK cells [36, 37].

It is of note that the ligands for these activating receptors were constitutively expressed by both M1 and M2 cells. In this regard, the crosstalk between activated NK cells and non-infected macrophages inducing IFN-γ secretion was recently shown to involve DNAM-1 and 2B4 [38, 39], while NKp46 and DNAM-1 contributed to the lysis of MΦ [38]. These observations support that the NK cell response against HCMV-infected monocytic cell types, which down-regulate HLA class I molecules, appears mainly driven by receptors specific for constitutively expressed ligands, consistent with the orthodox "missing self" concept.

Among other immune evasion strategies, HCMV inhibits the expression of ligands for activating NK cell receptors. In particular, several mechanisms have evolved to impair the expression of NKG2D-L in infected cells, thus effectively preventing the participation of this killer lectin-like receptor (KLR), as observed in moDC [29]. As observed in other cell types, expression of DNAM-1L (PVR and Nectin-2) was down-regulated in infected macrophages, likely involving the UL141 immunoevasin [28, 29, 32]. Moreover, upon HCMV infection, binding of NKp46-Ig and NKp30-Ig fusion proteins to MΦ was also reduced. In that case, non-infected bystander MΦ were affected, thus suggesting the indirect involvement of

soluble factors, rather than a conventional immune evasion mechanism selectively acting on infected cells. Molecular characterization of NKp46 ligand is warranted to characterize in detail the underlying mechanism(s). Together with the reported effect of type I IFN and IL-12 on NKG2D expression [40], these observations suggest the existence of negative regulatory feedback mechanisms, transiently interfering with the expression of potentially self-reactive receptor/ligand pairs at late stages of the anti-viral inflammatory response. Remarkably, a decreased CD48 expression was detected only in HCMV-infected MΦ, with a pattern similar to HLA class I down-regulation, thus suggesting the existence of a putative novel immunoevasion strategy targeting the 2B4 ligand.

It is of note that, despite the partial reduction of CD48, DNAM-1L and NKp46L expression at 48 h post-infection, infected MΦ triggered NK cell activation, as previously reported for infected moDC [29], supporting that the response may depend on the time-course of HCMV infection. This might contribute to explain the discrepancy with a previous report describing that HCMV-infected MΦ were resistant to NK cell cytotoxicity independently of MHC class I down-regulation and the expression of the UL18 HCMV class I homologue [41]. A number of additional experimental differences, such as the use of the AD169 HCMV strain by Odeberg et al [41], render difficult the comparison of both studies.

A major difference between M1 and M2 MΦ was their ability to stimulate IFN-γ production by NK cells. Despite that both HCMV-infected MΦ subsets comparably triggered NK cell degranulation, only M1-infected MΦ produced IL-12 and effectively activated NK cell-mediated IFN-γ secretion. This effect was partially inhibited by an anti-IL-12 mAb, supporting a role for the cytokine. It is of note that HCMV infection of M1 MΦ directly triggered IL-12 secretion, in contrast to the requirement of IFN-γ to promote IL-12 production in response to *Mycobacterium* [10, 34].

The possibility that inhibitory mechanisms may also contribute to the the low IFN-γ production by NK cells in response to infected M2 MΦ should be considered. In this regard, filtered supernatants from HCMV infected-M2 MΦ were observed to partially inhibit the ability of NK cells co-cultured with M1-infected MΦ to produce IFN-γ (data not shown). However, when rh-IL-10 (25 ng/ml) was added to the co-culture only a small reduction of NK cell IFN-γ secretion was observed (data not shown). Considering the low levels of IL-10 production detected in M2-infected MΦ (Figure 2A), it is unlikely that the regulatory cytokine may underlie the low IFN-γ production by NK cells. Yet, a contribution of the IL-10 viral homologue (cmvIL-10) or additional inhibitory mechanisms cannot be ruled out. Among them, Activin A has been described to inhibit NK cell IFN-γ production [42]; though Activin A appears preferentially released by M1 MΦ [31], its expression by HCMV-infected M2 should be explored. IFN-γ activates MΦ, enhancing antigen presentation and the synthesis of pro-inflammatory cytokines [43], promoting as well Th1 polarization and T-bet expression [44, 45]. Moreover, acting synergistically with IFN-α, produced upon M1 MΦ HCMV infection, IFN-γ may efficiently inhibit viral replication and dissemination [46].

Thus, our results strongly suggest that *in vivo* HCMV infection of M1 and M2-like MΦ might have rather different immunopathological consequences. In this regard, lung MΦ were shown to be immunosuppressive and displayed a poor antigen-presenting capacity [13]. HCMV-infected lung macrophages have been detected and shown to express proteins representative of all stages of viral replication cycle [47]. Interstitial pneumonitis remains the most severe complication caused by HCMV in allogeneic hemopoietic stem cell transplantation, with high a mortality rate [48, 49]. We hypothesize that the susceptibility of M2 MΦ to HCMV infection, associated to their poor IFN-α and pro-inflammatory cytokine secretion, together with their limited ability to trigger NK cell IFN-γ production might facilitate the replication and spread of the pathogen in tissues where this MΦ cell type is abundant.

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AUTHORSHIP CONTRIBUTION

NR designed and performed experiments, analyzed results, and wrote the manuscript; ML-B and MG designed the research, analyzed results and wrote the manuscript; GM helped in the experimental work and discussed results; AM and AA analyzed and discussed results; DB and GH helped in the experimental work.

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Chapter 4

Association of atherosclerosis with expression of the LILRB1 receptor by human NK and T cells supports the infectious burden hypothesis

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Association of atherosclerosis with expression of the LILRB1 receptor by human NK and T cells supports the infectious burden hypothesis

SHORT TITLE: Infectious burden and atherosclerosis

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ABSTRACT

Objective: The contribution of human cytomegalovirus (HCMV) to vascular disease may depend on features of the immune response not reflected by the detection of specific antibodies. Persistent HCMV infection in healthy blood donors has been associated with changes in the distribution of NK cell receptors (NKR). The putative relationship among HCMV infection, NKR distribution, subclinical atherosclerosis and coronary heart disease was assessed.

Methods and results: NKR expression was compared in acute myocardial infarction (AMI) patients (n=70) and a population-based control sample (n=209). The relationship between NKR expression and carotid intima-media thickness (CIMT) in controls (n=149) was also studied. HCMV infection was associated with higher proportions of NKG2C+ and LILRB1+ NK and T cells. In contrast, only LILRB1+ NK and CD56+ T cells were found to be increased in AMI patients, independent of age, sex, conventional vascular risk factors and HCMV seropositivity. Remarkably, LILRB1 expression in NK and T cells significantly correlated with CIMT in controls.

Conclusions: The association of overt and subclinical atherosclerotic disease with LILRB1+ NK and T cells likely reflects a relationship between the immune challenge by infections and cardiovascular disease risk, without attributing a dominant role for HCMV. Our findings may lead to the identification of novel biomarkers of vascular disease.

INTRODUCTION

Inflammation is a hallmark in the pathogenesis of atherosclerosis, which causes myocardial infarction and stroke [1;2]. Endothelial dysfunction is considered an initial event in the development of atherosclerotic plaques, as it promotes the migration of leucocytes and monocytes into the vessel wall, where macrophage interactions with T cells are believed to play an important pathogenic role [3]. Beyond the influence of conventional risk factors (i.e. smoking, hypertension, diabetes mellitus, hypercholesterolemia), infections have been also related to atherosclerosis [4-6]. Yet, the issue has remained controversial owing to the fact that the circumstantial evidences do not fulfil conventional criteria for causality, and the mechanisms whereby microbial pathogens might contribute to atherogenesis are uncertain. In fact, different bacteria and viruses have been associated with vascular disease, mainly based on seroepidemiological studies that provide only partial information on the host-pathogen relationship [7;8]. Moreover, infectious agents have been only occasionally isolated from vascular lesions [9], and clinical trials have failed to show any beneficial effect of antibiotic therapy on myocardial infarction recurrence or long-term complications in patients with chronic coronary disease [10;11]. The "infectious burden" (IB) hypothesis reconciled to some extent apparently contradictory studies, proposing that different microbial agents contribute to the risk of vascular disease in a cumulative manner; yet, the search for suitable biomarkers to further validate the IB hypothesis is warranted [12;13].

Human cytomegalovirus (HCMV) is believed to be involved in the development of atherosclerosis, mainly based on three lines of evidence: a) the epidemiological observation of a higher frequency of HCMV seropositivity in patients with atherosclerotic diseases as compared with healthy controls, not confirmed by all studies [14;15]; b) observations linking HCMV to vascular lesions in chronic graft rejection and coronary re-stenosis

post-angioplasty [16;17]; and c) the ability of murine CMV infection to accelerate the development of vascular lesions in Apo-E$^{-/-}$ mice, an effect also promoted by the inactivated virus, thus supporting an indirect contribution to the inflammatory process [18;19].

HCMV infection is highly prevalent and the virus remains in a lifelong latent state in healthy individuals, occasionally undergoing subclinical reactivations [20]. Beyond its pathogenic role in immunocompromised patients and congenital infection, HCMV seropositivity has been proposed as being linked to an accelerated immunosenescence and a shorter life-span [21;22]. It is conceivable that the putative role of HCMV in the pathogenesis of atherosclerosis may ultimately depend on features of the complex host-pathogen interaction not reflected by the simple detection of circulating specific antibodies [23-25].

In this regard, it has been shown that HCMV infection may alter to a variable extent the distribution of NK cell receptors (NKR). Increased proportions of NK and T cell subsets expressing CD94/NKG2C, an activating lectin-like NKR specific for the HLA-E class I molecule [26;27], were associated with a positive serology for HCMV [28-31]; moreover, NKG2C+ NK cells expanded *in vitro* in response to HCMV-infected fibroblasts [28;32].

In addition, increased proportions of LILRB1+ NK and T cells [28] were also detected in HCMV+ individuals. LILRB1 (ILT2, LIR-1, CD85j) is an inhibitory receptor expressed by different leukocyte lineages, which specifically interacts with HLA class I molecules and the UL18 HCMV glycoprotein [33;34] regulating cell activation. LILRB1 expression has been associated with late differentiation stages of T lymphocytes specific for different microbial pathogens [35-37].

In the present study, we addressed whether the impact of HCMV infection on the NKR distribution might reflect its putative role in the pathogenesis of atherosclerosis. To challenge this hypothesis, a population-based case-control study was designed comparing the expression of NKG2C and

LILRB1 in NK and T cells from patients studied within 72 h after acute myocardial infarction (AMI), and from control individuals without clinical evidence of cardiovascular disease. In a subsample of the latter, the relationship between NKR expression and carotid intima-media thickness (CIMT) was also assessed.

METHODS

Design and subjects

Two different designs were used to test our hypothesis:

a) A case-control study was carried out to assess the association between NKR expression and AMI. Cases were 70 patients (aged 34 to 87 years) with confirmed AMI (Hospital Trueta, Girona and Hospital del Mar, Barcelona, Spain). Controls were contemporarily participants in a population-based cohort study (REGICOR-HERMES, Girona, Spain)[1] undertaken in the same area (3 controls per case). To control for differences in sex and age we also carried out an age- and sex-matched case-control study in a subsample including 62 cases and 124 controls.

b) A cross-sectional study was designed to assess the association between NKR expression and carotid intima media thickness (CIMT) in a group of participants of the population-based cohort study free of clinical disease (n=149).

In cases, blood samples were obtained within the first 72 h after symptom onset (n=70) and also 6 months later to the acute event (n=53). All the biological samples were coded, shipped at 4ºC to a central laboratory and analysed within a period of 48h. Waist perimeter was measured at the middle point between the last rib and iliac crest. Height and weight were measured with calibrated instruments and subjects in underwear. Body mass index (BMI) was calculated as weight (kg)/ height $(m)^2$. Written informed consent was obtained from every donor, and the study protocol was approved by the local Ethics Committee (CEIC, Parc de Salut Mar).

Antibodies and reagents

Anti–NKG2C-PE monoclonal antibody (mAb) was from R&D Systems, Inc. Z199 (anti-NKG2A) mAb was kindly provided by Dr. A. Moretta (University of Genova). Z199 mAb was conjugated to fluorescein

isothiocyanate (FITC) (Sigma-Aldrich, St. Louis, MO). Anti-LILRB1-FITC, CD3-PerCP, CD56-APC, CCR7-PE-Cy7, CD8-PE and CD4-PE were from BD Biosciences Pharmingen (San Jose, CA). Anti-CD45RA-APC was from Immunotools (Friesoythe, Germany). Anti-CD27 mAb (clone 143-14) was kindly provided by Dr. R. Vilella (Hospital Clinic, Barcelona), and the HP-F1 anti-LILRB1 mAb was generated in our laboratory [2]. Indirect immunofluorescence analysis was carried out with FITC-tagged or PE-tagged F(ab')$_2$ rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark). In whole blood samples, erythrocytes were lysed using FACS lysis buffer (Becton Dickinson). A commercially available ELISA kit (Bioelisa CMV Colour; Biokit, Barcelona, Spain) was used to determine circulating antibodies against HCMV. High-sensitivity C-reactive protein (hs-CRP) was measured by immunoturbidimetry (ABX-Horiba Diagnostics, Irvine, CA) in an autoanalyser PENTRA-400 (ABX-Horiba Diagnostics, Irvine, CA).

Immunofluorescence and flow cytometry analysis

The expression of NKG2A, NKG2C and LILRB1 was analysed by flow cytometry in fresh peripheral blood samples, obtained by venous puncture in EDTA tubes. For multicolor staining the following procedures were used. Protocol 1: whole blood samples were incubated with anti–NKG2A-FITC; subsequently, samples were incubated with anti–NKG2C-PE, anti–CD56-APC, and anti–CD3-PerCP. In parallel, samples were incubated with anti-LILRB1, anti-CD3 and anti-CD56. After washing, erythrocytes were lysed. It is of note that in our first original report the analysis was restricted to the NKG2C^{bright} cell subset as indicated [3], whereas total NKG2C+ lymphocytes were considered in the present study. Protocol 2: PBMC were isolated by centrifugation on Ficoll-Hypaque (Axis Shield PoC AS). Indirect immunofluorescence staining was performed with anti-LILRB1 mAb and a FITC-tagged F(ab')$_2$ rabbit anti–mouse Ig antibody; subsequently, samples were incubated with anti–CD56-APC, CD3-PerCP and anti-CD4 or CD8-

PE. Protocol 3: PBMC were isolated and an indirect immunofluorescence staining was performed with anti-LILRB1 unconjugated mAb and washed cells were labeled with FITC-tagged $F(ab')_2$ rabbit anti–mouse Ig antibody; subsequently, samples were incubated with anti–CD8-PE, CCR7-PE-Cy7 and CD45RA-APC. Protocol 4: PBMC were isolated and an indirect immunofluorescence staining was performed with anti-CD27 unconjugated mAb and washed cells were labeled with PE-tagged $F(ab')_2$ rabbit anti–mouse Ig antibody; subsequently, samples were incubated with anti–LILRB1-FITC, CD3- PerCP and CD56-APC, and were analyzed by flow cytometry (FACSCalibur; Becton Dickinson). For the sake of precision, flow cytometry data analysis was performed by a single researcher (NR).

Carotid artery ultrasound

B-mode ultrasound imaging of the carotid arterial walls was used to non-invasively assess intima-media thickness according to standardized and validated imaging and image analysis protocols. These protocols have been described in detail elsewhere [4]. In summary, ultrasound communication infrastructure in Girona was set up and sonographers trained and certified by AMC Vascular Imaging (VI), Amsterdam, The Netherlands. An Acuson Aspen with an L7 5-12MHz linear array vascular transducer (Siemens, Erlangen, Germany) was used. A specifically designed REGICOR scan application protocol was developed for the ultrasound equipment to ensure standardization throughout the study. In this application protocol images, clips and demographic information is incorporated into DICOM (Digital Imaging and Communications in Medicine) files as to provide a secure and efficient handling and tracebility record of clinical information. Subjects were scanned in the supine position. Bilaterally, from a single latero-lateral transducer angle, the far walls of the common carotid were imaged by B-mode ultrasound. High resolution stills as well as a dynamic clips of each segment were saved. All images were analysed off-line, centrally in

Amsterdam using the eTrack image analysis program. Primary endpoint of the ultrasound study is the per subject average mean common carotid IMT (CIMT).

Statistical analysis

Normality plots were used to assess whether a continuous variable followed a normal distribution or not. Continuous normal distributed variables were summarized as means and standard deviations; continuous non-normal distributed variables were summarized as medians and first and third quartiles; categorical variables were presented as absolute frequencies and proportions. Student's t test was used to compare means for normal distributed variables and Mann-Whitney U test was used to compare medians for continuous non-normal distributed variables. Chi-squared test or exact Fisher test were used as appropriate to compare proportions. Spearman correlation was used to assess the association between continuous variables.

To estimate adjusted means, multivariate linear regression was fitted. A logarithmic transformation was done if the response variable distribution departed from normality.

For age- and sex-matched case-control analysis, an algorithm was applied with the following criteria: every case was matched to two controls of the same sex and with a similar age ($\pm$ 5 years).

Statistical significance was set up at p-values<0.05. Statistical analysis was done with R software, version 2.10.1.

RESULTS

The expression of NKR was analysed by flow cytometry in fresh blood samples from AMI patients (N=70) and controls (N=209). NK cells (CD3-CD56+), T lymphocytes (CD3+), and T cell subsets defined by CD56 expression were gated (Figure 1). Conventional risk factors for cardiovascular disease, HCMV seroprevalence and hs-CRP levels in cases and controls are displayed in Table 1. Considering the demographical differences observed, a subsample of age- and sex-matched cases (N= 62) and controls (N=124) was separately analysed (Table 1).

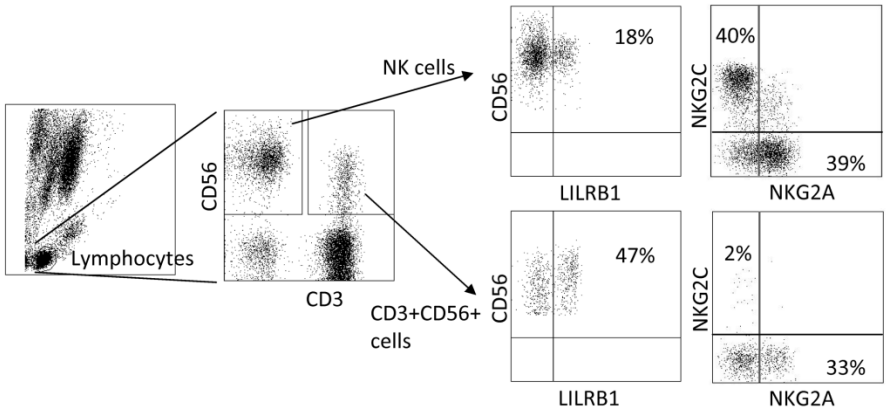


Figure 1. Analysis of NKG2A, NKG2C and LILRB1 expression. Whole blood from a healthy donor was stained with anti-LILRB1, anti-CD3, and CD56 mAbs. Samples were analyzed by flow cytometry and the proportions of LILRB1+ cells were calculated gating NK cells (CD3+ CD56-) as well as the CD3+ CD56+ T cell subset. Alternatively, whole blood was stained with anti-NKG2C, anti-NKG2A, anti-CD3 and anti-CD56 mAbs. Numbers correspond to the percentages of positive cells.

Table 1. Cardiovascular risk factors in AMI cases and controls

	Total controls N=209	**Total cases N=70**	**p-value**	**Matched § controls N=124**	**Matched § cases N=62**	**p-value**
Age (years) *	57.0 (11.4)	62.9 (12.3)	**<0.001**	60.5 (11.0)	60.7 (11.1)	0.910
Sex (% female) †	63 (30.1)	11 (15.7)	**0.018**	18 (14.5%)	9 (14.5%)	>0.999
Active Smoker or ex < 1 year (%)†	50 (24.3)	29 (41.4)	**0.006**	31 (25.2%)	28 (45.2%)	**0.006**
BMI (kg/m^2) *	27.8 (3.98)	26.7 (4.18)	**0.048**	27.7 (3.76)	26.8 (4.20)	0.140
Informed of hypertension†	78 (37.3)	47 (70.1)	**<0.001**	50 (40.3%)	40 (67.8%)	**0.001**
Informed of high blood glucose (%)†	25 (12.0)	23 (33.8)	**<0.001**	17 (13.7%)	17 (28.3%)	**0.017**
Informed of high cholesterol (%)†	77 (36.8)	35 (52.2)	**0.026**	50 (40.3%)	30 (50.8%)	0.180
hs-CRP (mg/dL) ‡	0.15 (0.05-0.34)	2.12 (1.06-5.23)	**<0.001**	0.15 (0.06-0.33)	1.89 (0.98-4.46)	**<0.001**
HCMV serology (HCMV-positive %) †	167 (79.9)	61 (88.4)	0.111	94 (75.8%)	54 (88.5%)	**0.042**

* Data are expressed as mean (SD). Statistical analysis according to the Student's t-test.

† Frequency (%).Statistical analysis according to the χ^2 test or Fisher exact test as appropriate.

‡ Data are expressed as median (1^{st}-3^{rd} quartiles). Statistical analysis according to the Mann-Whitney test.

§ Sex and age (±5 years) matched subsample of cases and controls

In bold significant p-values are marked.

Association of NKG2C and LILRB1 expression with HCMV seropositivity

As shown in Table 1, the frequencies of HCMV seropositive age- and sex-matched AMI patients and controls were significantly different (88.5% vs 75.8%; p=0.042). The influence of HCMV infection on the immunophenotype was analysed by pooling data from all cases and controls, adjusting for age, sex and cardiovascular risk factors, as well as for the incidence of AMI (Table 2).

In agreement with previous reports [28-31], the proportions of NKG2C+ NK cells and LILRB1+ NK and T cells were higher in samples from HCMV+ individuals that, conversely, contained lower proportions of NK cells bearing the NKG2A inhibitory receptor. LILRB1 and NKG2C expression were increased in both the CD56- and CD56+ T cell subsets, whereas differences in the distribution of NKG2A+ cells were only significant in the CD3+ CD56+ population. This minor T cell subset was also increased in HCMV+ subjects, and no differences in total NK and T cells were noticed. The association of these immunophenotypic features with a positive HCMV serology was independent of age, sex, cardiovascular risk factors and AMI, as potentially confounding factors. Similar results were obtained when the analysis was restricted to the subsample of age- and sex-matched cases and controls (Supplemental Table I). Regression analysis did not reveal any significant correlation between NKG2C+ NK cells and LILRB1+ NK cells (r=0.059), LILRB1+ T lymphocytes (r=0.024), nor LILRB1+ CD56+ T cells (r=0.007), supporting a dissociated expression of both receptor

Table 2. NKR expression analysis comparing HCMV positive and negative individuals adjusted by sex, age, cardiovascular risk factors* and AMI

Receptor †	Subset †	HCMV-negative ‡ N=50	HCMV-positive ‡ N=228	p-value §
	$CD3^+$ $CD56^-$	72.7 [69.2 ; 76.3]	73.2 [71.7 ; 74.8]	0.806
	$CD3^+$ $CD56^+$	1.03 [0.76 ; 1.39]	2.66 [2.34 ; 3.03]	**<0.001**
	NK	8.03 [6.37 ; 10.1]	7.09 [6.41 ; 7.83]	0.333
NKG2A	$CD3^+$	0.71 [0.50 ; 1.01]	1.01 [0.86 ; 1.19]	0.080
	$CD3^+$ $CD56^-$	2.32 [1.85 ; 2.92]	2.04 [1.83 ; 2.27]	0.318
	$CD3^+$ $CD56^+$	12.7 [9.06 ; 17.9]	7.67 [6.56 ; 8.97]	**0.009**
	NK	36.6 [32.7 ; 40.4]	31.4 [29.6 ; 33.2]	**0.018**
NKG2C	$CD3^+$	1.25 [0.92 ; 1.70]	1.80 [1.56 ; 2.07]	**0.037**
	$CD3^+$ $CD56^-$	0.29 [0.21 ; 0.40]	0.49 [0.42 ; 0.57]	**0.004**
	$CD3^+$ $CD56^+$	2.40 [1.57 ; 3.67]	5.26 [4.35 ; 6.36]	**0.001**
	NK	3.98 [2.88 ; 5.51]	7.54 [6.48 ; 8.77]	**0.001**
LILRB1	$CD3^+$	2.16 [1.65 ; 2.83]	5.28 [4.65 ; 5.98]	**<0.001**
	$CD3^+$ $CD56^-$	1.69 [1.30 ; 2.19]	4.10 [3.63 ; 4.63]	**<0.001**
	$CD3^+$ $CD56^+$	10.3 [7.99 ; 13.3]	20.7 [18.4 ; 23.2]	**<0.001**
	NK	12.6 [10.2 ; 15.7]	17.5 [15.8 ; 19.3]	**0.009**

*risk factors: smoking, BMI, informed of hypertension, informed of high glucose, informed of high cholesterol.
† Data are expressed as the proportions (%) of cells expressing every receptor within the defined lymphocyte subsets.
‡ Data are expressed as mean [IC95%].
§ Statistical analysis according to the Mann-Whitney test.
In bold significant p-values are marked.

Supplemental Table I. Descriptive analysis of NKR expression comparing age and sex-matched HCMV positive and negative individuals

	Subset*	HCMV-negative N=37	HCMV-positive N=148	p-value	Adjusted p-value ‡
	$CD3^+$ $CD56^-$	72,3 (67,3-77,9)	76,1 (66,1-82,3)	0,223	0.203
	$CD3^+$ $CD56^+$	1,16 (0,57-1,55)	2,64 (1,35-4,73)	**<0,001**	**<0.001**
	NK	9,88 (6,16-12,9)	6,93 (3,68-12,2)	0,129	0.094
NKG2A *†	$CD3^+$	0,88 (0,34-2,11)	1,16 (0,38-2,80)	0,334	0.407
	$CD3^+$ $CD56^-$	2,22 (1,05-3,54)	2,07 (1,29-3,55)	0,772	0.618
	$CD3^+$ $CD56^+$	14,3 (4,32-27,1)	8,42 (3,50-18,9)	0,179	0.178
	NK	33,4 (27,8-41,6)	30,1 (21,7-41,7)	0,099	0.196
NKG2C *†	$CD3^+$	0,91 (0,36-2,50)	1,65 (0,92-3,21)	**0,029**	**0.018**
	$CD3^+$ $CD56^-$	0,30 (0,09-0,74)	0,49 (0,18-1,12)	**0,032**	**0.023**
	$CD3^+$ $CD56^+$	1,64 (0,60-4,82)	4,97 (1,53-14,8)	**0,001**	**0.011**
	NK	3,80 (2,44-7,98)	7,69 (4,19-14,5)	**<0,001**	**0.015**
LILRB1 *†	$CD3^+$	2,57 (1,30-4,37)	5,76 (2,82-10,8)	**<0,001**	**<0.001**
	$CD3^+$ $CD56^-$	1,70 (0,93-3,44)	4,14 (2,27-7,52)	**<0,001**	**<0.001**
	$CD3^+$ $CD56^+$	10,0 (4,83-25,4)	25,5 (13,2-50,2)	**<0,001**	**<0.001**
	NK	14,9 (9,29-22,7)	17,8 (11,6-34,9)	**0,019**	**0.036**

* Data are expressed as the proportions (%) of cells expressing every receptor within the indicated lymphocyte subsets.
† Data are expressed as median (1^{st}-3^{rd} quartiles). Statistical analysis according to the Mann-Whitney test.
‡ Adjusted by informed of HTA, informed of high glucose and smoking.

Increased proportions of LILRB1+ NK and T cells in AMI patients

When NKR distribution was compared in age- and sex-matched cases and controls, the proportions of LILRB1+ NK and T cells, as well as the CD3+ CD56+ subset, were significantly increased in AMI patients; moreover, the relative numbers of total NK and T cells also differed (Table 3). Remarkably, NKG2C and NKG2A expression was undistinguishable between cases and controls (Table 3), in contrast to the effect of HCMV infection on these markers (Table 2). Similar results were obtained when total cases and controls were analysed (data not shown). The increased proportions in AMI patients of LILRB1+ NK cells, LILRB1+ CD56+ T cells, as well as total CD3+ CD56+ cells remained consistent after adjusting for cardiovascular risk factors, HCMV seropositivity (Table 3), and therapy with statins (24.3% cases and 13% controls, p=0.025). NKR expression was also compared in cases and controls according to their HCMV serological status. Among HCMV+ individuals, the proportions of CD3+ CD56+, LILRB1+ NK and CD56+ T cells were increased in AMI patients, while no significant differences were found between HCMV seronegative cases and controls (data not shown) Yet, the statistical power of this latter analysis was limited due to the few HCMV-negative cases (n= 7).

A low but significant correlation between the proportions of LILRB1+ NK and T cells ($r=0.2$; $p<0.001$) was noticed. Moreover, hs-CRP levels were elevated in AMI cases (Tables 1 and 3) correlating with LILRB1+ NK ($r=0.30$, $p=0.014$) and CD56+ T cells ($r=0.30$, $p=0.016$) (n= 69).

Table 3. Descriptive analysis of NKR expression comparing age and sex-matched AMI cases and controls

	Subset*	Control N=124	Case N=62 †	p-value	Adjusted p-value ‡	Adjusted p-value ¶
	$CD3^+$ $CD56^-$	71.4 (65.3-79.3)	78.7 (68.7-83.3)	**0.014**	**0.031**	**0.036**
	$CD3^+$ $CD56^+$	1.86 (1.03-3.06)	3.31 (1.56-5.98)	**0.003**	**0.002**	**0.010**
	NK	9.54 (4.34-13.0)	5.79 (3.65-11.7)	**0.047**	0.102	0.056
NKG2A *II	$CD3^+$	1.27 (0.38-2.72)	0.75 (0.34-2.59)	0.257	0.688	0.186
	$CD3^+$ $CD56^-$	2.12 (1.14-3.53)	2.08 (1.33-3.58)	0.832	0.763	0.857
	$CD3^+$ $CD56^+$	8.61 (3.51-18.9)	8.97 (4.16-20.5)	0.753	0.984	0.748
	NK	30.2 (23.6-41.4)	31.7 (22.6-42.3)	0.834	0.736	0.701
NKG2C *II	$CD3^+$	1.63 (0.77-2.89)	1.57 (0.76-3.58)	0.825	0.967	0.633
	$CD3^+$ $CD56^-$	0.48 (0.19-1.09)	0.42 (0.15-0.82)	0.447	0.730	0.222
	$CD3^+$ $CD56^+$	3.92 (1.35-13.2)	4.97 (1.49-15.4)	0.375	0.478	0.777
	NK	6.19 (3.55-12.1)	7.45 (4.06-15.1)	0.226	0.620	0.434
LILRB1 *II	$CD3^+$	4.52 (2.43-8.76)	5.19 (3.11-10.1)	0.223	0.184	0.403
	$CD3^+$ $CD56^-$	3.39 (1.61-6.71)	3.90 (2.78-6.61)	0.164	0.138	0.267
	$CD3^+$ $CD56^+$	18.5 (8.88-35.9)	30.3 (15.3-56.8)	**0.003**	**0.023**	**0.030**
	NK	17.0 (10.4-28.9)	24.4 (13.1-41.0)	**0.003**	**0.014**	**0.004**
Hs- CRP (mg/dl) II		0.15 (0.06-0.33)	1.89 (0.98-4.46)	**<0.001**	**<0.001**	**<0.001**
HCMV serology (HCMV-positive %)§		94 (75.8)	54 (88.5)	**0.042**	**0.038**	**-**

* Data are expressed as the proportions (%) of cells expressing every receptor within the indicated lymphocyte subsets.
† 8 cases remaining unmatched were excluded.
‡ Adjusted by informed of HTA, informed of high glucose and smoking.
§ Frequency (%). Statistical analysis according to the χ^2 test or Fisher exact test as appropriate.
II Data are expressed as median (1^{st}-3^{rd} quartiles). Statistical analysis according to the Mann-Whitney test.
¶ Adjusted by HCMV serology.

Relationship of CIMT with increased proportions of LILRB1+ NK and T cells

When samples obtained from cases (n=53) > 6 months after the acute event were analysed, differences in LILRB1 expression with controls persisted (data not shown), indirectly suggesting that the immunophenotypic profile was not attributable to the acute phase of AMI, and might predate the development of the disease. To address this issue, we studied the putative relationship between NKR expression and subclinical atherosclerosis, measuring carotid intima-media thickness (CIMT) in control individuals (n=149) (Table 4). Remarkably, the proportions of LILRB1+ NK and T cells directly correlated with CIMT. After adjusting for age, sex, cardiovascular risk factors and HCMV serology (Table 4) the correlation with CIMT remained significant for LILRB1 expression in NK and CD56- T cells. In contrast, the proportions of LILRB1+ CD56+ T lymphocytes and of total CD3+ CD56+ cells did not correlate with CIMT, at variance with the observations in AMI. Altogether the data further supported that LILRB1 expression by NK and T cells may be independently associated with atherosclerosis.

Hs-CRP concentrations have been previously related not only with acute coronary disease but also with subclinical atherosclerosis. In our analysis, hs-CRP levels significantly correlated with CIMT in controls (n= 149; r=0.252 p=0.002), but not with the proportions of LILRB1+ lymphocytes, in contrast to the observations in AMI.

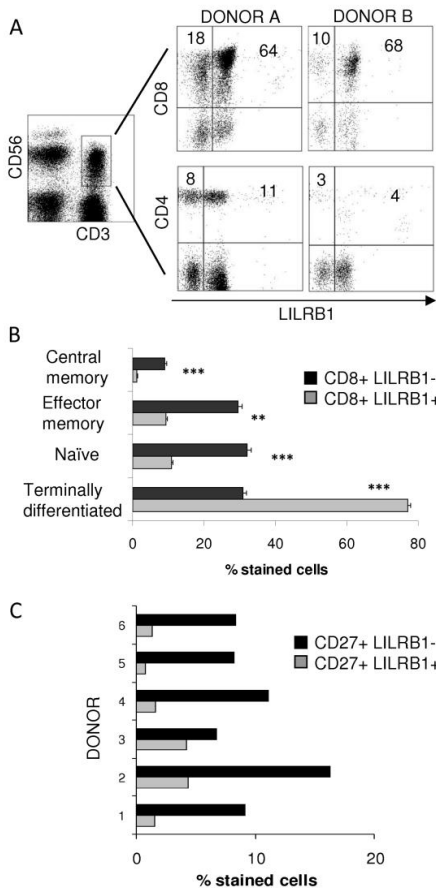
Table 4. Correlation between NKR expression and CIMT in control subjects

Receptor *	Subset *	Correlation † CCA IMT ‡§	Adjusted correlation II CCA IMT ‡§	Adjusted correlation ¶ CCA IMT ‡§
	$CD3^+ CD56^-$	**-0.284 (0.005)**	-0.165 (0.132)	-0.147 (0.183)
	$CD3^+ CD56^+$	-0.163 (0.113)	-0.055 (0.619)	-0.088 (0.424)
	NK	**0.207 (0.043)**	0.048 (0.661)	0.040 (0.715)
NKG2A	$CD3^+$	0.056 (0.516)	-0.035 (0.695)	-0.036 (0.685)
	$CD3^+ CD56^-$	**-0.241 (0.004)**	-0.103 (0.246)	-0.086 (0.339)
	$CD3^+ CD56^+$	**-0.189 (0.026)**	-0.134 (0.130)	-0.104 (0.244)
	NK	0.027 (0.754)	-0.064 (0.473)	-0.040 (0.650)
NKG2C	$CD3^+$	**-0.236 (0.004)**	-0.102 (0.241)	-0.110 (0.206)
	$CD3^+ CD56^-$	-0.014 (0.866)	-0.021 (0.813)	-0.051 (0.564)
	$CD3^+ CD56^+$	-0.054 (0.519)	-0.001 (0.994)	-0.032 (0.717)
	NK	0.057 (0.497)	-0.040 (0.645)	-0.079 (0.364)
LILRB1	$CD3^+$	**0.229 (0.006)**	**0.234 (0.006)**	**0.194 (0.025)**
	$CD3^+ CD56^-$	**0.203 (0.014)**	**0.278 (0.001)**	**0.242 (0.005)**
	$CD3^+ CD56^+$	**0.273 (0.001)**	0.094 (0.278)	0.044 (0.614)
	NK	**0.257 (0.002)**	**0.227 (0.008)**	**0.198 (0.022)**

* The proportions of cells expressing every receptor within the indicated lymphocyte subsets were considered as markers (predictors).
† Numbers correspond to the Spearman correlation (p-value) between CCA IMT and the % of receptor expressing cells.
‡ right and left common carotid artery (CCA) IMT mean.
§ n=149
II Partial correlation (p-value) of the markers with CIMT, adjusting for age, sex, systolic blood pressure, LDL/HDL ratio, smoking, blood pressure treatment and history of diabetes.
¶ Partial correlation (p-value) of the markers on CIMT, adjusting for age, sex, systolic blood pressure, LDL/HDL ratio, smoking, blood pressure treatment, history of diabetes and cytomegalovirus serology. In bold significant p-values are marked.

LILRB1+ NK and T cells display a late differentiation phenotype

Surface LILRB1 expression has been related to late stages of T cell differentiation [35]. Multicolour flow cytometry analysis performed in a limited number of donors confirmed that LILRB1 was co-expressed with CD45RA mainly in CD8+ T cells (Supplemental Figure I A and B). In the same line, LILRB1+ NK cells were predominantly found among the CD27-negative subset [40] (Supplemental Figure I C). Altogether, the data point out that LILRB1+ NK and T cells associated with atherosclerosis display a phenotypic profile corresponding to late differentiation stages in both lineages.



Supplemental Figure I. Phenotypic characterization of LILRB1+ T and NK cells. (A) PBMC were stained with anti-LILRB1, anti-CD3, anti-CD56 and either anti-CD4 or CD8 mAbs (protocol 2; see "Immunofluorescence and flow cytometry analysis"). LILRB1 expression was analyzed gating on CD3+ CD56+ cells. The staining pattern observed in two representative donors out of 16 performed is displayed. **(B)** PBMC were also stained with anti-LILRB1, anti-CD8, anti-CD45RA and anti-CCR7 mAbs (protocol 3; see "Immunofluorescence and flow cytometry analysis"). CD45RA and CCR7 expression was analyzed gating on CD8+ LILRB1+ and CD8+ LILRB1- T cells. The proportions (mean ± SEM) of central memory (CD45RA- CCR7+), effector memory (CD45RA- CCR7-), naïve (CD45RA+ CCR7+) and terminally differentiated (CD45RA+ CCR7-) CD8+ LILRB1+ and CD8+ LILRB1- T cells in PBMC samples from 16 different donors is shown. ** P<.01, *** P< .001. **(C)** NK cells were also analysed with anti-LILRB1, anti-CD27, anti-CD3 and anti-CD56 (protocol 4; see "Immunofluorescence and flow cytometry analysis"). CD27 expression was analyzed gating on LILRB1+ and LILRB1- NK cells. Data from an analysis representative of six donors analysed is shown.

DISCUSSION

Despite a wide number of studies addressing the role of HCMV in the pathogenesis of atherosclerosis and vascular disease, the issue remains open. This may be explained by a high prevalence of the persistent infection, an overlapping involvement of other microbial pathogens, and the possibility that its contribution may be indirect [24]. Moreover, the detection of HCMV-specific antibodies used in conventional studies is not informative on the complex host-pathogen relationship, which may be a determinant of cardiovascular risk at the individual level. On that basis, we addressed whether the influence of HCMV infection on the distribution of NKG2C and LILRB1 NKR might reflect its putative contribution to the pathogenesis of atherosclerosis.

Our results supported that increased proportions of circulating LILRB1+ NK and T cells were associated with AMI, independently of age, sex, conventional cardiovascular risk factors and HCMV infection. Remarkably, these immunophenotypic features appeared as well related to carotid plaque thickness, a measurement of preclinical atherosclerotic disease, in control participants, thereby suggesting that such profile might contribute to predict future cardiovascular disease development.

Though increased LILRB1+ NK cells were associated with both AMI and CIMT, some differences were noticed between both settings. In fact, CIMT correlated with the proportions of LILRB1+ CD56- T cells, whereas AMI appeared associated with LILRB1 expression in CD56+ T cells. Moreover, increased proportions of total T lymphocytes and CD56+ T cells were associated with AMI but not with CIMT. The CD56+ T cell subset includes cytotoxic CD8+ and some CD4+ cells, often bearing NK cell associated molecules [41]. In the same line, LILRB1 expression correlated with elevated hs-CRP levels in AMI patients but not in controls. The relationship of the different immunophenotypic features with the pathogenesis of atheroma

formation vs acute coronary disease deserve further attention; the possibility that some of the observed changes may simply encompass the development of vascular disease is not ruled out. Despite the persistence along time of the immunophenotypic features associated to AMI, the possibility that they might be secondary to the ischemic event could not be formally ruled out. Further studies in patients with stable CHD might contribute to clarify the issue. Moreover, analysing NKR expression in CD4 and CD8 T cell subsets, as well as the absolute numbers of the different cell populations would be of interest to refine the picture.

In agreement with previous studies [15], a higher frequency of HCMV+ samples was detected among AMI cases as compared to age- and sex-matched controls. Nevertheless, the associations of AMI and CIMT with increased LILRB1+ cells appeared independent of HCMV seropositivity. Moreover, AMI and CIMT were unrelated to the expression levels of NKG2C, a marker that was confirmed to be linked to this viral infection, in line with previous reports [28-31]. The dissociated expression of both NKR may be explained by the ability of other microbial pathogens to expand LILRB1+ cells [36;37]. On the other hand, HCMV infection may promote an increase of LILRB1+ cells without expanding in parallel NKG2C+ NK cells. In fact, a wide variability in the proportions of this subset was found among HCMV+ individuals [28] without a significant correlation with LILRB1+ cells. Studies in immunodeficiencies [29;30;42] suggest that the magnitude of the NKG2C+ NK cell expansion may be inversely related to the efficiency of the T cell-mediated response to the virus. In summary, despite that our results do not support a dominant role for HCMV, its contribution to the pathogenesis of atherosclerosis is not ruled out, and might be related with the quality of the T cell response to the infection, rather than to its influence on the distribution of the NKG2C+ NK cell subset. In this regard, the role of NK cells in atherosclerosis remains uncertain [1;43] but T lymphocyte

subsets appear to play a complex role in the development of vascular lesions [1-3], and NKR+ T cells have been identified in atheroma plaques [44].

The observation that NK and T cells use the same LILRB1 promoter region[45] suggests that its expression may be controlled by common signals in both lineages. LILRB1 is mainly detected in differentiated T cells specific for different pathogens, which display the CD28- CCR7low and CD45RA+ phenotype, and may co-express CD56 and NKR (i.e. CD94/NKG2, KIR) [35-37]. Circulating LILRB1+ T cells have been shown to be predominantly CD8+ but a subset of cytotoxic CD4+ CD56+ LILRB1+ cells expressing different NKR has been also identified in normal blood donors [46]. Our studies in a limited number of individuals confirmed this immunophenotypic profile. It is of note that the LILRB1 inhibitory receptor has been reported to dampen the response of terminally differentiated virus-specific T cells [37], whose expansion has been associated with immunosenescence [22]. Interestingly, Spyridopoulos et al. recently reported a marked reduction of telomere length in CD8+ T lymphocytes from coronary heart disease (CHD) patients that correlated with cardiac dysfunction, and was particularly evident in HCMV seropositive individuals suggesting as well a link with immunosenescence [47].

Similarly to T and B lymphocytes, activated NK cells may undergo clonal expansions and experience differentiation events that modify their phenotype and survival [48;49]. In this regard, LILRB1 is displayed by a variable fraction of CD56dim NK cells, being virtually undetectable in the CD56bright subset, reported to bear longer telomeres [50]. In the same line, CD27 expression has been related to early stages of mature NK cell differentiation [40], and most LILRB1+ cells were predominantly found among the CD27-negative population.

Altogether, these observations support that the increase of LILRB1+ NK and T cells associated with atherosclerosis correspond to an accumulation of differentiated lymphocytes, likely reflecting the pressure of

persistent/recurrent infections. Though weak, a correlation between LILRB1+ NK and T cells, suggests that both cell lineages might be responding to common challenges, presumably involving intracellular pathogens. In this context, the relationship of atherosclerosis with other late differentiation markers in NK and T cells should be considered. On the other hand, an association of hs-CRP levels with AMI and CIMT was observed, confirming previous reports [13;51]. It is conceivable that an increased production of the acute phase protein might reflect as well the influence of persistent infections.

Whether LILRB1+ NK and T cells participate in the pathogenesis of vascular lesions or merely constitute an indirect marker of the influence exerted by infections deserves further attention. Exploring the dominant antigen specificities of LILRB1+ and CD56+ T cells in individuals with atherosclerosis might eventually provide further insights on the nature of infectious agents contributing to the development of vascular lesions. As previously discussed, it is noteworthy that the lack of association of AMI and CIMT with an expansion of the NKG2C+ NK cell subset does not dismiss a significant contribution of HCMV to the role of the infectious burden in the pathogenesis of atherosclerosis.

Some limitations of the present study warrant attention. From a methodological standpoint, the analysis of leukocyte surface markers by flow cytometry requires the use of fresh or cryopreserved cells, as well as a precise standardization of technical protocols. Moreover, our observations are based on the analysis of a small population sample from a geographical area characterized by a low incidence of cardiovascular disease [38;52], and the study design does not allow the establishment of causality. Finally, it cannot be ascertained whether the immunophenotypic changes associated to AMI indeed predate the development of the acute episode; studies in stable CHD would be helpful to approach this issue.

In summary, our findings may have clinical implications, opening perspectives for research on the role of infections in atherosclerosis and potentially leading to the identification of novel biomarkers of vascular disease development, irrespective of classical risk factors.

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DISCLOSURE

An active license agreement for the commercialization of a LILRB1-specific monoclonal antibody (clone HP-F1), generated by ML-B, has been established by the Pompeu Fabra University with eBioscience.

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PART III
DISCUSSION AND CONCLUSIONS

Chapter 5

Discussion

HCMV does establish a life-long persistent infection that remains latent, undergoing occasional reactivation in immunocompetent individuals. Haematopoietic cells of the myeloid lineage are considered to provide a main reservoir for latent infection, favouring its dissemination. Viral reactivation and replication in epithelial cells allows the transmission to other individuals through secretions. Current evidence suggests that inflammatory cytokines may promote reactivation of latent HCMV in MΦ, allowing the virus to spread to other cells in the inflamed tissue. By influencing the regulation of various cellular processes including the cell cycle, apoptosis and migration as well as angiogenesis, HCMV may participate in disease development. On the other hand, a number of HCMV molecules are devoted to counteract central functions of the innate and adaptive immune responses. In this context, understanding the response of cells of the myelomonocytic lineage to HCMV infection and their interaction with NK and T cells deserves special attention.

In the first part of this work, we analysed the impact of HCMV infection on MΦ, showing that differences in the NK cell response to distinct HCMV-infected monocyte-derived cell types may be relevant in the immunopathology of HCMV infection. To characterize the interaction between NK cells and HCMV-infected MΦ, monocytes were differentiated *in vitro* to pro-inflammatory or anti-inflammatory MΦ. Low-passage HCMV isolates and endotheliotropic strains capable of infecting MΦ efficiently *in vitro* [122] were used. Our results indicate that NK cells specifically killed comparably both HCMV-infected M1 and M2 MΦ, involving 2B4, NKp46 and DNAM-1 receptors, but secreted high amounts of IFN-γ only in response to infected M1 MΦ.

Our results are in agreement with recently described data showing that NK cells efficiently respond against HCMV-infected immature monocyte-derived dendritic cells through NKp46 and DNAM-1 receptors [82]. In contrast to our results, HCMV-infected MΦ were previously reported to be resistant to

NK cell lysis, independently of MHC class I downregulation or expression of the UL18 HCMV class I homologue [63]. The basis for this discrepancy is unknown, but likely results from differences in the experimental settings, including the HCMV strain, the use of allogeneic vs autologous conditions and, especially, the day post-infection in which the assay was performed [54, 213-215]. Our previous studies revealed that by day three post-infection moDC became more resistant to NK cells than at the earlier time point (48 h) fixed for our assays. In this regard, the expression of DNAM-1 ligands (DNAM-1L), PVR and Nectin-2, progressively decreased presumably interfering with NK cell mediated recognition [82, 107, 108]. The HCMV glycoprotein UL141 has been described to sequester PVR and is also necessary for the down-modulation of Nectin-2 in infected fibroblasts [107, 108]. Though UL141 likely accounts for the down-modulation of DNAM-1L in infected MΦ, this question should be directly addressed infecting with an UL141 deletion mutant. A recently identified inhibitory receptor named TIGIT has been described to bind PVR with higher affinity than DNAM-1. TIGIT is expressed by NK and T cells and could also participate in recognition of PVR in the infected MΦ. The fact that blocking with anti-DNAM-1L mAbs inhibits NK cell degranulation indicates that TIGIT does not play a dominant role under the experimental conditions employed; yet, its role at later time points cannot be ruled out. In this regard, blocking with the anti-DNAM-1L mAbs at 72h post infection promotes NK cell activation against to HCMV-infected moDC [82].

NKp46 has been reported to recognize influenza hemagglutinins, contributing to activation of the NK cell response against influenza infected moDC [100], as well as to the lysis of MΦ by activated NK cells [30]; yet, the nature of its cellular ligands remains unknown and no HCMV viral protein has been shown to interact with NKp46. NKp30 has been involved in the crosstalk of activated NK cells with moDC but not with MΦ [30]. In line with our observations in HCMV-infected moDC [82], binding of NKp46-Fc

and NKp30-Fc soluble fusion proteins indicated that the ligands for both NCR are constitutively expressed on the surface of M1 and M2 MΦ and that their expression is down-modulated upon HCMV infection (Figure 1). Of note, the reduced NCR ligand expression affected both infected and not infected MΦ, suggesting the involvement of soluble factors secreted in response to HCMV infection.

The CD48 molecule recognized by 2B4 is a member of the CD2 family broadly expressed in most hematopoietic and endothelial cells. Engagement of 2B4 in mature human NK cells promotes NK cell activation [112]. In resting NK cells, 2B4 acts as a co-receptor but in IL-2 activated NK cells its engagement is sufficient to trigger NK cell cytotoxicity [113]. 2B4 ligation has been described to induce IFN-γ secretion in NK cells upon MΦ co-culture [30, 31]. We provide the first evidence supporting its involvement in the response to HCMV-infected MΦ. Interestingly, CD48 expression was down-regulated in HCMV-infected myeloid cells similarly to MHC class I molecules, suggesting the existence of a putative immune evasion mechanism targeting this molecule. It is of note that CD48 mRNA levels were reduced in M2 MΦ at 48h post-infection (G. Magri et al. unpublished results); on that basis we hypothesize that an HCMV miRNA might regulate CD48 expression at a posttranscriptional level, as described for NKG2D ligands [96, 97]. Further studies are required to address this issue using HCMV deletion mutants. Despite CD48 down-modulation, its expression was sufficient to promote NK cell cytotoxicity under our experimental conditions. We observed that by 48h post-infection, down-modulation of DNAM-1L, CD48 and NCR-L was more evident in M2 than in M1 MΦ (Figure 1); this may simply reflect differences in the kinetics of the infection between both types MΦ subsets.

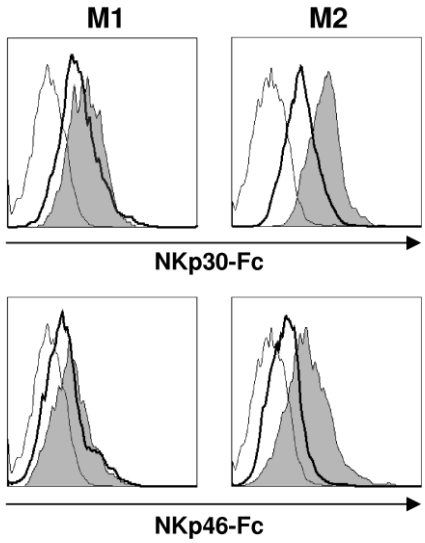

Figure 1. NKp30 and NKp46 ligands are constitutively expressed on M1 and M2 MΦ. NKp30 and NKp46 ligand expression on M1 and M2 MΦ was assessed by indirect immunofluorescence and flow cytometry using soluble recombinant NKp30-Fc and NKp46-Fc fusion proteins. Mock MΦ (filled histograms) and TB40/E MΦ (bold line, open histograms) were surface labeled at 48h post virus exposure. Staining with a human IgG1 is included as a control (thin open histograms). Results of a representative experiment are shown (% of IE1/IE2+ cells in M1 and M2 MΦ: 40%).

LPS-activated MΦ up-regulate MICA and ULBP1-3 molecules, engaging NKG2D and triggering NK cell activation [31]. In in our experimental setting an antibody against NKG2D did not block NK cell degranulation indicating that this receptor does not play a dominant role in the recognition of infected MΦ. In this regard, several evasion mechanisms impairing the expression of NKG2D-L in infected cells have been described in fibroblasts and moDC, and it is plausible that they may also operate in infected MΦ [82, 91-97].

HCMV-infected M1 MΦ secreted IL-12, IL-6, TNF-α and IFN-α, whereas activated M2 MΦ produced lower concentrations of TNF-α, IL-6 and IFN-α, secreted poorly IL-10 and failed to release detectable IL-12. M1 MΦ have been described to secrete in vitro IL-12 in response to *Mycobacterium* only in the presence of IFN-γ [27, 28] whereas HCMV infection was sufficient to

trigger IL-12 production. Altogether, our results indicate not only that M2 MΦ are more permissive to HCMV infection but, moreover, that they display a limited inflammatory response.

Although both HCMV-infected MΦ subsets triggered NK cell degranulation, only M1 MΦ effectively induced NK cell-mediated IFN-γ production. The viability of M1 and M2 infected MΦ, assessed by Annexin V and propidium iodide staining, was comparable in both MΦ subsets, ruling out that the differences observed could be due to a higher M2 mortality rate (data not shown). Only infected M1 MΦ produced IL-12 and NK cell IFN-γ production was partially blocked by an anti-IL-12 mAb, pointing out a key role of the cytokine. Yet, the participation of other cytokines is not ruled out, as anti-IL-15 or anti-IFNAR mAb partially blocked the secretion of IFN-γ by NK cells co-cultured with M1 HCMV-infected MΦ (data not shown). Though IL-15 has been described to mediate NK cell proliferation and type I IFNs enhance cytotoxic activity, [216] recent studies show that IL-15 also promotes NK cell-mediated IFN-γ production in response to autologous mature moDC [217]. IFN-β produced by NK cells when stimulated with IL-12 and a TLR3 agonist (polyI:C) induced, in an autocrine manner, the production of IFN-γ [218].

The low levels of IL-10 produced by infected M2 MΦ indirectly ruled out that this cytokine might interfere with the NK cell response. However, a contribution of its viral homologue cmv-IL-10, reported to suppress the synthesis of proinflammatory cytokines in human monocytes, cannot be excluded [219]; its expression in M1 and M2 MΦ should be directly compared. On the other hand, infected M1 MΦ produced higher levels of IFN-α than M2 MΦ, thus increasing their resistance to HCMV infection. Similar results have been obtained with other intracellular pathogens and M2 MΦ have been reported to be more susceptible to *Mycobacterium* and *Coxiella burnettii* infection than M1 MΦ [26, 28]. Type I IFNs (IFN-α and IFN-β) and type II IFNs (IFN-γ) are important components of the host immune

response to HCMV infection. Type I IFNs are produced by most cells in response to infection while IFN-γ is secreted by activated NK and T cells. Moreover, IFN-α/β and IFN-γ synergistically inhibit HCMV replication, supporting that IFN-γ produced by NK cells synergize with type I IFNs to prevent HCMV dissemination [220].

The fact that M2 MΦ secrete less IFN-α and their inability to trigger NK cell IFN-γ production might facilitate the spread of the virus in tissues were M2-like MΦ are abundant. An M2 MΦ-like functional profile has been described for tumour-associated and alveolar MΦ, that were shown to be immunosuppressive and displayed a poor antigen-presenting capacity [18, 20]. Evidence supporting that lung MΦ are infected by HCMV *in vivo* has been reported, and viral proteins representing all stages of HCMV infection were detected in tissue sections, suggesting that these cells supported the full viral replication cycle [221]. Thus, the poor pro-inflammatory response of M2 MΦ could partially explain the impact of HCMV infection in some tissues, particularly liver and lung. Hepatitis is a common consequence of systemic HCMV infection, and interstitial pneumonitis remains the most severe manifestation of HCMV disease in allogeneic hemopoietic stem cell transplant recipients [222].

Altogether, our results support important differences in NK cell response triggered by HCMV-infected M1 and M2 MΦ (Figure 2) and indicates that the plasticity in the human MΦ compartment may affect host defense against HCMV-infection, potentially explaining the dual role of MΦ in combating the viral infection while being the HCMV reservoir.

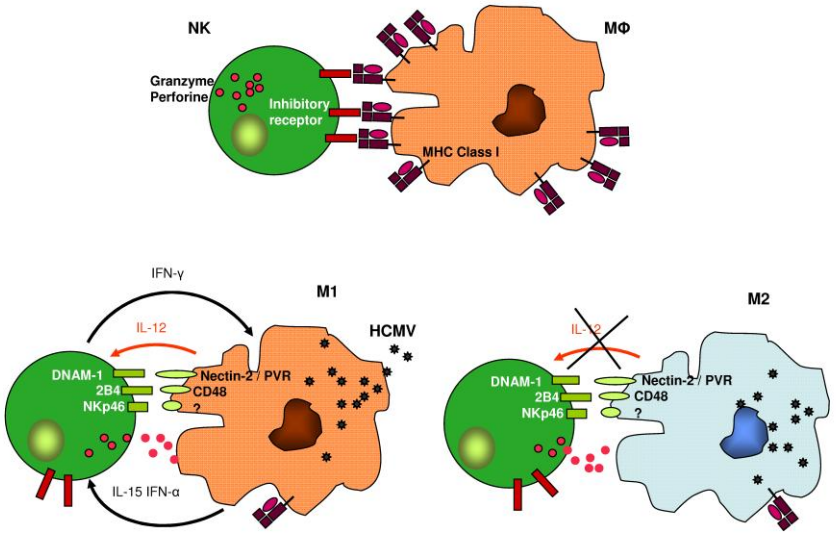

Figure 2. Model of crosstalk between NK cells and MΦ during HCMV infection.

HCMV infection has been associated to certain solid tumors [223], atherosclerosis [224] and some autoimmune diseases [225-227]. These pathologic conditions are often associated with an accumulation of infiltrating activated MΦ, which represent a major regulator of local inflammation but also a target of HCMV infection. In ApoE$^{-/-}$ mice, a conventional experimental model of atherosclerosis, lesion progression was correlated with the dominance of the M1 MΦ phenotype [228]. Moreover, MCMV infection was shown to activate MΦ to produce pro-inflammatory cytokines in ApoE$^{-/-}$ mice, and might promote the development of atherosclerosis through systemic and/or local immune activation [229]. Though foamy MΦ in human atheroma plaques are CD163-negative, MΦ in the plaque surface are weakly positive for CD163 [230], consistent with an M1 phenotype. Macrophages alter their phenotype and biological function in response to plaque lipids. Interaction of oxidized lipids with pattern recognition receptors and activation of the inflammasome by cholesterol crystals drive macrophages toward an inflammatory M1 phenotype [231]. In

contrast, other studies suggest that the M1 and M2 MΦ phenotypes respectively predominate in normal and pathological intima [232]. Atherosclerotic lesions in mice and in humans contain macrophages with various phenotypes, which play different roles in inflammation, and presumably the situation is more complex than that simply predicted based on the M1 vs M2 paradigm [233, 234]. HCMV-infected MΦ could potentially contribute to disease progression secreting inflammatory cytokines (i.e. IL-12, IL-18 and TNF-α) and activating IFN-γ secretion by NK and T cells [209]. IFN-γ produced locally in the atherosclerotic lesions may promote MΦ and endothelial activation, having a pro-atherogenic effect [179]. Furthermore, viral infection of MΦ may lead to an alterated lipid metabolism and an increased expression of the CD36 scavenger receptor, which increases lipid uptake by the infected cells [126-128].

The putative role of HCMV infection in the pathogenesis of atherosclerosis has remained somehow controversial, being mainly supported by seroepidemiological studies and an occasional detection of the virus in vascular lesions. It is conceivable that the putative role of HCMV in the pathogenesis of atherosclerosis may depend on features of the complex host-pathogen interaction not simply reflected by the detection of anti HCMV antibodies [235]. In the second part of this thesis we addressed whether the impact of HCMV infection on the NKR repertoire might reflect its putative role in the pathogenesis of atherosclerosis. A prospective case-control study was designed comparing the expression of NKG2A, NKG2C and LILRB1 in NK and T cells from patients studied after an acute myocardial infarction (AMI), and a cohort of control individuals. Moreover the relationship between NKR expression and carotid intima-media thickness (CIMT) was assessed in a cohort of individuals without clinical evidence of cardiovascular disease.

Our results indicate that a positive HCMV serology was associated to higher proportions of circulating NKG2C+ and LILRB1+ NK and T cells, as

previously described [65, 83, 84]. It is of note that also the CD3+ CD56+ T cell subset was also increased in HCMV+ subjects, whereas no differences in total NK and T cells were observed. By contrast, only LILRB1+ NK and T cells as well as the CD3+ CD56+ subset were found increased in patients with AMI independently of age, gender, conventional vascular risk factors and HCMV serology. Similar results were obtained in an analysis restricted to age and sex-matched individuals. In order to assess whether the increase of LILRB1+ cells predates the development of AMI we designed a study measuring the carotid plaque thickness in a cohort of control individuals. Remarkably, higher LILRB1 expression levels in NK and T cells significantly correlated with CIMT and the association was found to be independent of the HCMV serology. Though increased LILRB1+ NK cells were associated to both AMI and CIMT, some differences were noticed between both settings. In fact, CIMT correlated with the proportions of LILRB1+ CD56- T cells, whereas AMI appeared associated to LILRB1 expression in CD56+ T cells, concurring with a significant expansion of the latter cell subset. In the same line, increased proportions of total T lymphocytes, CD56+ T cells and reduced NK cells were associated to AMI but not to CIMT. These observations may reflect distinct evolutionary stages of the same process or, alternatively, differences in the relationship of the immunophenotypic profile with the pathogenesis of atheroma formation vs. plaque instability and rupture, which triggers acute coronary disease. In this regard, it is of note that LILRB1 expression correlated with elevated CRP levels in AMI patients but not in controls.

In line with previous studies [201], a higher frequency of HCMV+ donors was detected among AMI cases as compared to age- and sex-matched controls, however the increase of LILRB1+ lymphocytes in AMI cases vs. controls remained statistically significant after adjusting by HCMV serology. These results could be explained by the fact that other pathogens and pro-inflammatory stimuli may also contribute to the increase of LILRB1+ NK

and T cells, in line with the "infectious burden" (IB) hypothesis that proposes that different microbial agents may contribute to plaque development and vascular disease in an additive way.

The fact that NKG2C expression did not appear associated to atherosclerosis was intriguing. A marked expansion of NKG2C+ cells associated to HCMV infection has been noticed in immunodeficient patients, suggesting that the magnitude of this effect may be inversely related to the efficiency of anti-viral T cell-mediated response [50]. We recently reported that a marked NKG2C+ NK cell expansion was detectable in HCMV infected children [66] and we hypothesized that the variable increase of NKG2C+ cells in healthy HCMV+ individuals may depend, among other factors, on the age of the primary infection. A wide variability in the magnitude of the NKG2C+ NK cell subset expansion can be detected in HCMV+ individuals, but no correlation between the relative numbers of NKG2C+ and LILRB1+ cells was substantiated [65, 86]. Such dissociated influence of the viral infection on the expression of both receptors might be explained not only by the ability of other microbial pathogens to expand LILRB1+ cells [236, 237], but also by differences in the mechanisms underlying the influence of HCMV infection on these molecules [86] In fact, an in vitro expansion of the NKG2C+ NK cell subset was observed only in response to infected fibroblasts, whereas the effect on LILRB1+ lymphocytes was as well noticed upon stimulation with inactivated virus preparations [88]. In summary, these observations support that HCMV infection may eventually promote an increase of LILRB1+ cells in some individuals without necessarily expanding in parallel NKG2C+ NK cells.

According to their phenotype, LILRB1+ T cells mainly correspond to CD8+ terminally differentiated cells and, similarly, most LILRB1+ NK cells were CD27-negative. These observations suggest that the increase of LILRB1+ NK and T cells associated with atherosclerosis may correspond to an expansion of terminally differentiated lymphocytes, presumably accumulated

under the pressure of infections [56, 236-238]. Thus, the relationship to atherosclerosis of other phenotypic markers of terminal NK and T cell differentiation should be explored.

The nature of the mechanism(s) underlying LILRB1 up-regulation in NK cells is uncertain. Preliminary results co-culturing HCMV-infected MΦ and autologous PBMCs showed an increase of LILRB1 in the NK cell compartment after 3 days (Figure 3A). However, the effect was not reproducible in every donor and the increase of LILRB1 was not observed in the CD8+ T cell compartment (Figure 3B). Moreover, purified NK cell populations did not up-regulate LILRB1 expression in response to HCMV-infected MΦ (data not shown), pointing out the requirement of additional signals. It is plausible that LILRB1 up-regulation in NK cells may constitute a regulatory mechanism to further prevent, in concert with other inhibitory receptors, their response against cells expressing normal levels of HLA class I molecules. Recent studies shows that IL-15 and IL-2 can increase LILRB1 expression in PBMCs and in purified NK cells at 72h of treatment [239]. LILRB1 expression can be driven by the activity of two distinct promoters, a proximal and a distal promoter located 13 kb further upstream that leads to inclusion of an additional exon [58]. NK cells preferentially employ the distal promoter, which accounts in part for the lower levels of expression compared to myeloid cells but, upon IL-2 or IL-15 stimulation a switch in promoter choice occurs [239]. We have an indirect evidence that IL-15 is produced by infected MΦ (blocking with an anti-IL-15 mAb reduces NK cell IFN-γ production) and it is possible that IL-15 is modulating LILRB1 expression in our settings. Healthy donors with fluctuating levels of LILRB-1 on their NK cells have been described [239]. IL-15 is a cytokine expressed in human and mouse atherosclerotic lesions that has a pro-atherogenic effect [240, 241] and serum IL-15 levels are significantly higher in both acute and chronic patients of coronary heart disease than in controls [242]. IL-15 could contribute to the increased expression of LILRB1 in NK and T cells

observed in AMI patiens, however further studies are warranted to address if that mechanism occurs *in vivo*.

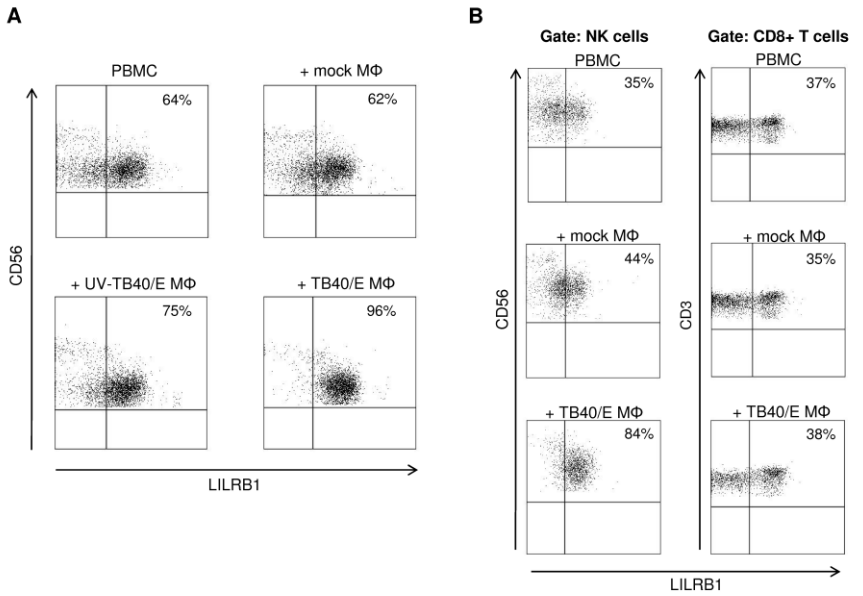

Figure 3. LILRB1 upregulation in PBMCs co-cultured with HCMV-infected MΦ. A) PBMCs were co-cultured with autologous mock, UV-TB40/E or TB40/E-infected MΦ and the NK cell phenotype was checked by flow cytometry at day 3. **B)** PBMCs were co-cultured with autologous HCMV-infected MΦ and NK cells and CD8+ T cells phenotype was checked by flow cytometry at day 3. NK cells were defined as CD3- CD56+ and CD8+ T cells as CD3+ CD8+.

Another open question is whether LILRB1+ cells may have a role in the lesion development or constitute an indirect marker of the influence exerted by the infections. Little is known about the role of NK cells in atherosclerosis however, NK and T cells bearing NKR have been detected in lesions [178, 243]. Further studies checking the presence of LILRB1+ NK cells in atherosclerotic plaques should be performed in order address this issue. On the other hand, *LILRB1* polymorphisms have been associated to higher expression levels of the receptor by NK cells [244]. A study addressing the association of *LILRB1* polymorphism with CIMT might be also of interest.

Our work suggests that the association of overt and subclinical atherosclerotic disease with increased LILRB1+ NK and T cells, which display a terminally differentiated phenotype, likely reflects a relationship between the immune challenge by infections and cardiovascular disease risk, without attributing a dominant role for HCMV in the population studied. Our findings might potentially lead to the identification of novel biomarkers of vascular disease development related to the infectious burden.

Chapter 6

Conclusions

1. M1 and M2 MΦ differed in their sensitivity and pattern of response to HCMV infection. M1 MΦ secreted IL-6, TNF-α, IFN-α and IL-12. By contrast, infected M2 MΦ produced low levels of proinflammatory cytokines, IL-10 and IFN-α, being IL-12 undetectable. A relative resistance of M1 MΦ to infection was partially attributable to IFN-α production.

2. HCMV-infected M1 and M2 MΦ comparably triggered NK cell-mediated degranulation, but only M1 MΦ efficiently promoted IFN-γ secretion, an effect partially related to IL-12 production.

3. Based on the antagonistic effect of specific mAbs, 2B4, NKp46 and DNAM-1 NK cell receptors played a dominant role in NK cell activation upon recognition of autologous HCMV-infected M1 and M2 MΦ.

4. HCMV-infected MΦ down-regulated CD48 expression comparably to HLA class I molecules, suggesting the existence of a novel viral immune-evasion mechanism targeting the 2B4 ligand.

5. Differences in the NK-cell mediated response to HCMV-infected M1 and M2 MΦ may be relevant in the pathogenesis of natural infection.

6. Increased proportions of NK and T cells expressing NKG2C and LILRB1 receptors were associated with HCMV seropositivity in acute myocardial infarction (AMI) patients and control subjects.

7. AMI patients displayed higher proportions of CD56+ T lymphocytes and LILRB1+ NK and T cells than control subjects.

8. The expression of LILRB1 by NK and T cells in control subjects significantly correlated with carotid intima-media thickness, a marker of subclinical atherosclerosis.

9. The immunophenotypic profile found associated with atherosclerosis corresponds to an expansion of NK and T cells at late differentiation stages, presumably reflecting the pressure of pathogens on the immune system, in line with the infectious burden hypothesis for the pathogenesis of vascular disease.

ANNEX 1

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ANNEX 2

Abbreviations

ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen presenting cell
APOE	Apolipoprotein E
CAD	Coronary artery disease
CIMT	Carotid intima-media thickness
CRP	C-reactive protein
CVD	Cardiovascular disease
DAP10	10kDa DNAX adaptor protein
DAP12	12kDa DNAX adaptor protein
DC	Dendritic cells
DNAM-1	DNAX accessory molecule 1
DNAM-1L	DNAM-1 ligands
ER	Endoplasmic reticulum
GM-CSF	Granulocyte-macrophage colony stimulating factor
HCMV	Human Cytomegalovirus
HIV	Human Immunodeficiency virus
HLA	Human leukocyte antigen
IE	Immediate early
IFN	Interferon
IFNAR	Interferon receptor chain 2
Ig	Immunoglobulin
IL	Interleukin
ILT	Ig-like transcripts
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motive
KIR	Killer Ig-like receptor

LDL	Low density Lipoprotein
LDLR	Low density Lipoprotein Receptor
MΦ	Macrophages
mAb	Monoclonal antibody
MCMV	Murine Cytomegalovirus
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MIC	MHC class I chain related
miRNA	microRNA
moDC	Monocyte-derived dendritic cell
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKG2	Natural killer group 2
NKR	Natural killer cell receptors
ORF	Open reading frames
PBMC	Peripheral blood mononuclear cells
PTK	Protein Tyrosine Kinase
PVR	Poliovirus receptor
SMCs	Smooth muscle cells
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptors
TNF	Tumor necrosis factor
UL	Unique long
ULBP	UL-16 binding protein
US	Unique short

ANNEX 3

Publications

1. **Romo N**, Fitó M, Gumá M, Sala J, García C, Ramos R, Muntasell A, Masiá R, Bruguera J, Subirana I, Vila J, de Groot E, Elosua R, Marrugat J, López-Botet M. Association of atherosclerosis with expression of the LILRB1 receptor by human NK and T cells supports the infectious burden hypothesis. Arterioscler Thromb Vasc Biol. 2011 31:2314-21.

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