Role of the LILRB1 HLA class I-specific inhibitory receptor in the regulation of macrophage function

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THESIS ABSTRACT

In the present work the regulatory role played by the HLA class I (HLA-I)-specific LILRB1 inhibitory receptor in human macrophages (MΦ) was addressed. *In vitro* differentiated monocyte-derived M1 and M2 MΦ expressed different LILRB1 levels. We provide experimental evidence supporting that LILRB1-HLA-I interaction regulated the MΦ activation threshold, controlling cytokine secretion under steady state as well as in response to tumor cells and to signaling through activating receptors. On the other hand, our observations support that LILRB1 binding to cells occurred independently of total HLA-I expression levels, correlating with HLA-I dimerization. Such conformational change was detectable in type I IFN-treated MΦ and associated to an enhanced LILRB1-HLA-I interaction, thus providing a potential regulatory mechanism to control MΦ activation.

RESUM DE LA TESI

En aquest projecte hem analitzat la participació del receptor inhibidor específic per HLA de classe I: LILRB1, en la regulació de la funció dels macròfags humans (MΦ). Els macròfags M1 i M2 diferenciats *in vitro* expressen nivells diferents de LILRB1. Aportem evidències experimentals que recolzen el paper de la interacció LILRB1/HLA-I com element regulador del llindar d'activació en la homeòstasi del macròfag, influint en la secreció de citocines en condicions basals així com en resposta a cèl.lules tumorals i a la senyalització de receptors activadors dependents de motius ITAM. D'altra banda, les nostres observacions indiquen que el reconeixement de cèl.lules diana per part de LILRB1 es dóna independentment del nivell d'expressió de les molècules del HLA en la seva superfície, però correlaciona amb la dimerització d'aquestes molècules. Aquest canvi conformacional, detectat en MΦ tractats amb interferons de tipus I, s'associa a un increment de la interacció entre LILRB1 i HLA-I, suggerint un possible mecanisme regulador de l'activació del MΦ.

PREFACE

Macrophages (MΦ) are tissue resident innate immune cells which, according to their location, display a wide phenotypic heterogeneity and exert specialized functions essential for homeostasis and host defense. MΦ express different surface inhibitory receptors, including LILRB1 and LILRB2 specific for HLA-I class I molecules (HLA-I). Other inhibitory receptors enable Natural Killer (NK)-cells to selectively react against tumor and virus-infected cells displaying altered HLA-I expression. Information regarding the function of LILRB1 and LILRB2 in MΦ is limited. Our work provides novel insights on the regulatory role played by LILRB1 in MΦ, under steady state and experimental pathological conditions.

CONTENTS

Chapter 1

INTRODUCTION

1 - Macrophage biology.

Since the identification of macrophages (MΦ), during the description of phagocytosis by Elie Metchnikoff [1], these cells where defined as important players in the immune response and homeostasis. MΦ are strategically located throughout the portals of entry from the outside environment (skin, lungs and gut), immune privileged sites (brain, testis and eyes) and in organs specialized in the elimination of circulating senescent cells and toxins (spleen and liver), playing important immune surveillance activities such as phagocytosis, antigen presentation and immune regulation [2]. MΦ have an important plasticity and, depending on the microenvironment, display different phenotypes and specific functions (Fig. 1).

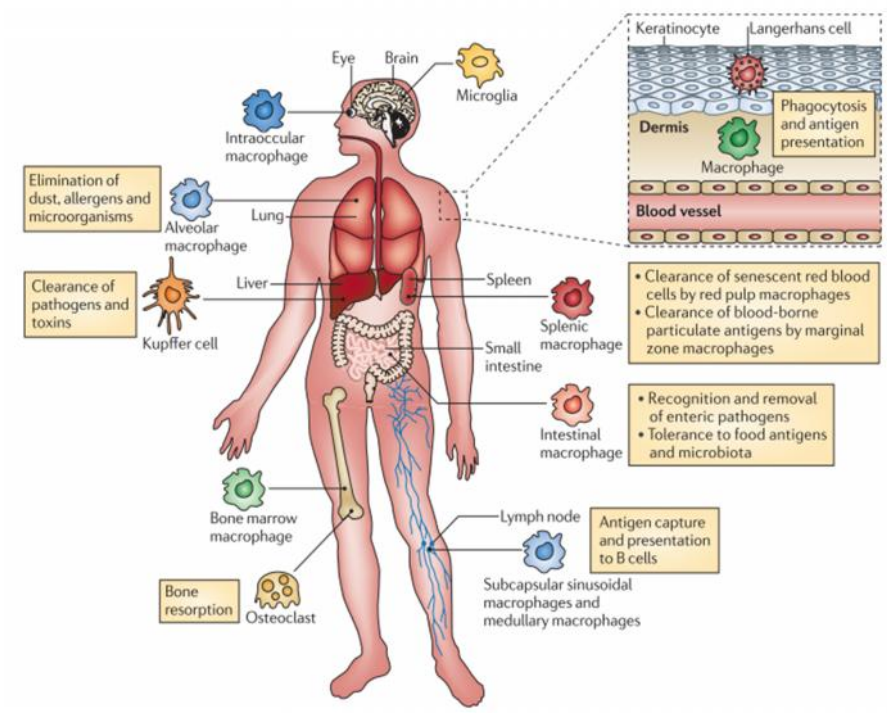

Figure 1 - Tissue macrophages perform important homeostatic functions. Murray PJ et al; *Nature Reviews Immunology*; 2011 [2].

1.1 - Differentiation and polarization.

Most tissue resident MΦ are believed to be derived from circulating monocytes which extravasate into the tissue in steady state conditions or in response to inflammation and it has been proposed that different monocyte subsets give rise to specific types of MΦ [3]. Moreover, it has been recently demonstrated in mice that MΦ derived from cells in the yolk sac or fetal liver contribute to the adult pools of Langerhans cells and microglia, co-existing with MΦ with a hematopoietic origin [4].

In mice, two monocyte subpopulations (inflammatory and resident monocytes) have been identified based on their maturation status prior to tissue migration [5]. The inflammatory monocytes, defined as $CCR2^{+}CX_3CR1^{low}GR1^{+}$, are quickly recruited to inflamed tissues once they exit the bone-marrow into the bloodstream. The resident monocytes, defined as $CCR2^{-}CX_3CR1^{hi}GR1^{-}$, have a maturation process in the blood prior to the tissue recruitment [6] and contribute to the blood vessels integrity and the maintenance of tissue-resident MΦ in steady state. Apart from tissue MΦ replenishment by monocytes, it has been shown that *in situ* proliferation of tissue-resident colony-forming cells, giving origin to mature tissue MΦ, can also occur [7,8].

In humans, two subsets of monocytes have also been identified based on the surface expression of CD14 and CD16 (classical $CD14^{hi}CD16^{-}$ and non-classical $CD14^{+}CD16^{+}$ monocytes). However, no parallelism with murine monocyte subpopulations has been observed regarding their capacity to originate specific functional MΦ subsets [9].

Inflammation or infection results in resident MΦ activation triggering cytokine and chemokine production. Exposure of MΦ to microbial products and cell-derived cytokines can induce different activation states in these cells.

Based on T-cell biology concepts, MΦ have been classified as M1 (classically activated – TLR ligands and IFN-γ) or M2 (alternatively activated – IL4/IL13) [10].

M1 MΦ are characterized by the 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), the production of reactive nitrogen and oxygen intermediates, strong antimicrobial and tumoricidal activity, and the support of Th1 responses. In contrast, M2 MΦ secrete predominantly the anti-inflammatory cytokine IL-10, express scavenger and mannose receptors, contribute to Th2 responses, display enhanced phagocytosis capacity, eliminate pathogens and promote tissue repair [11,12].

M1 and M2-like MΦ have been identified throughout the body. Alveolar MΦ and tumor-associated MΦ (TAM) share some functional characteristics with the M2 MΦ subset such as immunosuppression and poor antigen-presentation capacity [13,14]. On the other hand, M1-like MΦ are key mediators in several autoimmune diseases, including rheumatoid arthritis and inflammatory bowel disease [15,16].

Although this classification is still used, it is considered to be a very simplified way to define MΦ polarization. Based on MΦ functions (host defence, wound healing and immune regulation), three basic MΦ populations have been recently identified [17]. Taking into account the hallmarks of MΦ (plasticity and diversity), a model of MΦ activation has been proposed to define the MΦ polarization process (Fig. 2).

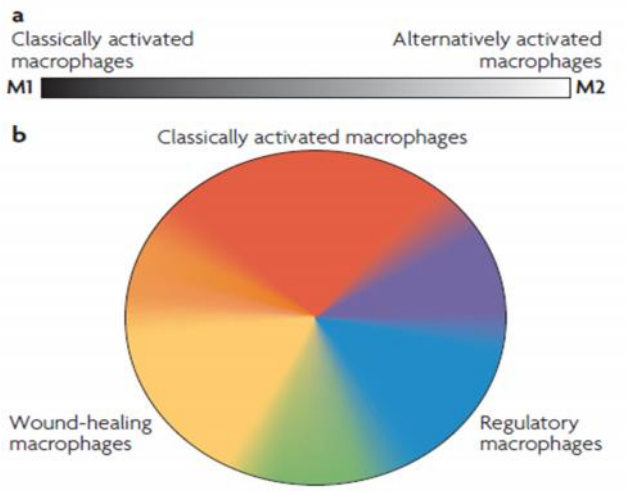

Figure 2 – Colour wheel of macrophage activation. Mosser DM et al; *Nature Reviews Immunology*; 2008 [17].

1.2 - Macrophage inhibitory receptors.

MΦ express surface and intracellular receptors that regulate a range of functions including differentiation, growth and survival, adhesion, migration, phagocytosis, cytokine secretion and antigen presentation. The capacity of these receptors to properly recognize both endogenous and exogenous ligands is crucial for MΦ function in homeostasis and host defense. The expression of several pattern recognition receptors (PRR) like the C-type lectin receptors Dectin-1 and DC-SIGN, the Toll-like receptors TLR2 and TLR4, the NOD-like receptors (NLR) and RIG-like helicases (RLH), allow MΦ to directly recognize conserved pathogen-associated molecular patterns (PAMP) expressed by microorganisms [18]. Besides direct sensing, MΦ can also uptake and kill pathogens through the recognition of opsonized particles and immune complexes by complement and Fc receptors [19,20].

In addition to exogenous pathogen recognition, MΦ can also sense endogenous molecules associated with damaged self through danger-associated molecular patterns (DAMP) receptors. The oxidative phosphorylation is essential for metabolism and signal transduction. Nevertheless, this process produces reactive oxygen species that damage proteins, lipids and DNA. Oxidized LDL is recognized by several MΦ scavenger receptors, including CD36, SR-A1 and -A2, SR-B1, MARCO, LOX-1 and PSOX [21]. Recently, it has been demonstrated that TLR4 is also able to recognize oxidized cholesteryl esters and phospholipids [22]. For the recognition and elimination of apoptotic cells, MΦ express surface glycoproteins that include phosphatidyl serine receptor (PSR), the vitronectin receptor αvβ3-integrin, the complement receptors 3 and 4 (CR3 and CR4), the β2-glicoprotein I receptor and the scavenger receptors SR-A and CD36 [18].

The immune response is tightly regulated by a balance between activating and inhibitory signals and the disruption of this balance may cause autoimmune disorders, allergy and infectious diseases. An inappropriate MΦ response can lead to chronic inflammation, hyperproduction of cytokines or cause severe tissue damage through an excess of ROS production. In order to regulate their function, MΦ express several inhibitory receptors that, upon ligand binding, interfere with activating signaling cascades. The majority of inhibitory receptors expressed on human MΦ possess immunoreceptor tyrosine-based inhibitory motifs (ITIM) within the cytoplasmic tail. The interaction of ITIM-bearing receptors with their ligands promotes the phosphorylation of tyrosine residue(s) within ITIM motifs by Src family tyrosine kinases, allowing the recruitment of SH2 domain-containing inositol (SHIP) or tyrosine (SHP) phosphatases, promoting a local and transient inhibition of signaling through activating receptors [23] (Fig. 3).

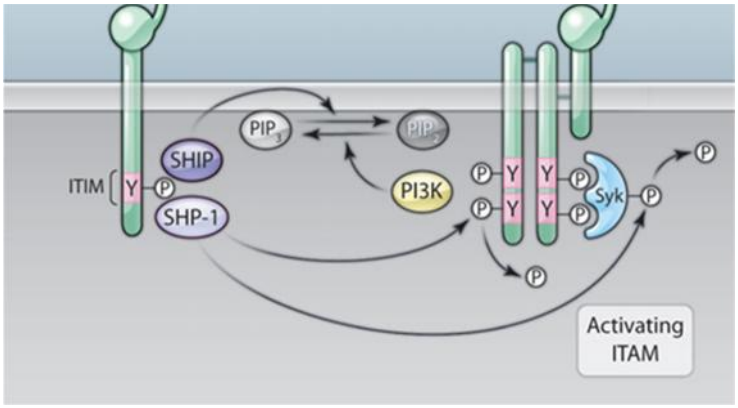

Figure 3 – Model of ITIM-mediated inhibition. Ivashkiv LB et al; *Science Signaling*; 2011 [23]**.**

The binding of SHIP phosphatases to phosphorylated ITIM results in phosphatidylinositol (3,4,5)-triphosphate (PIP_3) and inositol (1,3,4,5)-tetrakisphosphate (IP_4) hydrolysation, leading to the impairment of Pleckstrin Homology (PH)-domain containing molecules (such as Bruton Tyrosine Kinase (Btk) or Protein Kinase B (PKB)) recruitment to the cell membrane. On the other hand, the tyrosine phosphatases SHP-1 and SHP-2 contribute to the negative regulation of cell activation through the dephosphorylation of members of the syk family of kinases (such as ZAP-70 or syk) and also pITAM motifs in different activating receptors [24].

According to their structure, the inhibitory receptors expressed by human MΦ can be divided in three groups: Immunoglobulin Superfamily molecules, Sialic Acid-Binding Immunoglobulin-like Lectins and C-type Lectins (Table 1).

Family	Receptor	Ligand	ITIM motifs	Inhibitory mechanism
Ig Superfamily	CD200R	CD200	NO	Binds to Dok1 or Dok-2 adapter protein and recruits RasGAP which mediates the inhibition of the Ras/ErK pathways.
	CD300a (IRp60)	PS and PE	3	Recruits SHP-1, SHP-2 and SHIP phosphatases.
	CD300f (IREM-1)	ND	2	Recruits SHP-1 phosphatase.
	CEACAM1 (CD66a)	CEACAM1/ CEACAM5	2	Recruits SHP-1 and SHP-2 phosphatases.
	FcγRIIb (CD32b)	IgG	1	Recruits SHIP phosphatase.
	LILRB1 (CD85j)	HLA-I and UL18	4	Recruits SHP-1 phosphatase.
	LILRB2 (CD85d)	HLA-I	3	Recruits SHP-1 phosphatase.
	LILRB3 (CD85a)	ND	4	Recruits SHP-1 phosphatase.
	LILRB4 (CD85k)	ND	3	Recruits SHP-1 phosphatase.
	PECAM-1 (CD31)	PECAM-1/ CD177/CD38	2	Recruits SHP-2 phosphatase.
	SIRP-a (CD172a)	CD47	2	Recruits SHP-1 and SHP-2 phosphatases.
Siglec	Siglec-11	Sialic acid	2	Recruits SHP-1 and SHP-2 phosphatases.
C-type lectins	CLEC4A (DCIR)	Mannose and fucose	1	Recruits SHP-1 and SHP-2 phosphatases.
	CLEC12A (MICL)	ND	1	Recruits SHP-1 and SHP-2 phosphatases.

Table 1. Surface inhibitory receptors expressed on human MΦ.

Immunoglobulin superfamily (IgSF) receptors:

CD200R. The CD200 receptor is mainly expressed in cells of the myeloid lineage such as monocytes, dendritic cells (DC), MΦ, mast cells and granulocytes, but is also present on B- and T-lymphocytes [25]. This receptor signals through the direct binding to the adaptor molecules downstream of tyrosine kinase 1 or 2 (Dok1 and Dok2) followed by a recruitment and activation of Ras GTPase-activating protein (RasGAP) resulting in the inhibition of Ras-Erk signaling [26]. CD200R has been shown to play an important role on the regulation of microglia activation upon interaction with neurons. CD200 deficiency is correlated with increased inflammatory responses in murine models of multiple sclerosis and Parkinson´s disease [27]. Moreover, it has been shown that monocyte-derived MΦ from individuals with Parkinson´s disease display dysfunctional CD200R signaling [28].

CD300a (IRp60). CD300a, also known as inhibitor receptor protein 60(IRp60), is the first member of the CD300 multigene family of receptors. CD300a is expressed on myeloid lineages, all NK Cells and in subsets of B and T Cells [29]. This receptor possess three classical and one non-classical (switch motif) immunereceptor tyrosine-based inhibitory motifs (ITIM). Upon tyrosine phosphorylation of the ITIMs, different phosphatases including SHP-1, SHP-2 and SHIP, are recruited depending on the cell type [30]. Recently it was reported that CD300a recognizes phosphatidylserine (PS) and phosphatidylethanolamine (PE), and consequently negatively regulates the uptake of late apoptotic/necrotic cells by monocyte-derived MΦ [31].

CD300f (IREM-1). CD300f, or inhibitor receptor expressed by myeloid cell 1 (IREM-1), contain five tyrosine-based motifs in the intracellular domain. Two of them fit with classical ITIM (Y205 and Y249), and two (Y236 and Y263) are within motifs reported to bind the p85α regulatory subunit of phosphatidylinositol 3-kinase (PI3K) [32,33]. Upon ITIM phosphorylation, this receptor is able to recruit SHP-1 phosphatase. Although the ligand for CD300f is still unknown, it has been recently shown that the mouse CD300f functional orthologue (CLM-1) binds to PS [34], suggesting that this receptor, like CD300a, should recognize phospholipids and therefore regulate the uptake of apoptotic cells.

CEACAM1 (CD66a). The carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is expressed in a vast variety of cell types including epithelial, endothelial and hematopoietic cells. This receptor mediates intercellular adhesion through homophilic and heterophilic interactions (CEACAM1-CEACAM5) [35]. Several pathogenic viruses and bacteria contain adhesins that are able to interact with CEACAM1 influencing the outcome of the immune response. It has been shown that *N. gonorrhoeae* Opa proteins supress the activation and proliferation of $CD4^+$ T cells through CEACAM1 [36]. Different splicing of *CEACAM1* transcript can originate either a long isoform containing two ITIM, that upon phosphorylation can recruit SHP-1 and SHP-2 phosphatases, or short isoforms lacking tyrosine-based signalling motifs [37]. The ratio between the long and short isoforms varies depending on the cell type and activation state [38]. It has been shown that IL2 treatment induces CEACAM1 expression on human NK cells that, upon cross-linking, interferes with NKG2D-mediated lysis of tumour cells [39].

FcγRIIb (CD32b). The Fcγ receptor IIb (FcγRIIb**)** is the only FcγR that has an inhibitory function. This receptor is expressed by B cells, DCs, MΦ, activated neutrophils, mast cells and basophils [40]. Three different FcγRIIb isoforms were identified [41]. FcγRIIb1 and FcγRIIb2 encode membrane proteins containing an ITIM within the intracellular domain. FcγRIIb3 is a soluble protein lacking both transmembrane (TM) and cytoplasmic domains. Surface isoforms of FcγRIIb inhibit the signalling cascade of activating FcγRs through the recruitment of SHIP [42]. The balance between activating and inhibitory FcγRs-mediated signals regulate the immunological effects of Ab and immunocomplexes (IC) recognition [43]. Alterations on surface expression of both activating or inhibitory FcγRs, influence cell activation threshold upon IC stimulation resulting in enhanced or reduced inflammatory responses [44]. Moreover, using THP-1 cells, it has been demonstrated that FcγRIIb expression is correlated with a decreased capacity to phagocyte IgG-opsonized particles [45].

LILR (CD85). This family of receptors contain four inhibitory (LILRB1-4) receptors that are expressed on human MΦ. LILRs will be described in more detail on Section 2.

PECAM-1 (CD31). Platelet/endothelial cell adhesion molecule 1 (PECAM-1) is expressed by platelets, endothelial cells, bone marrow precursor cells, myelomonocytic cells, granulocytes and a subset of lymphocytes [46,47]. PECAM-1 possess a large cytoplasmic tail containing two ITIM motifs that upon phosphorylation can provide a docking site to SHP-2 phosphatase [48]. Like other adhesion molecules (e.g. CEACAM1) this receptor displays the capacity to form either homophilic or heterophilic (CD177 and CD38) interactions [46]. MΦ can discriminate between apoptotic and viable cells through a homophilic PECAM-1 interaction. This receptor promotes

selective detachment signals, preventing the ingestion of viable cells by MΦ [49].

SIRP-α (CD172a). Signal regulatory protein α (SIRP-α) is mainly expressed in myeloid leukocytes including granulocytes, monocytes, MΦ and DCs. SIRP-α is a type-1 transmembrane protein that contains two ITIM motifs in the cytoplasmic domain [50]. Upon binding to CD47, a protein that is ubiquity expressed, SHP-1 and SHP-2 phosphatases are recruited initiating the negative signalling events [51]. SIRP-α interaction with CD47 prevents red-blood cells (RBC) phagocytosis by mice splenic red pulp MΦ [52].

Sialic acid-binding immunoglobulin-like lectins (Siglec):

Siglecs are a family of sialic acid-binding immunoglobulin-like lectins that promote cell-cell interactions and regulate cell functions of both innate and adaptive immune system. These receptors are type-I transmembrane proteins and can be divided in two subsets accordingly to their sequence similarity. Siglec-1, -2, -4 and -15 are distantly related (~25-30% sequence identity) while CD33-related Siglecs (-3, -5, -6, -7, -8, -9, -10, -11 and -14) share ~50-99% identity [53]. Each Siglec has a unique specificity for sialyated ligands [54]. Human MΦ express Siglec-1 and Siglec-11. Siglec-1, or Sialoadhesin, lack inhibitory signaling cytosolic motifs and is thought to mediate adhesion events [55]. Siglec-11 contains one ITIM motif and one ITIM-like motif within the cytoplasmic tail and is able to interact with both SHP-1 and SHP-2 [56]. Using mice microglia transduced with human Siglec-11, it has been shown that cross-linking of this receptor inhibits the production of pro-inflammatory mediators upon LPS stimulation [57].

C-type lectin receptors:

CLEC4A (DCIR). C-type lectin domain family 4 member A (CLEC4A), also known as dendritic cell immune receptor (DCIR), is a type II membrane protein expressed on antigen-presenting cells (APC) and granulocytes. The cytoplasmic domain of CLEC4A contains an ITIM motif that after tyrosine phosphorylation recruits SHP-1 and SHP-2 phosphatases [58]. This receptor recognizes mannose and fucose [59] and the binding to these glycans is modulated by the glycosylation of CLEC4A carbohydrate recognition domain (CRD) [60]. CLEC4A engagement, using a mAb, induces receptor internalization into the lysosome and inhibition of TLR8-mediated cytokine secretion by monocyte-derived DC [61].

CELC12A (MICL). Myeloid inhibitory C-type lectin-like receptor (MICL) is predominantly expressed by neutrophils, eosinophils, monocytes, MΦ and DC and is down-regulated during inflammation and cellular activation [62]. MICL contains an ITIM motif that after tysosine phosphorylation is able to interact with SHP-1 and SHP-2 phosphatases [63]. The ligand for MICL is still unknown but it has been recently shown that the murine homolog (mMICL) recognizes an endogenous ligand expressed on cells isolated form heart, lung, liver, spleen and kidney, but not microbial ligands [64].

Besides regulation of ITAM-mediated cell activation, recent reports have been shown that ITIM signaling can also interfere with TLR stimulation. Apart from the previously described Siglec-11 and CLEC4A, other ITIM-bearing receptors such as CEACAM1 [65] and CD300F [66] can negatively modulate TLR signaling cascades, mediated both by MyD88 and TRIF, through the recruitment of SHP-1 phosphatases. Moreover, it has been recently reported that pathogenic entero bacteria express an ITIM-containing protein (Tir) that, upon host SHP-1 recruitment, can prevent TLR-induced pro-inflammatory cytokine production [67], suggesting that bacteria have

developed ITIM-based immune escaping strategies to counteract TLR-mediated host cell activation.

2 - Leukocyte immunoglobulin-like receptors (LILR).

Leukocyte Immunoglobulin-like receptors (LILR), also known as CD85, ILT, LIR and MIR, are encoded on human chromosome 19q13.4 in close linkage to the KIR locus and, with one exception, are predominantly expressed on monocytes, MΦ and DC. This family of receptors belonging to the IgSF, contain either two or four homologous extracellular C2-type Ig-like domains [68]. According to their transmembrane and cytoplasmic domains, these receptors can be divided in three groups (Fig. 4):

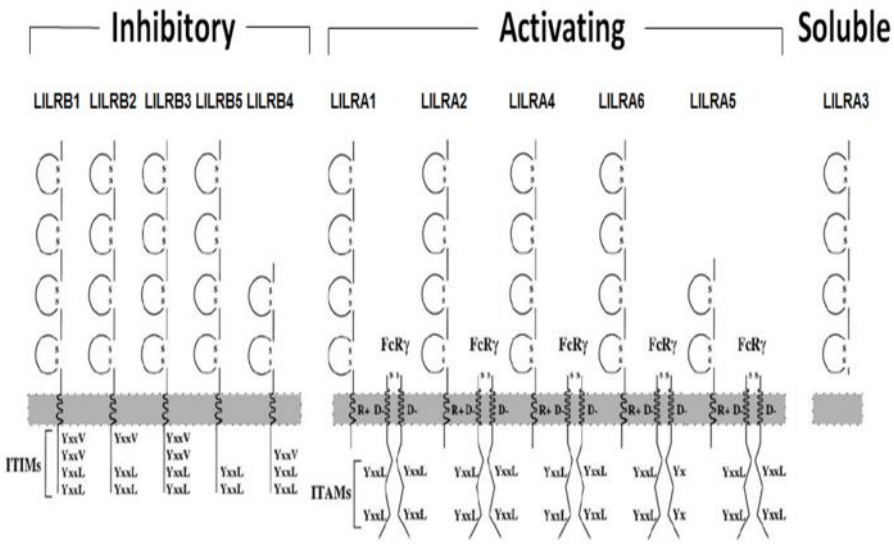

Figure 4 - LILR receptor family. Adapted from Colonna M. et al; *Journal of Leukocyte Biology*; 1999[68].

Inhibitory - The inhibitory LILR (LILRB) possess a long cytoplasmic tail containing two, three or four ITIM motifs. Upon tyrosine phosphorylation, these receptors are able to interfere with activating signalling through the recruitment of SHP-1 phosphatase [68–71].
The best characterized inhibitory LILR are LIRB1 and LILRB2. Both receptors recognize classical and non-classical HLA-I molecules through the membrane-distal Ig-like domains D1 and D2 [72,73]. The ligands for LILRB3, LILRB4 and LILRB5 have not been yet identified.

Activating – The activating LILR (LILRA) contain a short cytoplasmic domain with no signalling motifs. In order to transduce activating signals, these receptors associate with the ITAM-bearing FcεR-I γ chain through the transmembrane positive charged amino acid arginine [74–76].
Based on the analysis of LILRB1 amino acid residues that interact with HLA-I, and comparing with the other LILR, it has been proposed that the activating receptors LILRA1 and LILRA2 should be also able to recognize HLA-I molecules [77,78]. LILRA1 recognizes HLA-B27 in both classical and β2m-free conformations [79]. More recently, it has been shown that this receptor binds with low affinity to several HLA-I molecules and with an overall preference for HLA-C alleles. Furthermore, the removal of β2m increased the LILRA1 binding capacity to HLA-I [80]. Regarding LILRA2, no binding to HLA-I has been reported. The LILRA2 extracellular crystal structure revealed structural shifts of the corresponding HLA-I-binding amino acid residues explaining its inability to recognize HLA-I [81].

Soluble – LILRA3 is the only soluble LILR lacking both transmembrane and cytoplasmic domains. Its function is unknown but it was proposed that LILRA3 could act as an antagonist of other LILR by preventing the binding to a common ligand [76]. Recently it has been shown that LILRA3

recognizes both classical and non-classical HLA-I molecules with reduced affinities compared with LILRB1 and LILRB2 [82].

2.1 - Inhibitory LILR expressed on MΦ.

LILRB1 - LILRB1, also known as CD85j, ILT2 or LIR-1, is expressed on the surface of monocytes, MΦ, DC, B cells and a subset of NK and T cells [83,84]. Monocytes display a higher cell surface expression of LILRB1 than NK and T cells. It has been described that monocytes and lymphocytes use different promoters to drive LILRB1 expression. The lymphocyte promoter maps 13kb upstream of the monocyte promoter, giving origin to the inclusion of a distant exon at the 5´- untranslated region that represses LILRB1 protein translation, resulting in lower expression levels in NK and T cells [85].

Structure and ligand recognition:

This inhibitory receptor is composed by an extracellular region with four Ig-like domains (D1-D4), a TM domain and a cytoplasmic tail containing four ITIM motifs. Upon ligand binding, LILRB1 is able to recruit SHP-1 phosphatases through ITIM phosphorylation [83].

LILRB1 recognizes a broad range of HLA-I molecules. The extracellular D1 and D2 domains contact with the highly conserved HLA-I α3 domain and β2m [77], explaining the reported promiscuity regarding HLA-I binding. Other HLA-I-specific receptors, such as TCR and KIR, recognize the polymorphic α1 and α2 domains, what provides an exquisite selectivity in their capacity to interact with allelic variants of different HLA-I molecules (Fig. 5).

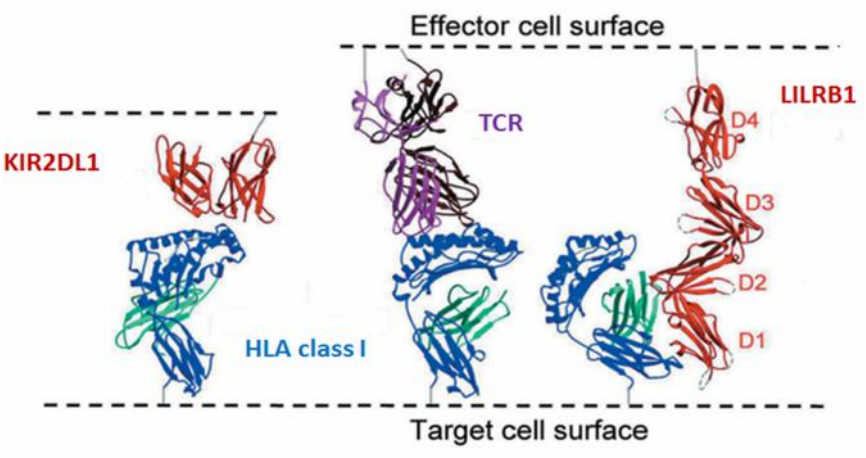

Figure 5 – KIR2DL1, TCR and LILRB1 interactions with HLA-I. Adapted from Willcox BE et al; *Nature Immunology*; 2003 [77].

Surface plasmon resonance analysis, using the LILRB1 ectodomain, demonstrated that LILRB1 binds to HLA-G with higher affinity than to classical HLA-I molecules [86]. HLA-G molecule possesses a cysteine residue at the position 42 (α1 domain) allowing the formation of dimers by a disulphide bridge. This C42 is unique to HLA-G and is determinant for LILRB1 recognition[87]. Comparing LILRB1 binding to HLA-G+ cells, it has been shown that this receptor binds preferentially to the dimeric form of HLA-G [88]. Furthermore, HLA-G dimers are able to trigger LILRB1 signalling more efficiently than monomers [89].

Another interesting feature regarding LILRB1 ligand binding is its capacity to form *cis* interactions with HLA-I. Using fluorescence resonance energy transfer (FRET) analysis, it has been shown that LILRB1, expressed by *in vitro* derived human osteoclasts, can interact with HLA-I molecules located on the same cell surface [90]. In order to perform a *cis* interaction, it has been proposed that the Ig-like domains D1 and D2 alter their orientation to form a horseshoe-shaped configuration [91] (Fig. 6).

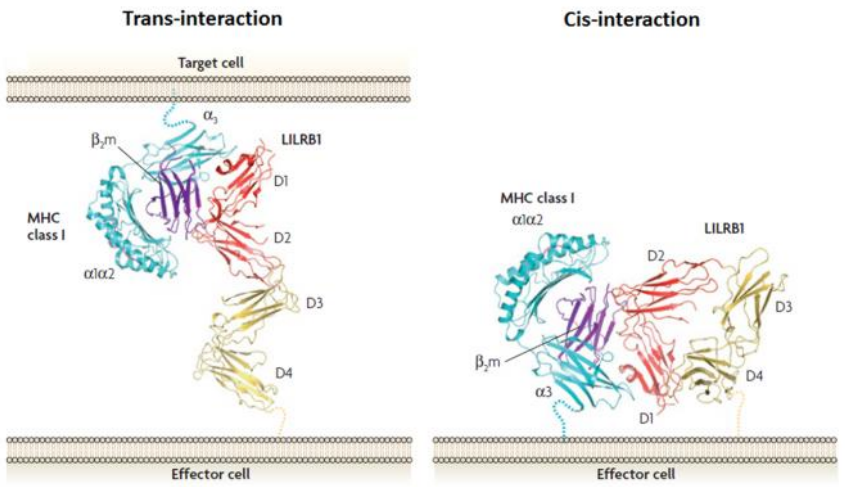

Figure 6 – Hypothetical models for trans and cis interactions of LILRB1 with HLA-I ligands. Adapted from Held W. et al, *Nature Reviews Immunology*; 2008 [91].

In addition to HLA-I binding, LILRB1 is also able to recognize with very high affinity the human cytomegalovirus (HCMV) -encoded protein UL18 [84,92]. HCMV encodes several proteins capable to interfere with HLA-I synthesis and migration towards the cell surface (US2, US3, US6, US10 and US11), avoiding a proper viral antigen presentation to cytotoxic T cells [93,94]. On the other hand, the down-modulation of HLA-I surface expression promotes the response against infected-cells by NK cells expressing HLA-I specific inhibitory receptors such as LILRB1, NKG2A or KIR. The UL18 HLA-I-like protein [95] is believed to inhibit the response of NK and T cells expressing LILRB1. It was reported that UL18 transfection repressed the lysis of the HLA-I deficient cell line 721.221 by NK cells [96]. However, using *in vitro* HCMV infection models, UL18 surface expression is almost undetectable [97,98]. In fact, UL18 cytoplasmic tail contains two motifs that are homologous to consensus sequences that mediate intracytoplasmic retention [99], questioning that during natural HCMV

infection UL18 interacts with LILRB1 expressed on the surface of immune cells.

Biological function:

The LILRB1 interaction with HLA-G has been proposed to have an important role on the induction of feto-maternal tolerance during pregnancy [100]. HLA-G is mainly expressed on the surface of extravillous trophoblasts (EVT) [101]. These specialized fetal cells, that invade the uterine implantation site and directly contact with maternal cells, do not express HLA-A or HLA-B, expressing low levels of HLA-C [102] and HLA-E, avoiding the induction of immune responses through fetal antigenic presentation to maternal T lymphocytes. Moreover, the maternal uterine leukocytes that contact with EVT are predominantly composed by NK cells (which express the CD94/NKG2A inhibitory receptor for HLA-E and KIR that recognize HLA-C) and decidual MΦ [103] that can be negatively regulated by LILRB1 interaction with HLA-G expressed on EVT cells [104,105].

Several functional studies have demonstrated the LILRB1 capacity to negatively modulate signals transduced by activating receptors, resulting in inhibition of different effector functions such as cytokine secretion, cell proliferation or cell differentiation. In human monocytes it has been shown that the co-engagement of LILRB1 with the Fcγ receptor CD64 prevents tyrosine phosphorylation of Fc receptor γ chain and intracellular Ca^{2+} mobilization [106]. LILRB1 ligation using an anti-LILRB1 mAb, modulates monocyte-derived DC differentiation giving origin to a cell population resistant to CD95-mediated cell death, altered cytokine production and a poor capacity to stimulate T-cell proliferative responses [107]. Furthermore, LILRB1 constitutive binding to HLA-I molecules can suppress *in vitro* osteoclast development induced by RANKL and M-CSF [90]. Besides

myelomonocytic cell regulation, this inhibitory receptor can also modulate lymphocyte functions. LILRB1 engagement inhibit both TCR- and BCR-mediated responses [83,108,109]. In addition, LILRB1 cross-linking interferes with class switching events driven by B-cell activation through CD40 in combination with IL4 [110]. Regarding NK cell function, it has been shown that LILRB1 is able to regulate their cytotoxic activity in response to different tumor cell lines [83,111]. Moreover, IFN-γ production by the NKL NK cells line induced upon recognition of the HLA class I-deficient 721.221 cell line, was inhibited by interaction with different HLA-I molecules transfected on target cells [112].

LILRB2. The inhibitory receptor LILRB2, also known as CD85d, ILT4 or LIR-2, is mainly expressed by myelomonocytic cells. LILRB2 contains four Ig-like extracellular domains, a TM domain and a cytoplasmic tail with three ITIM motifs. Upon ligand binding, tyrosine residues within ITIM motifs are phosphorylated allowing the recruitment of SHP-1 phosphatase [83,113].

Even though LIRB2 and LIRB1 have a high amino acid sequence homology (81%), these two receptors display important differences regarding HLA-I recognition. While LILRB1 only binds to HLA-I molecules containing β2m, LILRB2 is able to recognize HLA-I free heavy chains (FHC) [80,114]. Structural analysis have shown that LILRB2 forms a more intimate contact with HLA-I α3 domain than β2m while LILRB1 preferentially binds to β2m (Fig. 7).

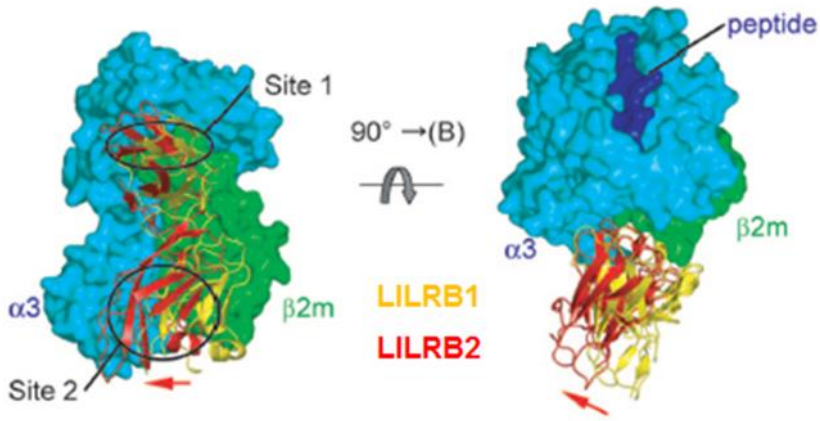

Figure 7 – Structural comparison of the LILRB2/HLA-G and LILRB1/HLA-A2 complexes. Adapted from Shiroishi M. et al; *Proc. Natl. Acad. Sci.*; 2006 [114].

LILRB2 recognizes HLA-B27 FHC monomers and homodimers with higher affinity than classical HLA-I heterotrimers (heavy chain, β2m and peptide) [115]. HLA-B27 is strongly associated with the development of ankylosing spondylitis (AS), an inflammatory arthritic disorder triggered by the activation of immune cells [116]. Based on the increased surface expression of HLA-B27 FHC in patients with spondyloarthropathy in comparison with healthy individuals [117] a putative role of LILRB2 role in the disease course has been hypothesized.

Biological function:

LILRB2 signalling has been shown to be associated with the generation of tolerogenic DC by inducing MHC-II, CD80 and CD86 down-modulation [118]. This specialized type of DC was first reported when interaction of $CD8^+$ T suppressor cells (Ts) with antigen presenting cells (APC) resulted in the down-modulation of co-stimulatory molecules influencing their capacity

to trigger subsequent T cell responses [119]. A treatment used for the generation of DC that induce T cell hyporesponsiveness is the combination of vitamin D3 and dexamethasone. DC maturation with LPS in the presence of these tolerogenic agents promotes IL-10 secretion and LILRB2 mRNA up-regulation [120]. Moreover, it has been recently demonstrated that DC, *in vitro* differentiated in the presence of IL-10 (called DC-10), are potent inducers of type 1 T regulatory (Tr1) cells. These cells are characterized by the capacity to suppress immune responses through IL-10 and TGF-β secretion in an antigen-specific manner. Interestingly, DC-10 express high levels of LILRB2 and the blockade of this inhibitory receptor, using a mAb, impairs the generation of the anergic and immunosuppressive Tr1 cells [121]. The mechanism whereby LILRB2 promotes T cell anergy is not fully understood but it is believed that besides the low expression of co-stimulatory molecules, the impairment of HLA-I binding may play an important role. Moreover, it has been shown that LILRB2, like LILRB1, competes with CD8 for HLA-I binding and can act as a CD8-antagonist controlling cytotoxic T cell priming [86,122]. In the later study, the authors compared the ability to prime $CD8^+$ T cells of Langerhans cells and dermal $CD14^+$ DC. Of note, their allogeneic T cells priming capacity was inversely associated with LILRB2 surface expression. Langerhans cells ($LILRB2^-$) induced both T cell IFN-γ and TNF-α production, whereas the dermal DC ($LILRB2^+$) were not able to produce these effects. In addition, the blockade of LILRB2, but not LILRB1, using an antagonistic mAb, enhanced the dermal DC capacity to promote the generation of polyfunctional $CD8^+$ T cells.

LILRB3. LILRB3, also known as CD85a, ILT5 or LIR-3, is mainly expressed on the surface of myeloid cells and some T cells. This molecule contains four Ig-like extracellular domains, a TM domain and a cytoplasmic tail with four ITIM motifs [68,75,83].

LILRB3 is the most polymorphic inhibitory receptor belonging to the LILR family [83,123]. Recently it has been demonstrated that LILRB3 disparity between donors and recipients, in hematopoietic stem cell transplantations, can induce antibody-mediated responses [124]. Interestingly, in the same report, the authors showed that LILRB3-specific antibodies, produced upon hematopoietic stem cell transplantation, are able to mediate killing of LILRB3+ leukemic cells of both myeloid and lymphoid origin.

The ligand for LILRB3 remains unknown but it has been demonstrated that this inhibitory receptor is constitutively phosphorylated and associated with SHP-1 phosphatase in osteoclasts, suggesting that LILRB3 recognizes an endogenous ligand [90]. LILRB3 cross-linking, using a mAb, results in osteoclast development inhibition. Another functional assay has shown that the co-engagement of LILRB3 with Fcε receptor I or LILRA2 down-regulates the basophil responses triggered by these activating receptors [125].

LILRB4. LILRB4, also known as CD85k, ILT3 or LIR-5, is the only inhibitory receptor, belonging to the LILR family, containing just two extracellular Ig-like domains. LILRB4 is expressed on the surface of monocytes, MΦ and DC [70] and, like the other LILRB members, it transduces inhibitory signals through the recruitment of SHP-1 phosphatase upon tyrosine phosphorylation within ITIM motifs [68,75].

A LIRB4 ligand has not been yet identified, however the crystallization of LILRB4 ectodomain indicated that there are substantial differences regarding the LILRB1, LILRB2 regions interacting with HLA-I. Bioinformatics prediction of residues at a putative protein interface allowed the identification of three possible interaction surfaces in LILRB4 located on the D1 domain, D1D2 hinge region and D2 domain respectively [126].

Different functional assays have revealed the LILRB4 capacity to interfere with signalling events triggered by CD11b, HLA-DR and FcγR-III stimulation in monocytes [70]. More recently, using the human THP-1 monocytic cell line, it has been shown that LILRB4 cross-linking inhibits FcγR-I-induced phosphorylation of multiple signalling molecules including Lck, Syk, LAT and Erk [127]. The expression of this receptor has been shown to be increased in tolerogenic DC [128]. Interestingly, LILRB4 overexpression in DC is associated with a reduced transcription of NF-kB-dependent co-stimulatory molecules resulting in a decreased stimulation capacity of T-cell proliferation and maturation [129].

Chapter 2

HYPOTHESIS AND AIMS

It is known that NK-cell function is tightly regulated by a balance between activating and inhibitory signals [130]. NK cells express at the cell surface different inhibitory receptors specific for HLA-I molecules (namely KIR, NKG2A and LILRB1) that recruit phosphatases upon ligand binding, preventing their activation against HLA-I sufficient healthy cells. The down-regulation of HLA-I in viral infected or transformed cells interferes with inhibitory receptor signalling, facilitating the activation of NK cell effector functions against target cells (Fig. 8).

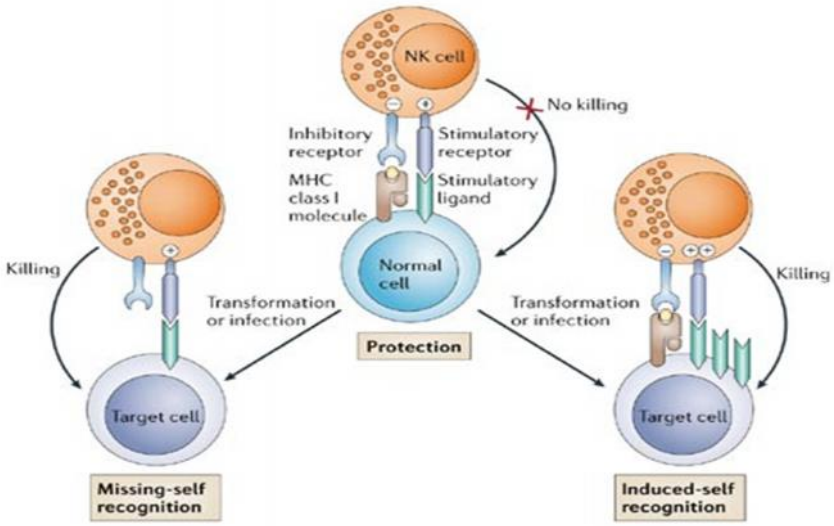

Figure 8 – The balance of inhibitory and stimulatory signals received by a NK cell determines the outcome of interactions with target cells. Raulet & Vance; *Nature Reviews Immunology*; 2006 [131].

We hypothesized that LILRB1 and LILRB2 may play in MΦ a role similar to that shown for HLA-I-specific inhibitory NK-cell receptors, regulating the MΦ activation threshold.

In this work, we addressed the regulatory role of LILRB1 and LILRB2 inhibitory receptors on the regulation of human monocyte-derived MΦ function.

The main objectives of the project were:

1 - To establish a suitable experimental system to approach the study of LILRB1 and LILRB2 interaction with HLA-I in *in vitro* differentiated primary human MΦ.

2 - Characterization of LILRB1/2 and their ligands expression on different monocyte-derived MΦ subtypes.

3 - Role for LILRB1 and LILRB2 interaction with HLA-I on the regulation of MΦ function.

3.1 - Role for LILRB1 and LILRB2 interaction with HLA-I on the preservation of MΦ tolerance to normal self.

3.2 - Role for LILRB1 interaction with HLA-I on MΦ response to tumour cells, HCMV-infected cells or aged platelets.

Chapter 3

MATERIALS AND METHODS

Cells and antibodies.

THP-1, 721.221 and 721.221 single-transfected with HLA-I molecules (G1m, B7, B21, B35, B51, A2 and A3) were grown in RPMI-1640/glutamax source medium (Invitrogen Life Technologies, Paisley, UK) supplemented with 10% (v/v) heat-inactivated FBS (Lonza, Cologne, Germany), penicillin (100 U/mL), and streptomycin (10 μg/mL). HEK293T and MRC-5 cell lines were grown in complete Dulbecco minimal essential medium (DMEM, Invitrogen Life Technologies).

Human PBMC were separated from buffy-coat units by Ficoll-Paque PLUS centrifugation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and washed extensively with PBS (400 x g 7 min, 350 x g 6 min, 300 x g 6 min and 200 x g 11 min) for platelet removal. Monocytes were isolated from total PBMC by negative selection using EasySep™ human monocytes enrichment kit without CD16 depletion (StemCell Technologies, Seattle, WA, USA) following the manufacturer´s data sheet. Using CD14 as a monocyte marker, cell preparation purity was controlled by flow cytometry analysis and varied between 80-90%.

Monocyte-derived M1 and M2 MΦ were differentiated during 7 days by culturing isolated monocytes in 10cm plates ($0{,}5x10^6$/mL, final volume 10mL) with RPMI complemented with 10% (v/v) heat-inactivated low-endotoxin FBS (Lonza) and supplemented at days 0, 2 and 5 with 100 ng/ml rhGM-CSF (PeproTech, London, UK) or 10ng/mL rhM-CSF (Immunotools, Friesosythe, Germany) respectively. After differentiation, MΦ were detached by adding 2,5 mL of Accutase cell detachment solution (Millipore, Boston, MA, USA) for 30 min at 37 ºC. CD14 and CD163 surface expression were used as M1 and M2 differentiation markers. All the solutions used were sterile, endotoxin-free and prepared with clinic water (Grifols, Barcelona, Spain).

In some experiments M2 MΦ,M1 MΦ and THP-1 cells were treated with IFN-α2a (1000U/mL, Roche Pharmaceuticals, Basel, Switzerland), IFN-β1a (1000U/mL, Merck, Darmstadt, Germany), IL-10 (10ng/mL, Peprotech) and IL-6 (25ng/mL, PeproTech) at 37ºC for 24h followed by immunofluorescence and biochemical analyses to evaluate LILRB1-Fc binding and HLA-I expression.

The mAbs used for immunophenotyping, western blot analyses, immunofluorescence and functional assays are described in Table 2:

Antibody	Specificity	Host species	Subtype	Application
HP-F1	LILRB1	mouse	IgG1	FACS/Functional assays
27D6	LILRB2	rat	IgM	FACS/Functional assays
HP-1F7	HLA-I	mouse	IgG1	FACS/Functional assays
A6136	HLA-I	mouse	IgM	FACS/Functional assays
W6/32	HLA-I	mouse	IgG2a	FACS
HC10	HLA-I (unfolded)	mouse	IgG2a	FACS/WB
HCA2	HLA-A, -G (unfolded)	mouse	IgG1	WB
G233	HLA-G	mouse	IgG2a	FACS
Mem/G9	HLA-G	mouse	IgG1	FACS
4H84	HLA-G (unfolded)	mouse	IgG1	WB
PM6/248	CD41	mouse	IgG1	Functional assays
MB45	CD42b	mouse	IgG1	FACS
M5E2	CD14	mouse	IgG2a	FACS
GHI/61	CD163	mouse	IgG1	FACS
CB16	CD16	mouse	IgG1	FACS
FUN-2	CD32	mouse	IgG2b	FACS
10.1	CD64	mouse	IgG1	FACS
TS1/18	LFA-1	mouse	IgG1	Functional assays
9E10	Myc	mouse	IgG1	FACS/Functional assays
21C7	TREM-1	mouse	IgG1	Functional assays
8B1.2	CMV IE1/IE2	mouse	IgG2a	Immunofluorescence

Table 2 - List of mAbs used in this study and their respectively specificity, host species, subtype and application.

Antibody purification and fragmentation.

The HP-1F7 (anti-HLA-I; mouse IgG1), HP-F1 (anti-LILRB1; mouse IgG1) and TS1/18 (anti LFA 1; mouse IgG1) hybridomas were grown in complete RPMI until obtain a final volume of 320mL ($0{,}5x10^6$ cells/mL). 48h prior to supernatant (SN) harvest, cells were washed with PBS and fresh incomplete RPMI with no antibiotics was added. The obtained cell-free SN was concentrated using a 30KDa pore centrifugal unit (Millipore) and purified by affinity chromatography. The concentrated SN was passed through a BioRad chromatography column containing 0,125mg of protein G-sepharose (GE Healthcare) following the protocol explained in detail in Current Protocols in Immunology (Unit 2.7.6). After Ab elution, the content was concentrated and dialyzed in PBS using 30KDa filter units. Subsequently, the collected Ab was quantified by Bradford and the purity was validated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining.

In order to remove the Fc fragments, Abs were diluted in sodium acetate buffer (pH 3,5) and digested with pepsin (Sigma-Aldrich, Saint Louis, MO, USA) using a 1:20 (pepsin:antibody) ratio for 1h at 37ºC.To equilibrate the pH and stop the digestion, 2M Tris solution was added. Fragmented Abs were dialyzed in PBS. Ab quantity and purity were measured by Bradford and SDS-PAGE respectively.

LILRB1-Fc and LILRB2-Fc production and purification.

Preparations of plasmid DNA were made using the Endo-free Maxi-prep Kit (QIAgen). LILRB1-Fc and LILRB2-Fc (kindly provided by Prof. John Trowsdale, University of Cambridge, UK) were obtained by transfecting HEK293T cells with Signal pIgplus vector containing the coding sequence of each inhibitory receptors ectodomain using calcium phosphate.

18h post-transfection, the culture medium was replaced by fresh FBS-free medium (EX-cell ACF CHO Medium, Sigma-Aldrich) supplemented with 5mM L-glutamine (Life Technologies). After 6 days, supernatants were harvested and fusion proteins purified by affinity chromatography using protein A-Sepharose CL-4B (GE Healthcare) and analysed by SDS-PAGE and Coomassie blue staining. Fusion protein concentration was measured by Bradford assay.

The specificity of the obtained fusion proteins was checked using a competition assay. PBMC were immunostained for 30 min on ice with mAbs specific for LILRB1 (HP-F1) or LILRB2 (27D6) in the absence or presence of increasing concentrations of LILRB1-Fc or LILRB2-Fc (1, 5 or 10μg) and Abs binding to monocytes was evaluated by flow cytometry analyses.
Fusion protein binding to HLA-I molecules was analyzed by FACS using 721.221 and HLA-B7 transfected (.221-B7) cells.

For acid stripping experiments .221-B7 cells were incubated for 2 min on ice with a citrate phosphate buffer (0,13 M citric acid; 0,06M disodium phosphate; 1% BSA; pH 3), followed by DMEM incubation and three washes with PBS. The influence of acid-treatment on HLA-I surface conformation was confirmed by immunostaining with mAbs recognizing the classical HLA-I heterotrimeric conformation (HP-1F7) or HLA-I free-heavy chains (HC10; kindly provided by Prof. José A. López de Castro, Center for Molecular Biology Severo Ochoa, Spain) by flow cytometry..

Flow cytometry.
Cells were incubated with 10μg/ml human-aggregated IgG to block FcγR and subsequently labelled with mAb specific for the following surface molecules: CD14-PE (BD Biosciences PharMingen, San Jose, CA, USA), CD163-PE and CD16-FITC (eBioscience, San Diego, CA, USA), CD32-

FITC and CD64-FITC (Biolegend, San Diego, CA, USA) and HLA-I-APC (ImmunoStep, Salamanca, Spain). The unconjugated HP-F1 (anti-LILRB1) and HP-1F7 (anti-HLA-I) mAbs were generated in our laboratory and have been previously characterized [83]. The 27D6 mAb (anti-LILRB2) was kindly provided by prof. M. Colonna (Washington University, USA). To measure HLA-G surface expression, the mAbs Mem-G9 (Exbio, Praha, Czech Republic; mouse IgG1) and G233 (kindly provided by Dr. Ashley Moffett, University of Cambridge, UK; mouse IgG1) were used. An anti-myc mAb (clone 9E10; mouse IgG1) was used on flow cytometry analysis as negative control. In indirect immunostaining, PE-conjugated fragmented rabbit anti-mouse IgG/M (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and Alexa488-conjugated rabbit anti-Rat IgM (Molecular Probes, Merelbeke, Belgium) were used.

To perform cell staining with the fusion proteins, cells were pre-treated with heat-inactivated rabbit serum (30uL, Invitrogen) to block FcR. Subsequently, cells were incubated with LILRB1-Fc or LILRB2-Fc (3ug) followed by PE-conjugated anti-human IgG (Jackson ImmunoResearch). Cells incubated with the PE-conjugated secondary Ab alone were used as staining negative control. Flow cytometry analyses were performed with a FACSCalibur instrument and data were processed using the FlowJo software (Tree Star, Ashland, OR, USA).

To compare LILRB1, LILRB2 and HLA-I expression and LILRB1-Fc and LILRB2-Fc binding to the different myelomonocytic cell types, we calculated the Staining Index (Specific staining Geo Mean / Negative control Geo Mean).

LILRB1 silencing by siRNA nucleofection.

M2 MΦ were transfected using the Neon transfection system (Life Technologies), following the manufacturer's data sheet. Briefly, M2 MΦ were washed with PBS, and 2,5x10^5 cells were resuspended in T buffer containing 50 nM siRNAs [Human LILRB1 ON-TARGETplus SMARTpool siRNA or Control Non-Targeting siRNA (Thermo Scientific Dharmacon, Cramlington, UK)], transferred into a gold tip and electroporated by 1 pulse at 1.850V for 30 ms, followed by 24h incubation in culture media without antibiotics in 24-well plates. The efficiency of LILRB1 silencing was evaluated by flow cytometry by measuring MΦ LILRB1 surface expression. Untreated cells and MΦ transfected with Non-Targeting siRNA were used as negative controls.

M2 MΦ functional assays.

The regulation of LILRB1 and LILRB2 on steady-state MΦ function was evaluated by incubating M2 MΦ (0,5x10^6/mL, final volume 100uL) with HP-F1 F(ab) (10µg), HP-1F7 F(ab')2 (10µg), 27D6 (concentrated SN, 100uL) and A6136 (concentrated SN, 100uL) mAbs or the fusion proteins LILRB1-Fc (purified, 10µg), LILRB2-Fc (purified, 10µg) and IREM1-Fc (purified, 10µg) for 24h at 37ºC. Subsequently, SN were harvested and IL-6, IL-10, TNF-α and IL-12p40 secretion were analysed using commercial ELISA kits (eBioscience) following the manufacturer's instructions.

M2 macrophage (0,5x10^6/mL, final volume 100uL) responses against HLA-I deficient 721.221 or HLA-I transfected B-LCL cell line were analysed in M2: B cell (1:5) co-cultures for 24h at 37ºC. Next, SN were harvested and IL-6 secretion was measured by ELISA as previously explained.

For the evaluation of MΦ function regulation by LILRB1 upon ITAM-mediated cell activation, M2 MΦ (0,5x10^6/mL, final volume 100uL) were

cultured in pre-coated plates with anti-TREM-1 (10μg, kindly provided by Dr. Marco Colonna), human IgG1 (10μg) or PBS in the absence or presence of the fusion proteins LILRB1-Fc (10μg) or IREM-1-Fc (10μg) for 24h at 37ºC. Subsequently, SN were harvested and IL-6, IL-10 and TNF-α secretion were analysed by ELISA.

HCMV stock preparation.

TB40/E HCMV and BAC-TB40/E 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 SN 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 by centrifugation for 90 min at 27.000 x g at 15ºC. Pelleted virus was resuspended in DMEM supplemented with 3% FBS 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).

MΦ **recognition of HCMV-infected autologous M2** MΦ.

M2 MΦ (0,5x10^6/mL; final volume 500μL) were treated with medium alone (mock), TB40/E (MOI 5 or 10), UV-inactivated TB40/E (MOI 5), BAC-TB40/E (MOI 30) or UV-inactivated BAC-TB40/E (MOI 30) at 37ºC in 24-well plates. 48h post infection, cells were harvested and cytospin glass slides prepared by centrifugation (7x10^4 cells/100μl) for 3 minutes at 500 rpm, using a Cytospin4 Cytocentrifuge (Thermo Scientific). 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 (Chemicon, Temecula, CA, USA) for 60 min followed by Alexa 448-conjugated F(ab')2 goat anti-mouse Ig (Invitrogen) and counterstained with DAPI. The percentage of IE1/IE2

positive cells was calculated using Leica DM6000B fluorescence microscope, and cell images were analysed with the Leica FW4000 Fluorescence Workstation software (Leica, Bensheim, Germany).

M1 MΦ (0,5x10^6/mL) were co-cultured with autologous mock-, HCMV-, UV-HCMV-, BAC-HCMV- or UV-BAC-HCMV-treated M2 MΦ (ratio 1:1; 96-well plate; final volume 200uL) at 37ºC. 24h post co-culture, SN were harvested and cytokine production measured by ELISA.

Platelet isolation and immunostaining.

Blood samples were obtained from healthy adult individuals using Vacutainer® tubes containing citrate (BD). Upon centrifugation (250 x g for 8 min at room temperature without break), plasma-reach platelets (PRP) was collected in 15mL plastic tube and washed (1000 x g for 15 min) with an acid-citrate-dextrose solution (pH 6,5) containing 5mM adenosine (Sigma-Aldrich) and 3mM theophylline (Sigma-Aldrich). Subsequently, platelet pellet was carefully and slowly resuspended (to prevent platelet activation) in washing buffer and transferred into a new 15mL tube. After 3 washes, platelets were resuspended in 1mL of HANKS buffer, counted using the automated hematology analyzer XT-2000*i* (Sysmex, Lincolnshire, IL, USA) and adjusted to a final concentration of 100x10^6/mL.

To analyze HLA-I and CD42b surface expression, 1x10^6 platelets were incubated with a FITC-conjugated anti-HLA-I (Immunotools) or PE-conjugated anti-CD42b (Immunotools) respectively. Flow cytometry analyses were performed using adapted parameters (FSC-H and SSC-H log scale) and platelets were gated based on their size and complexity.

Platelet phagocytosis by M2 MΦ.

For CFDA (Vybrant® CFDA SE Cell Tracer Kit, Invitrogen) labeling, $100x10^6$ platelets (1mL) were incubated with 0,5μM CFDA for 8 min at room temperature. The reaction was stopped by adding an equal volume of FBS, platelets were washed 3x in PBS/10% FBS and resuspended in HANKS buffer. Platelet CFDA staining was validated by FACS analyses using unlabeled platelets as negative control.

Platelet phagocytosis experiments were performed by incubating M2 MΦ ($100x10^5$ cells) with CFDA-labeled platelets for 1h at 37ºC in flat 96-well plates using two different MΦ:platelet ratios (1:10 and 1:100). After incubation, MΦ were washed with PBS/2mM EDTA to eliminate platelets attached to the MΦ surface. Next, platelet phagocytosis was determined by flow cytometry analyses. MΦ incubated with medium alone or unlabeled platelets were used as negative controls. In some experiments, CFDA-labeled platelets were pre-incubated with anti-HLA-I (HP-1F7 entire or pepsin digested) or anti-CD41 (PM6/248; GeneTex) mAb prior to the co-culture with M2 MΦ.

Western blotting.

Whole-cell lysates (WCL) were prepared by resuspending cells ($10x10^6$/mL) in lysis buffer (150 mM NaCl, 50mM Tris (pH 7,4), 1% NP40, 1% PMSF, 1mM protease inhibitor cocktail (Sigma-Aldrich; P8340) and 50 μM of iodoacetamide) for 1h on ice and subsequently centrifuged at 14.000 x g for 20 min. Lysates were resolved on non-reducing or reducing 10% SDS-PAGE and transferred onto PVDF membranes (Immobilon, Millipore) followed by overnight incubation with blocking solution (PBS containing 0.01% Tween 20 and 5% non-fat dry milk). 4H84 (BD Biosciences), HC10 and HCA2 (kindly provided by Dr. Hidde Ploegh, Harvard Medical School, MA, USA) mAbs were used for immunoblotting with a horseradish

peroxidase-conjugated goat anti-mouse IgG (Amersham Biosciences, Piscataway, NJ, USA) developed by Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Measurement of Reactive Oxygen Species.

ROS levels were measured in untreated, IFN-β1a (1000U/mL) or Diamide (1mM, Sigma-Aldrich) treated THP-1 by labeling with 10μM of 5-(and-6)-carboxy-2´,7´-difluorodihydrofluorescein (DCFH-DA, Life Technologies), incubated at 37ºC for 30 min, washed and analysed by flow cytometry. The DCFH-DA cleavage by intracellular esterases forms DCFH, a molecule that upon ROS oxidation becomes fluorescent allowing its detection by flow cytometry.

Statistical analysis.

Statistical analyses were performed by the Student *t*-test using GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA). Results were considered significant at the two-sided *P* level of 0,05.

Chapter 4

RESULTS

1 - Development of tools and strategies to approach the study of LILRB1 and LILRB2 interaction with HLA-I in *in vitro* differentiated primary human MΦ.

In order to study the role of LILRB1 and LILRB2 on the regulation of MΦ function, we used different tools to antagonise the interaction of these inhibitory receptors with HLA-I molecules. This is a conventional approach employed to study the function of NK-cell receptors specific for HLA class I molecules on which the team has an extensive experience. Nevertheless, considering the functional characteristics of MΦ, we anticipated a number of potential problems that might complicate the interpretation of results namely: signalling by non-specific binding of monoclonal antibodies (mAbs) to FcγR-I and II; MΦ activation in response to contaminating PAMPs present in the different biologicals; overlapping effects of other inhibitory receptors specific for different ligands.

1.1 - Blocking monoclonal antibodies.

Previous reports from our group [83,111] have shown that LILRB1 interaction with HLA-I in NK cells can be prevented by the HP-F1 (anti-LILRB1) and HP-1F7 (anti-HLA-I) mAbs. Regarding LILRB2, it has been shown that the 27D6 (anti-LILRB2) mAb is able to abrogate the binding of HLA-G tetramers to LILRB2$^+$ cells [132].

Human MΦ express at the cell surface several FcγR (namely CD16, CD32 and CD64) that can interact with the Fc fragment of IgG isotype Abs. Unlike the 27D6 mAb, which is a rat IgM, both HP-F1 and HP-1F7 are mouse IgG1 Abs so, in order to ensure that no direct recognition of these Abs through FcγR could occur, we digested the Abs using pepsin to generate F(ab')2 fragments Fig. 9a).

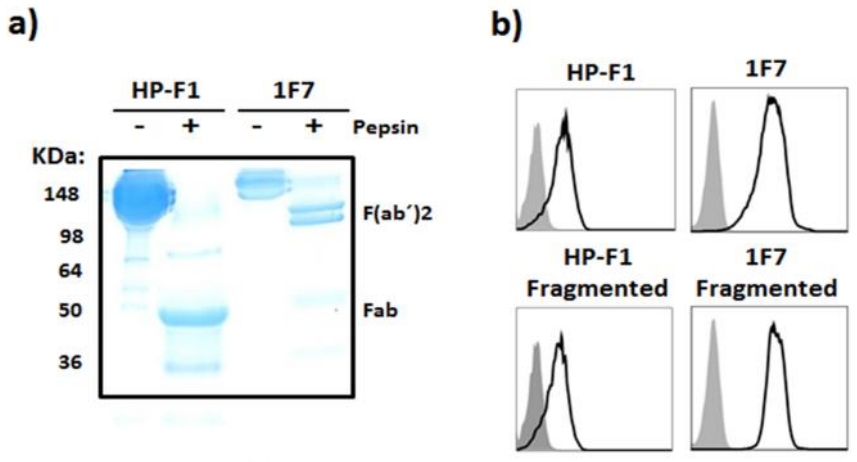

Figure 9 – Pepsin digestion of the mAbs HP-F1 and HP-1F7: (a) anti-LILRB1 (HP-F1) and anti-HLA-I (HP-1F7) Abs (entire or pepsin-digested) were resolved on a non-reducing 10% SDS-PAGE and stained with Comassie blue. (b) The antigen-binding capacity of the fragmented Abs was compared with the entire Igs by flow cytometry, using PBMC and selecting the monocyte gate (isotype controls - filled histograms; specific staining - bold lines).

In the case of HP-1F7, pepsin digestion resulted in F(ab´)2 Ab fragments (~110KDa) whereas HP-F1 digestion always resulted in Fab fragments (~50 KDa), despite that we extensively tested different antibody:pepsin concentrations and incubation times.

The Ab specificity after pepsin digestion was confirmed by immunofluorescence analysis on freshly isolated PBMC by flow cytometry (Fig. 9b). On that basis, both mAbs could be used in functional assays to assess the effect of blocking LILRB1 interaction with HLA-I in MΦ function. Considering that MΦ are able to sense a wide variety of PAMP, special care was taken during the Ab production and purification steps to avoid possible contaminations (the hybridomas were grown in sterile conditions and all the solutions were prepared using endotoxin-free clinic water).

1.2 - Fusion proteins.

Another approach implemented to antagonize the LILRB1 and LILRB2 interaction with HLA-I relied on the use of fusion proteins (LILRB1-Fc and LILRB2-Fc). Both LILRB fusion proteins are composed by the receptor extracellular region (4 Ig-like domains) fused to a human IgG1 Fc fragment mutated to decrease their interaction with FcγR [80]. The binding of these fusion proteins to HLA-I molecules mimics the interaction between the inhibitory receptors and their ligands.

Upon the production and purification of the fusion proteins as detailed in methods, their conformation was checked using a competition assay. PBMC were labelled with anti-LILRB1 (HP-F1) and anti-LILRB2 (27D6) mAbs in the absence or presence of increasing concentrations of LILRB1-Fc or LILRB2-Fc fusion proteins (Fig. 10a). Both Abs recognize epitopes located in the receptor ectodomains and should specifically recognize the fusion proteins if properly folded. Incubation of LILRB1-Fc with HP-F1 prevented mAb binding to monocytes in a dose-dependent manner (Fig. 10a). The same effect was observed upon incubating LILRB2-Fc with 27D6 mAb. As expected, the incubation of LILRB1-Fc with 27D6 and LILRB2-Fc with HP-F1 did not prevent the binding of these Abs to monocytes.

Next, we analysed the specificity of both fusion proteins by comparing their binding capacity to HLA-I deficient cells (721.221) and the derived HLA-B7 transfectants (.221-B7). As expected, both fusion proteins recognized the HLA-I transfected but not the parental cell line (Fig. 10b). Of note, we observed a more pronounced binding of the LILRB1-Fc in comparison with LILRB2-Fc despite of using saturating protein amounts for both of them.

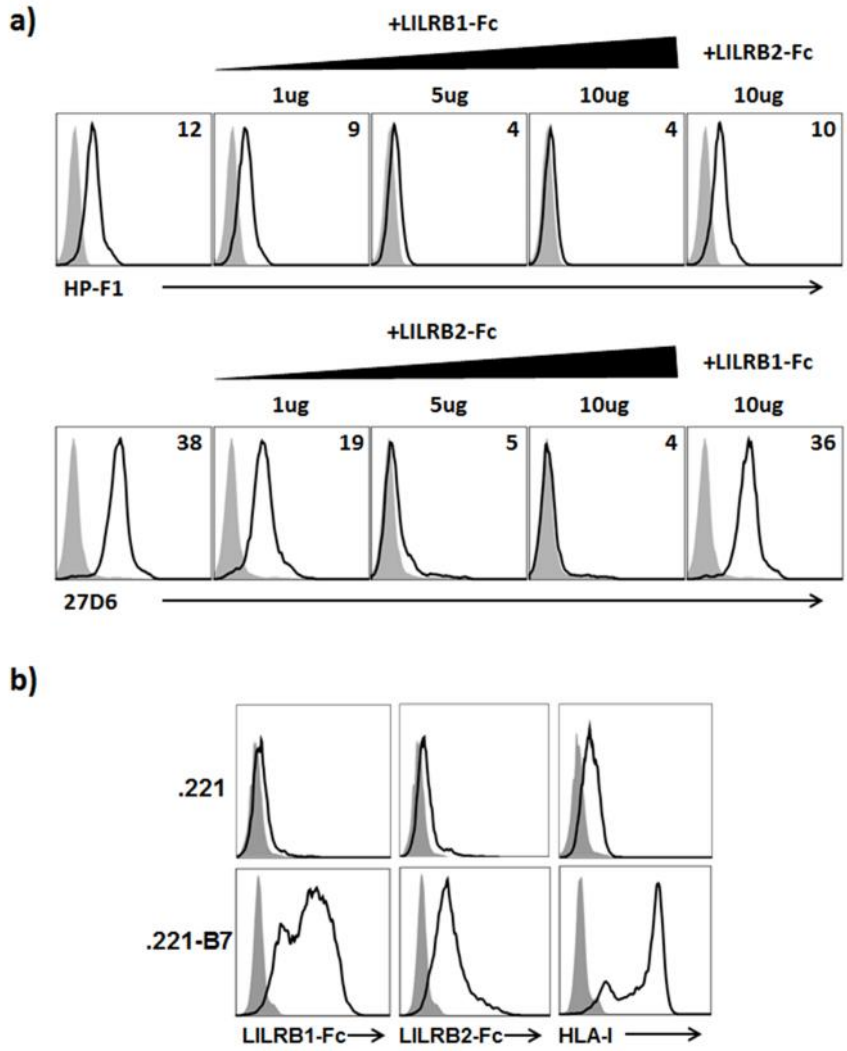

Figure 10 - Specificity of LILRB1-Fc and LILRB2-Fc fusion proteins: (a) PBMC were immunostained with mAbs specific for LILRB1 (HP-F1) or LILRB2 (27D6) in the absence or presence of increasing concentrations of LILRB1-Fc or LILRB2-Fc fusion proteins. Abs binding to monocytes was evaluated by flow cytometry analysis in the different conditions (isotype controls - filled histograms; specific staining - bold lines). Numbers depict the positive staining geo mean (b) .221 and .221-B7 cells were stained with the LILRB-Fc followed by a secondary PE-α-human IgG Ab. Negative controls (cells incubated only with the secondary Ab) are represented with filled histograms and specific staining with bold lines.

Knowing that LILRB2, but not LILRB1, is able to recognize β2m-free HLA-I heavy chains (FHC) [114], β2m was removed from 221-B7 cells by incubation in pH 3.0 citrate buffer and the binding of both fusion proteins was evaluated. The effect of the acid treatment was controlled by flow cytometry by monitoring the enhanced expression of surface FHC and the parallel decrease in HLA-I conventional conformers using the mAbs HC10 and HP-1F7 respectively (Fig. 11). In agreement with published observations, LILRB1-Fc engagement was prevented by acid treatment of .221-B7 transfectants whereas no significant alterations were detected for LILRB2-Fc binding

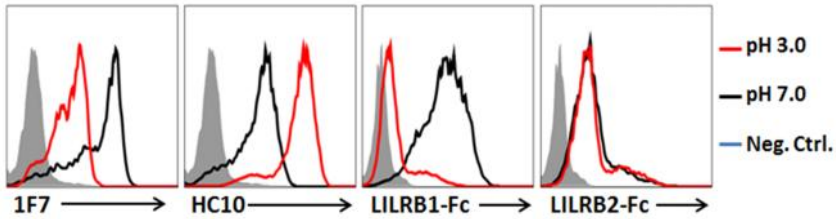

Figure 11 - Acid stripping effect on LILRB1-Fc and LILRB2-Fc binding. .221-B7 cells were either untreated (pH 7.0) or treated with citric acid (pH 3.0) for 2 min. prior to immunostaining with the HP-1F7 and HC10 mAbs and with LILRB fusion proteins. Histograms showing the staining with negative controls (filled histogram) and specific staining in untreated (black bold line) or acid treated cells (red bold lines).

Altogether these results confirmed the correct conformation and specificity of both fusion proteins.

2 - Characterization of LILRB1/2 and their ligands expression on different monocyte-derived MΦ subtypes.

We used *in vitro* differentiated monocyte-derived MΦ to characterize the impact of their functional polarization towards pro-inflammatory (M1) and anti-inflammatory (M2) profile in the expression of LILRB1/2 receptors and their ligands.

2.1 – Characterization of *in vitro* differentiated M1 and M2 MΦ phenotype and cytokine secretion profiles.

Monocytes were isolated from PBMC by negative selection and subsequently differentiated towards M1 and M2 MΦ by supplementing the culture medium with rhGM-CSF and rhM-CSF respectively. After 7 days, MΦ differentiation was evaluated by flow cytometry analyses of CD14 and CD163 surface expression (Fig. 12).

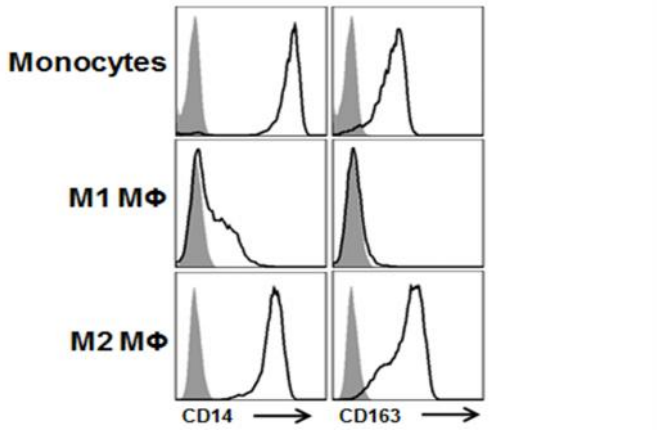

Figure 12 - CD14 and CD163 surface expression in monocytes, M1 and M2 MΦ: MΦ surface expression of CD14 and CD163 was evaluated by flow cytometry (isotype controls - filled histograms; specific staining - bold lines).

As previously reported [133], differentiation towards M1 MΦ was encompassed by the down-modulation of surface CD14 and CD163 expression in comparison to the levels detected in the precursor monocytes and M2 MΦ.

To confirm the functional polarization of the obtained MΦ subtypes, we analysed the cytokine secretion profile upon LPS stimulation (Fig. 13).

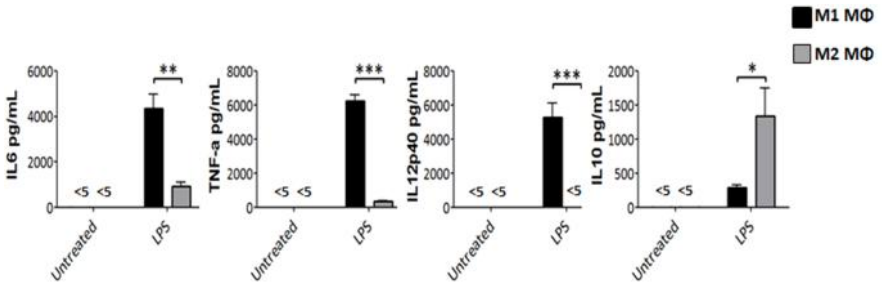

Figure 13 - M1 and M2 MΦ cytokine secretion upon LPS stimulation. The MΦ subtypes were either cultured with medium alone (untreated) or stimulated with 1ug/mL of LPS for 24h. Bar graphs (M1 MΦ –black bars; M2 MΦ – grey bars) displaying the secretion of IL-6, TNF-α, IL12p40 and IL-10 in cell-free supernatants. Data represent mean ± SEM of the combined results of three different donors. Significance was calculated using a student *t* test (* $p<0,05$; ** $p<0,01$ and *** $p<0,001$).

After LPS stimulation, M1 MΦ secreted higher levels of the pro-inflammatory cytokines IL-6, TNF-α and IL12p40 while M2 MΦ mainly secreted the anti-inflammatory cytokine IL-10, in agreement with the consensus definition of functionally polarized M1 and M2 MΦ [11,12].

Both MΦ subtypes expressed the FcγR I (CD64) and FcγR II (CD32) (Fig. 14) thus confirming the importance of using fragmented antibodies in the functional assays, to avoid direct stimulation through FcγR.

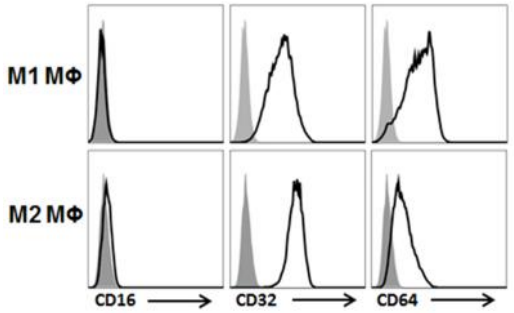

Figure 14 - M1 and M2 MΦ surface expression of FcγR. MΦ were immunostained with FITC-conjugated anti-CD16, anti-CD32 and anti-CD64 mAbs. The FcγR expression was evaluated by FACS analyses (isotype controls - filled histograms; specific staining - bold lines). Data correspond to results from a representative donor out of three tested.

2.2 – M1 and M2 MΦ surface expression of LILRB1 and LILRB2 receptors.

Surface expression of LILRB1 and LILRB2 in MΦ was respectively evaluated by immunostaining with HP-F1 and 27D6 mAbs. We compared the expression of both inhibitory receptors on MΦ and monocytes (Fig. 15). Freshly isolated peripheral blood monocytes presented a homogeneous surface expression of LILRB1 and LILRB2. *In vitro* differentiation to M2 MΦ did not modify the expression of these two receptors whereas differentiation towards M1 MΦ resulted in a lower surface expression of both LILRB1 and LILRB2.

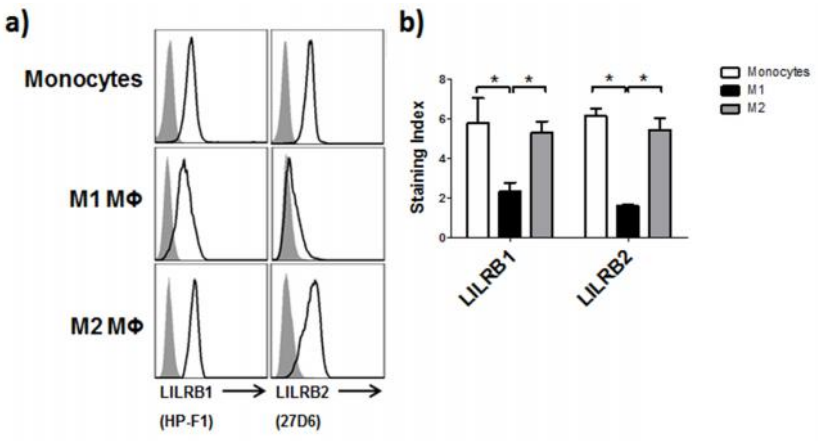

Figure 15 - LILRB1 and LILRB2 surface expression in monocytes, M1 and M2 MΦ. LILRB1 (HP-F1) and LILRB2 (27D6) expression were determined by indirect immunofluorescence staining analysed by flow cytometry. (a) Results from a representative donor (isotype controls - filled histograms; specific staining - bold lines). (b) Bar graphs displaying the average LILRB1 and LILRB2 Staining Index in four different donors. Significance was calculated using a student *t* test (* $p<0,05$).

2.3 – Evaluation of LILRB1 and LILRB2 ligands in M1 and M2 MΦ.

LILRB1 and LILRB2 original data on HLA-I recognition was mainly based on results obtained with HLA-I transfectants and soluble molecules assayed in surface plasmon resonance methodology. Remarkably, little is known regarding the capacity of these receptors to recognize HLA-I molecules on human primary cells.

Thus, we used LILRB1-Fc and LILRB2-Fc fusion proteins to analyse their binding to primary monocytes, M1 and M2 MΦ by flow cytometry. Albeit all cell types were derived from the same donor and expressed similar levels of surface HLA-I, LILRB1-Fc preferentially bound to M2 MΦ (Fig. 16). Moreover, we could not detect any binding of LILRB2-Fc to monocytes or MΦ.

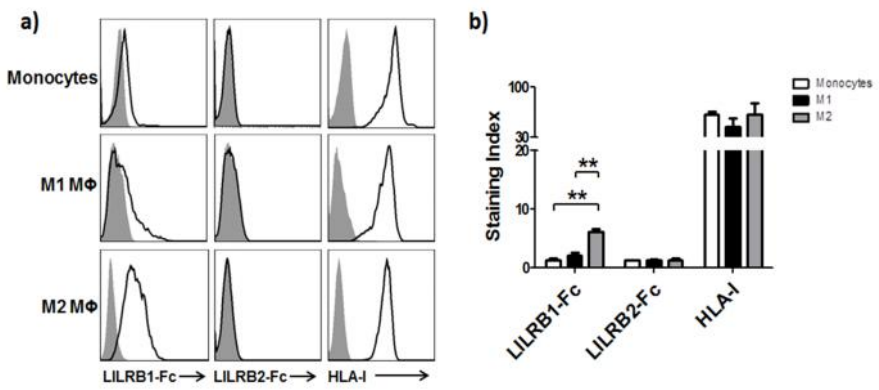

Figure 16 – HLA-I surface expression and LILRB1-Fc and LILRB2-Fc binding to monocytes, M1 and M2 MΦ. LILRB1-Fc and LILRB2-Fc engagement and HLA-I (HP-1F7) expression were determined by indirect immunofluorescence staining analysed by flow cytometry. (a) Data correspond to results from a representative donor (negative controls - filled histograms; specific staining - bold lines). (b) Bar graphs displaying the average Staining Index of LILRB1-Fc, LILRB2-Fc and anti-HLA-I of four different donors. Significance was calculated using a student *t* test (** $p<0,01$).

These results indicated that LILRB1-Fc binding did not exclusively depend on total levels of surface HLA-I expression or on the expression of distinct HLA-I alleles, suggesting that upon *in vitro* differentiation M2 MΦ display a high affinity ligand for LILRB1. Experiments addressing the molecular basis for these observations were undertaken and will be described in "Section 4".

3 - Role for LILRB1 and LILRB2 interaction with HLA-I on the regulation of MΦ function.

Once the proper tools and differentiation conditions were defined, we addressed the role of LILRB1 and LILRB2 on the regulation of MΦ function in different settings. Taking into account the LILRB1-Fc binding profile and the surface expression of both LILRB1 and LILRB2 receptors, functional assays to evaluate the role of these inhibitory receptors were performed using M2 MΦ on monocyte-derived MΦ.

3.1 - Role for LILRB1 and LILRB2 interaction with HLA-I on the control of MΦ activation upon self-interaction.

The expression of HLA-I specific inhibitory receptors suggests a mechanism of MΦ function regulation similar to NK cell recognition of missing self. In order to study if LILRB1 and LILRB2 receptors regulate the MΦ activation threshold, we used different strategies to interfere with LILRB1-2/HLA-I interactions.

3.1.1 MΦ activation upon HLA-I blockade.

First, we evaluated M2 MΦ activation upon HLA-I blockade using either anti-HLA-I specific mAbs or the LILRB1-Fc and LILRB2-Fc fusion proteins. The capacity of two different anti-HLA-I Abs (HP-1F7 and A6136) to block LILRB1 and LILRB2 interaction with HLA-I molecules was measured by flow cytometry using the .221 cell line transfected with HLA-G (.221-G1m).

Although both mAbs recognized HLA-G molecules (Fig. 17a), only a pre-incubation with the HP-1F7 Ab was able to prevent LILRB1-Fc engagement to this cell line (Fig. 17b). The binding of LILRB2-Fc fusion protein to

HLA-I molecules was barely detected and not influenced by the incubation with HLA-I specific mAbs.

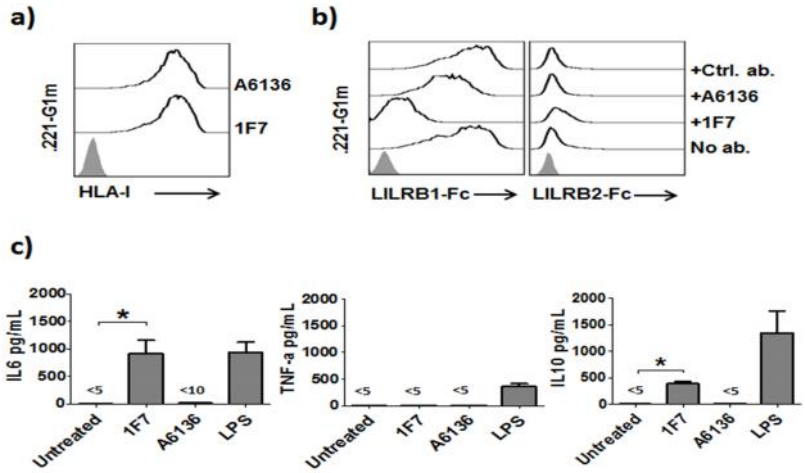

Figure 17 - Effect of HLA-I blockade on M2 MΦ function, using anti-HLA-I Abs: (a) Histograms displaying .221-G1m cells immunostained with the anti-HLA-I Abs HP-1F7 and A6136 (b) Immunofluorescence analysis of LILRB1-Fc and LILRB2-Fc binding to untreated (No ab) or.221-G1m cells pre-incubated with HP-1F7, A6136 or anti-Myc Ab. (c) Bar graphs displaying IL-6, TNF-α and IL-10 secretion upon M2 MΦ incubation with anti-HLA-I Abs: HP-1F7 F(ab´)2 and A6136 at 37ºC for 24h. Untreated and LPS-treated (1ug/mL) MΦ were used as negative and positive controls, respectively. Data correspond to mean ± SEM of three different donors, significance was calculated using a student *t* test (* p<0,05).

We next analysed the effect of disrupting the interaction between LILRB1 and HLA-I on M2 MΦ cell function at basal state (Fig. 17c). Upon incubation with fragmented HP-1F7 mAb, M2 MΦ secreted IL-6 and IL-10 while the incubation with A6136 did not induce cytokine secretion.

To confirm and validate the effect of HLA-I blockade on MΦ activation, we used LILRB1-Fc and LILRB2-Fc to interfere with HLA-I and LILRB interaction. LILRB1-Fc induced the secretion of both IL-6 and IL-10 (Fig. 18). However, the levels of cytokine secretion were slightly lower compared with HP-1F7 F(ab´)2 incubation. Neither incubation with LILRB2-Fc nor

IREM-1-Fc (control fusion protein with a mutated human IgG1 Fc domain to decrease FcR binding) induced MΦ cytokine secretion.

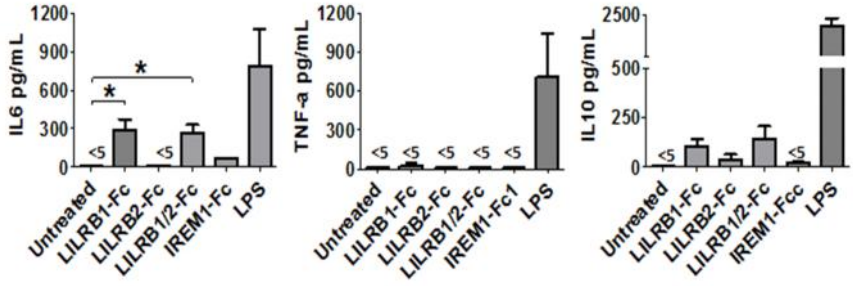

Figure 18 - Effect of HLA-I blockade on M2 MΦ function, using LILRB fusion proteins: Bar graphs displaying IL-6, TNF-α and IL-10 secretion upon M2 MΦ incubation with LILRB1-Fc, LILRB2-Fc, LILRB1-Fc + LIRB2-Fc and the control fusion protein IREM1-Fc at 37ºC for 24h. Untreated and LPS-treated (1ug/mL) MΦ were used as negative and positive controls, respectively. Data correspond to mean ± SEM of three different donors, significance was calculated using a student *t* test (* $p<0,05$).

Thus, both HLA-I blocking experiments supported that LILRB1 may regulate MΦ function in steady state conditions. MΦ express several surface activating receptors that recognize endogenous molecules and upon ligand binding recruit the ITAM-bearing adaptors DAP12 or FcR gamma chain. Therefore, the lack of LILRB1 signalling might allow MΦ activation through constitutive activating signals.

3.1.2 MΦ activation upon LILRB1 and LILRB2 blockade.

To address the role of each specific LILRB inhibitory receptor on the regulation of MΦ function, M2 MΦ were incubated with LILRB1 and LILRB2 antagonistic Abs HP-F1 and 27D6 for 24h. Contrary to what we were expecting, incubation with single or the combination of both Abs of did not resulted in cytokine secretion (Fig. 19).

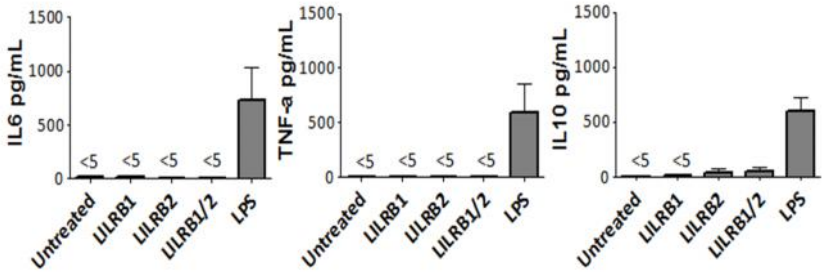

Figure 19 - Effect of LILRB1 and LILRB2 blockade on M2 MΦ function, using antagonistic Abs: Bar graphs displaying IL-6, TNF-α and IL-10 secretion upon M2 MΦ incubation with anti-LILRB1 (HP-F1 F(ab)), anti-LILRB2 (27D6) as single agents or in combination at 37ºC for 24h. Untreated and LPS-treated (1ug/mL) MΦ were used as negative and positive controls respectively. Data correspond to mean ± SEM of three different donors.

The inability of anti-LILRB2 IgM to promote cytokine secretion from steady state MΦ could reflect the absence of LILRB2 ligand as indicated by the undetectable binding of LILRB2-Fc to M2 MΦ. On the other hand, the lack of MΦ activation after LILRB1 blockade could result from the reduced capacity of monovalent HP-F1 F(ab') to block LILRB1/HLA-I interaction.

Taking into account the experimental limitations explained before, we decided to use a different strategy to evaluate the participation of LILRB1 on MΦ homeostasis. To this end, we used small interfering RNA (siRNA) to specifically down-modulate LILRB1 expression in M2 MΦ. After optimizing the electroporation conditions (voltage, pulse width and pulse number) to properly transfect M2 MΦ, the cells were treated with LILRB1-specific siRNA for 48, 72 or 96h before the analysis of surface LILRB1 expression by immunofluorescence (Fig. 20).

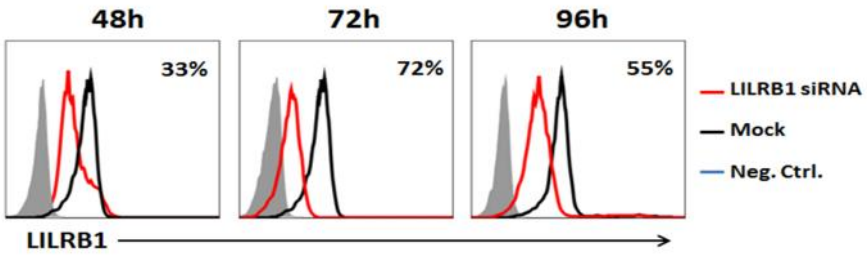

Figure 20 - Kinetics of LILRB1 surface expression after siRNA transfection. M2 MΦ were electroporated in the absence (mock) or presence of 100nM of LILRB1 siRNA and surface LILRB1 was monitored by immunostaining with HP-F1 mAb at 48, 72 or 96h post transfection. Histograms representing the staining with isotype controls (filled histogram) and specific staining in mock-treated cells (black bold line) and LILRB1 siRNA treated cells (red bold line). Numbers indicate the LILRB1 surface expression reduction for each time point.

The maximum reduction of LILRB1 expression (72%) was observed at 72h after MΦ transfection. Even though we did not reach a complete LILRB1 down-regulation from the MΦ surface, we decided to address whether LILRB1 down-modulation would promote MΦ activation. We transfected M2 MΦ with either LILRB1 or non-sense (NS) siRNA for 72h and monitored IL-6 secretion for the last 24h. As expected, LILRB1 but not NS siRNA transfection induced the specific down-modulation of LILRB1 receptor expression. No significant alteration in surface HLA-I was observed upon MΦ transfection (Fig. 21a).

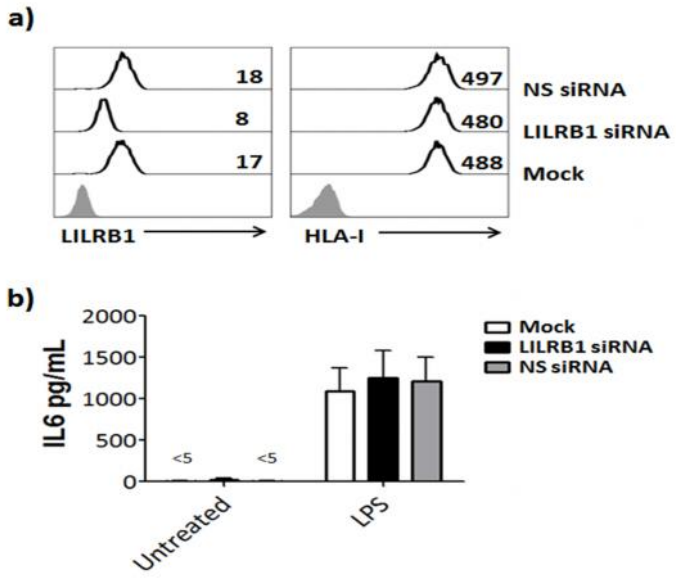

Figure 21 - Effect of LILRB1 down-modulation by siRNA on MΦ activation. M2 MΦ were transfected in the absence (mock) or presence of 100nM of siRNA (LILRB1 or NS) for 72h. (a) Flow cytometry analysis of LILRB1 and HLA-I surface expression. Data correspond to results from a representative donor (isotype controls - filled histograms; specific staining - bold lines). Insets indicate the fluorescence geo mean. (b) IL-6 secretion of mock- (white bars), LILRB1 siRNA- (black bars) or NS siRNA- (grey bars) transfected M2 MΦ, cultured with medium alone (untreated) or supplemented with 1ug/mL of LPS for 24h. Data correspond to mean ± SEM of three different donors.

Despite the considerable surface LILRB1 down-modulation no cytokine secretion was observed (Fig. 21b). It is conceivable that the remaining surface LILRB1 molecules could still repress MΦ activation or, alternatively, that another unidentified inhibitory receptor specific for HLA-I could be involved. LPS stimuli induced measurable secretion of IL-6 confirming the capacity of siRNA treated M2 MΦ to produce cytokines in response to activating stimuli.

Combining all the results related with MΦ tolerance to normal self, we could demonstrate that HLA-I blockade in steady state M2 MΦ induced cytokine secretion (IL-6 and IL-10). This effect was reproduced upon MΦ incubation with the LILRB1-Fc but not LILRB2-Fc fusion protein, supporting the involvement of LILRB1 in the regulation of basal MΦ activation threshold through the interaction with HLA-I molecules. Yet, experiments directly addressing LILRB1 involvement in such phenomenon were unsuccessful.

3.2 - Role for LILRB1 interaction with HLA-I on MΦ response to transformed cells.

The loss of HLA-I surface expression by tumor cells constitutes an escape mechanism of T-cell mediated immune surveillance [134]. These alterations may favour not only the NK cell response but also the interaction of MΦ with tumor cells.

3.2.1 - HLA-I expression by tumor cells regulates MΦ activation.

To assess the role of HLA-I recognition on the regulation of MΦ activation in response to tumor cells, we incubated M2 MΦ with the HLA-I deficient B-LCL (721.221) or a transfectant expressing HLA-G (.221-G1m), which constitutes an optimal LILRB1 ligand. Both HLA-I surface expression and LILRB1-Fc binding to .221-G1m cells were confirmed by flow cytometry (Fig. 22a).

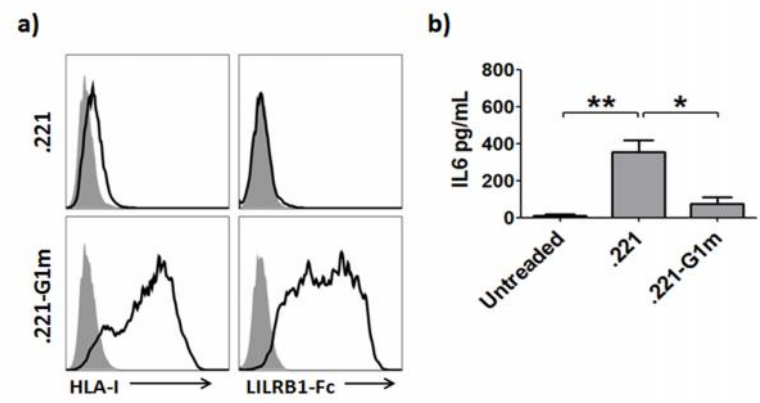

Figure 22 - Effect of HLA-I surface expression on B-LCL in MΦ activation. (a) Flow cytometric analysis of .221 and .221-G1m cells immunostained with an anti-HLA-I Ab (HP-1F7) or the LILRB1-Fc fusion protein. Histograms representing the staining with negative controls (filled histogram) and specific staining (bold line) are shown. (b) Bar graphs displaying IL-6 secretion upon M2 MΦ incubation with medium alone (untreated) or co-cultured with .221 or .221-G1m cells for 24h at 37º C. Data correspond to mean ± SEM of three different donors, significance was calculated using the student *t* test (* $p<0,05$ and ** $p<0,01$).

Interaction with the HLA-I-deficient cells triggered M2 MΦ IL-6 secretion. The response was inhibited upon co-culture with target cells transfected with HLA-G, indicating that HLA-I molecules repressed MΦ activation (Fig. 22b). M2 MΦ did not secrete TNF-α nor IL-10 in response to the tumor cells (data not shown).

Pre-incubation of .221-G1m cells with HP-1F7 F(ab´)2 mAb did not revert MΦ inhibition (data not shown). The lack of MΦ response upon target cell HLA-I blockade could be related to the rapid turnover of surface HLA-I on target cells, facilitating the interaction of newly synthesized, non-blocked molecules with MΦ LILRB1.

To further evaluate the involvement of LILRB1/HLA-I interaction on the effect observed, we performed co-culture experiments using 721.221 cells transfected with different HLA-I alleles. We selected two HLA-B transfected cell lines: .221-B7 and .221-B35 displaying similar surface HLA-I expression

and different LILRB1-Fc binding (Fig. 23a). MΦ IL-6 secretion in response to .221 cells was repressed by HLA-B7 but not by HLA-B35 transfectants, correlating with their LILRB1-Fc binding capacity (Fig. 23b).

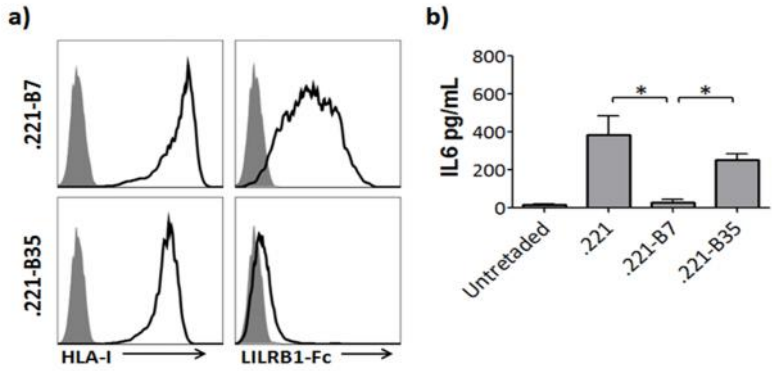

Figure 23 - Influence of HLA-I alleles on B-LCL recognition by MΦ. (a) Flow cytometric analysis of .221-B7 and .221-B35 cells immunostained with an anti-HLA-I Ab (HP-1F7) or the LILRB1-Fc fusion protein. Histograms representing the staining with negative controls (filled histogram) and specific staining (bold line) are shown. (b) Bar graphs displaying IL-6 secretion upon M2 MΦ incubation with medium alone (untreated) or co-cultured with .221, .221-B7 or .221-B35 cells for 24h at 37º C. Data correspond to mean ± SEM of three different donors, significance was calculated using the student *t* test (* $p<0,05$).

Thus, these results suggest that only HLA-I alleles recognized by LILRB1 modulate the MΦ response towards transformed cells, regardless of total HLA-I surface expression levels.

3.2.2 - Impact of *cis* and *trans* interaction on LILRB1/HLA-I regulation of MΦ function.

After showing that tumor HLA-I expression may regulate MΦ activation through a *trans* interaction with LILRB1, we decided to address whether MΦ function could also be regulated by a LILRB1/HLA-I *cis* interaction. To discriminate between LILRB1/HLA-I *trans* and *cis* interaction we interfered with cell adhesion events by using an antagonistic Ab specific for

lymphocyte function-associated antigen 1 (LFA-1). By culturing .221 cells in the presence of this mAb, the cell clustering that characterizes the growth of this cell line was inhibited indicating that this mAb was able to interfere with cell-cell interactions (Fig. 24a).

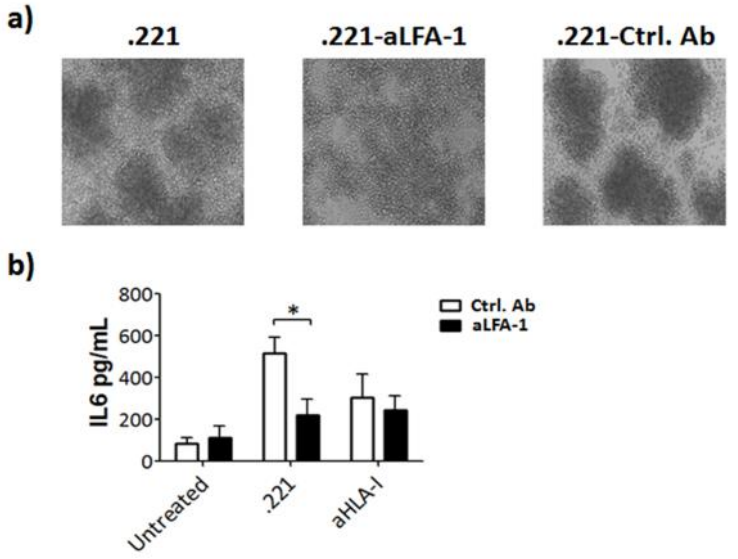

Figure 24 - Effect of cell interaction blockade on MΦ activation. a) Optical microscopy analysis of .221 cells cultured with medium alone or incubated with the anti-LFA-1 (TS1/18) or control (aMyc) mAbs for 24h. (b) Bar graphs displaying IL-6 secretion upon M2 MΦ (white bars – MΦ treated with the control Ab; black bars – MΦ treated with anti-LFA-1) incubation with medium alone (untreated), co-cultured with .221 cells or incubated with a fragmented anti-HLA-I Ab (HP-1F7 F(ab´)2) for 24h at 37º C. Data correspond to mean ± SEM of three different donors, significance was calculated using a *t* student test (* $p<0,05$).

Pre-incubation with anti-LFA-1 mAb significantly reduced MΦ IL-6 secretion in response to .221 cells, indicating its capacity to interfere with *trans* cell-cell interactions (Fig. 24b). On the other hand, no major alterations were observed on MΦ activation upon HLA-I blockade in MΦ pre-incubated with the anti-LFA-1 or a control Ab. These results suggest that MΦ function may be regulated by a LILRB1 *cis* interaction with HLA-I molecules.

3.3 - Role for LILRB1/HLA-I interaction on the MΦ response to HCMV-infected cells.

Human Cytomegalovirus (HCMV) encodes for several proteins US2, US3, US6, US10 and US11) capable to interfere with HLA-I surface expression [93,94]. The infection of M2 MΦ by HCMV induces a down-modulation of surface HLA-I expression [133]. On the other hand, HCMV encodes for UL18, an HLA-I-like glycoprotein which constitutes a high-affinity ligand for LILRB1. The aim of this set of experiments was to determine whether HLA-I loss could have an impact on MΦ recognition of HCMV-infected cells.

3.3.1 - Correlation between HCMV infection and LILRB1 binding.

The effect of HCMV infection on HLA-I surface expression and LILRB1-Fc binding was assessed by flow cytometry after 48h of M2 MΦ treatment with decreasing HCMV MOI (Fig. 25a). 90%, 57% and 18% of MΦ down-regulated HLA-I at MOI 10, 5 and 1, respectively, indirectly reflecting their infection rate as confirmed by IE1/IE2 immunofluorescence analysis (data not shown). Next, we incubated M2 MΦ with medium (mock), TB40/E (MOI 5) or UV-inactivated TB40/E (UV-HCMV) for 48h prior to the immunostaining analysis, in order to check whether LILRB1 binding to M2 MΦ could be affected by HCMV infection (Fig. 25b). Infected and non-infected cells were gated based on their HLA-I surface expression. Correlating with HLA-I expression, LILRB1-Fc poorly bound to HCMV-infected cells while the binding of the fusion protein to non-infected cells was increased compared to mock-treated cells (Fig. 25c).

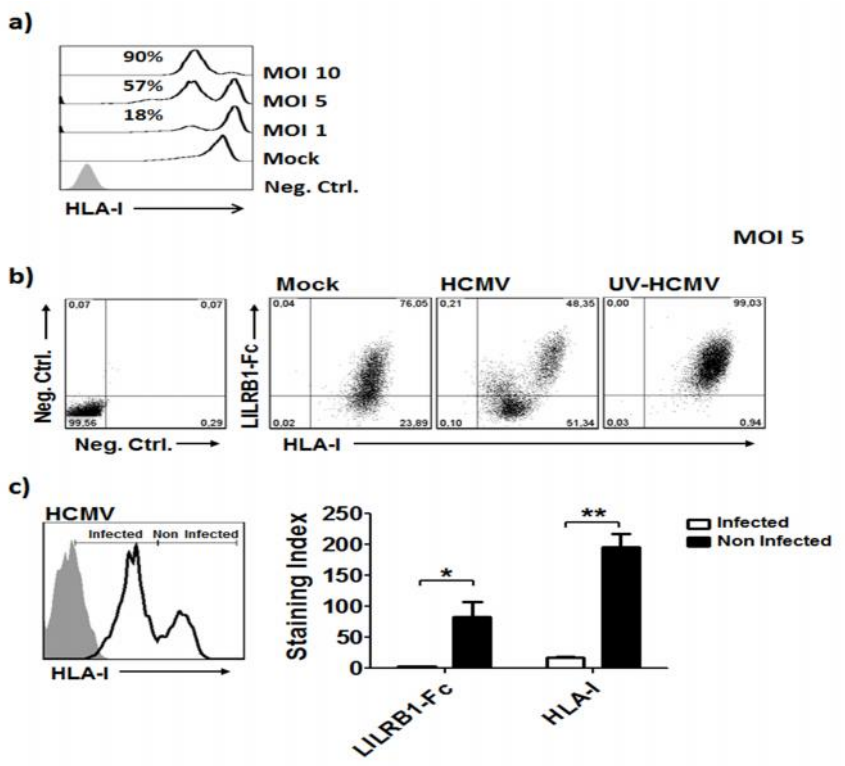

Figure 25 - HCMV infection induces changes on surface HLA-I and LILRB1-Fc binding to M2 MΦ: Immunofluorescence analysis of M2 MΦ treated with HCMV. (a) M2 MΦ were mock-treated or incubated with increasing MOI (1, 5 and 10) of the HCMV strain TB40/E. 48h post-treatment, HLA-I surface expression was analysed by FACS. Isotype control (filled histogram) and specific staining (bold line) are shown. (b) HLA-I expression and LILRB1 fusion protein engagement in M2 MΦ untreated (mock), incubated with TB40/E (HCMV) or UV-inactivated TB40/E (UV-HCMV) for 48h as determined by flow cytometry Results from a representative donor. (c) Based on HLA-I surface expression level (left histogram), M2 MΦ treated with HCMV were classified as "Infected" (low HLA-I expression) or "Non-Infected" (high HLA-I expression). Mean Staining Index ± SEM for LILRB1 binding and surface HLA-I from data in four different donors, significance was calculated using a student *t* test (* $p<0,05$ and ** $p<0,01$).

Remarkably, the limited interaction of LILRB1-fc with infected MΦ questions that UL18 may be significantly expressed at the cell surface, in line with previous studies on the NK-cell response to HCMV-infected fibroblasts [135].

3.3.2 - M1 MΦ response against autologous HCMV-infected M2 MΦ.

To study the MΦ recognition of HCMV-infected cells, we set up an experimental system co-culturing mock-treated or TB40/E-treated M2 MΦ as target cells of autologous M1 MΦ. M2 MΦ infection was monitored by anti-IE1/IE2 intracellular staining, ranging from 90% to 97% in all experiments (Fig. 26a).

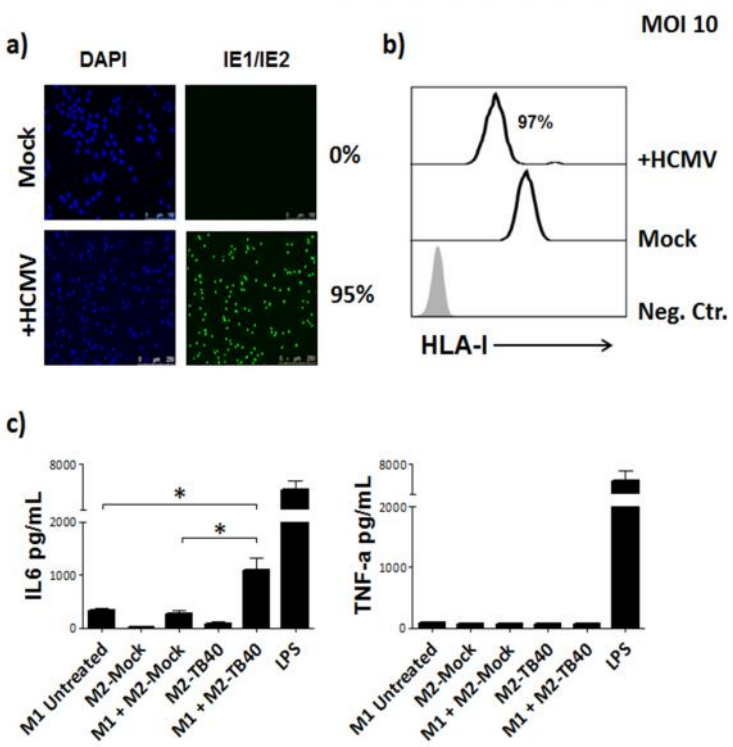

Figure 26 - Co-culture of M1 MΦ with autologous HCMV-infected M2 MΦ: M2 MΦ were either mock-treated or infected with TB40/E for 48h. (a) Immunofluorescence microscopy analysis of cells stained with an anti-IE1/IE2 mAb (green) and counterstained with DAPI (blue). (b) FACS analysis of cells immunostained with an anti-HLA-I mAb. Data correspond to results from a representative donor (isotype controls - filled histograms; specific staining - bold lines). (c) IL-6 and TNF-α secretion upon M1 MΦ co-culture with autologous mock-treated or HCMV-infected M2 MΦ at 37ºC for 24h. Untreated and LPS-treated M1 MΦ were used as negative and positive controls respectively. Data represents mean ± SEM of three different donors, significance was calculated using a student *t* test (* $p<0,05$).

As expected, the percentage of IE1/IE2$^+$ cells correlated with the percentage of cells that down-modulated surface HLA-I expression (Fig. 26b).

After incubation of M1 MΦ with autologous mock-treated or HCMV-infected M2 MΦ, SN were collected and cytokine secretion analyzed by ELISA. M1 MΦ secreted IL-6, but not TNF-α, in response to infected-cells while no cytokine secretion was observed in response to the mock-treated cells (Fig. 26c). Neither IL-6 nor TNF-α were detected in either mock-treated or HCMV-infected M2 MΦ. These results suggest that M1 MΦ recognized autologous M2 MΦ cells that down-modulated HLA-I surface expression upon HCMV infection.

3.3.3 - Addressing the role of surface HLA-I down-modulation on MΦ response against HCMV-infected cells.

To facilitate HCMV mutagenesis, the genomes of AD169 and TB40/E strains were cloned into a bacterial artificial chromosome (BAC) vector [136,137]. The BAC vector was integrated into the viral genome between the US1 and US7 genes, thus the resulting HCMV construct lacks the US2-US6 viral genes. Since in the HCMV BAC constructs some of the genes located in the deleted region interfere with HLA-I expression, we decided to compare the M1 MΦ response against TB40/E- and BAC-TB40/E- infected M2 MΦ in order to study a possible role of surface HLA-I expression on this response.

To obtain similar rates of MΦ infection different amounts of wt HCMV (MOI 5) and BAC-HCMV (MOI 30) were used. Treatment with BAC-HCMV induced morphological changes when compared with the HCMV-treated cells. Infection rates between both virus strains were comparable as indicated by IE1/IE2 staining (Fig. 27a). Unlike wt HCMV, the infection

with BAC-HCMV did not induce any significant change on surface HLA-I expression in M2 MΦ (Fig. 27b).

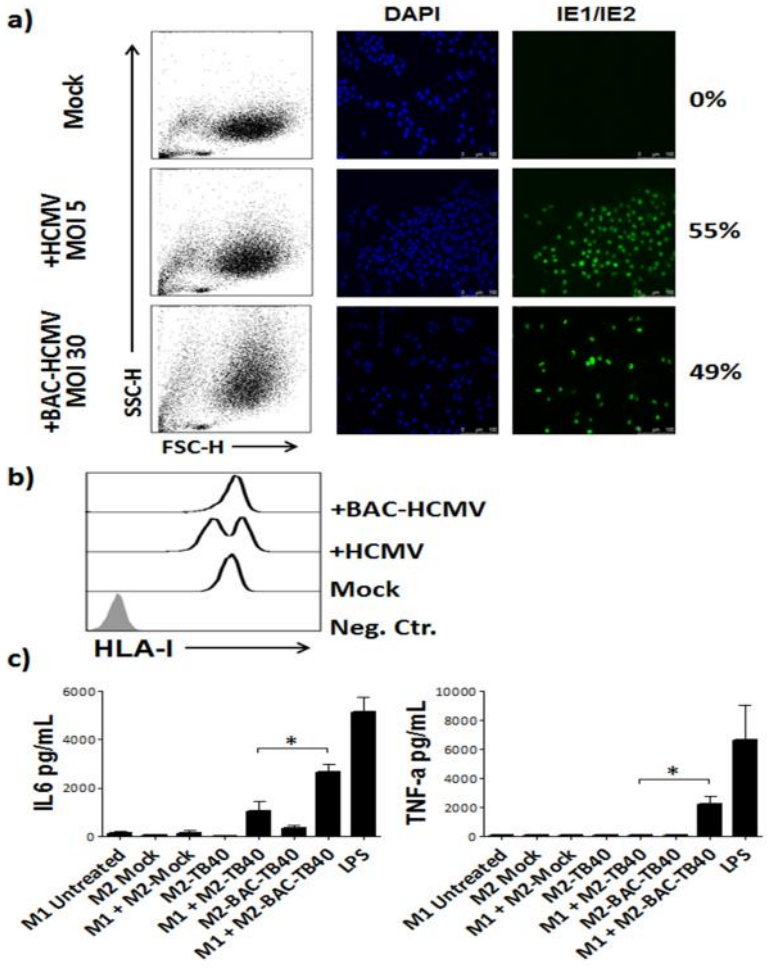

Figure 27 - Co-culture of M1 MΦ with autologous HCMV- and BAC-HCMV-infected M2 MΦ: M2 MΦ were either mock-treated, incubated with TB40/E or BAC-TB40/E for 48h. (a) Cell size and complexity were analysed by flow cytometry. HCMV infection was validated by immunofluorescence microscopy analysis (IE1/IE2 staining and DAPI counterstaining) (b) HLA-I surface expression monitored by FACS. Data correspond to results from a representative donor (isotype controls - filled histograms; specific staining - bold lines). (c) Bar graphs displaying IL-6 and TNF-α secretion upon M1 MΦ co-culture with autologous mock-treated, HCMV-treated or BAC-HCMV-treated M2 MΦ at 37ºC for 24h. Untreated and LPS-treated M1 MΦ were used as negative and positive controls respectively. Data represents mean ± SEM of three different donors, significance was calculated using a student *t* test (* $p<0,05$).

Contrary to what we expected, M1 MΦ secreted high levels of IL-6 and TNF-α in response to the BAC-HCMV (Fig. 27c). The high MOI used to infect M2 MΦ with BAC-HCMV may be the explanation for the induction of TNF-α secretion by M1 MΦ. Alternatively, the possibility that an activating receptor might recognize HLA-I modified by the viral infection was also considered.

The incubation with a high amount of viral stock may lead to the binding of viral particles to the M2 MΦ cell membrane. These virions presented in the M2 MΦ surface could be directly recognized by M1 MΦ through PRR. To explore this possibility we evaluated the M1 MΦ response against UV-inactivated HCMV and BAC-HCMV-treated autologous cells (Fig. 28).

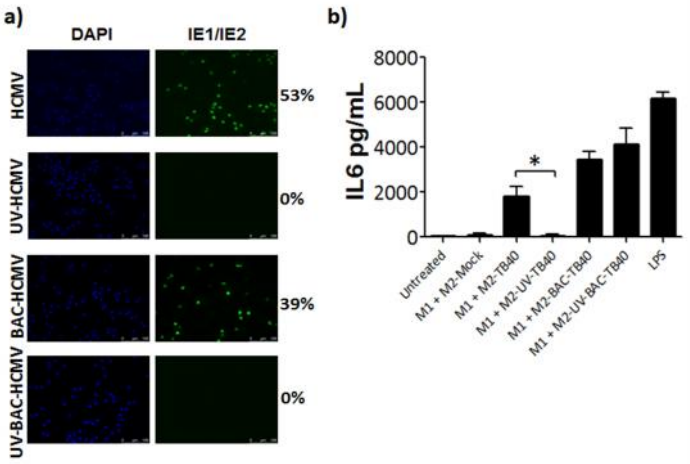

Figure 28 - Co-culture of M1 MΦ with autologous UV-inactivated HCMV- and BAC-HCMV-treated M2 MΦ: M2 MΦ were mock-treated or incubated with both active and UV-inactivated forms of TB40/E (MOI 5) or BAC-TB40/E (MOI 30) for 48h. (a) Immunofluorescence microscopy analysis (IE1/IE2 staining and DAPI counterstaining) (b) Bar graphs displaying IL-6 secretion upon M1 MΦ co-culture with autologous mock-treated, HCMV-treated, UV-HCMV-treated, BAC-HCMV-treated or UV-BAC-HCMV-treated M2 MΦ at 37ºC for 24h. Untreated and LPS-treated M1 MΦ were used as negative and positive controls respectively. Data represents mean ± SEM of experiment triplicate, significance was calculated using a student *t* test (* $p<0,05$).

Unlike HCMV-infected cells, M2 MΦ treated with either infective or uv-inactivated BAC-HCMV induced comparable IL-6 secretion from M1 MΦ, thus supporting that this response might be mediated by a direct recognition of the viral particle.

To avoid the contamination by HCMV virions resulting from the requirement of using high MOI, we tested the capacity of M1 MΦ to respond against HCMV-infected fibroblast cell line MRC-5. These cells were selected due to their high permissiveness for HCMV infection, thus high infection rates with at low MOI. Unfortunately, we did not detect M1 MΦ cytokine secretion in response to HCMV-infected MRC-5 cells even though we achieved infection rates ranging from 95% to 99% using MOI 1, (data not shown).

Thus, although MΦ can recognize HCMV-infected autologous cells which down-regulated surface HLA-I, the specific contribution of LILRB1/HLA-I recognition to the MΦ response against HCMV-infected cells remains unanswered.

3.4 - Role for LILRB-1/HLA-I interaction on the regulation of MΦ activation through ITAM-bearing receptors.

Ligand engagement of LILRB1 results in the phosphorylation of the tyrosine contained within ITIM motifs, promoting the recruitment of SHP-1 phosphatases that interfere with the early signaling events of ITAM-bearing receptors. We analyzed whether the blockade of LILRB1 interaction with HLA-I could modulate the MΦ response to activation triggered by ITAM-bearing receptors.

3.4.1 - Suppression of LILRB-1/HLA-I interaction enhances ITAM-dependent MΦ activation.

To evaluate the impact of LILRB1 signaling during cell activation, we stimulated MΦ through different ITAM-bearing receptors: TREM-1 and FcγR in the absence or presence of the LILRB1-Fc fusion protein (Fig. 29a). Culturing M2 MΦ in anti-TREM-1 or hIgG coated plates promoted IL-6 and IL-10 secretion which was clearly enhanced by the addition of LILRB1-Fc in the co-culture. TNF-α secretion was induced by FcγR stimulation and increased by the incubation with LILRB1 fusion protein.

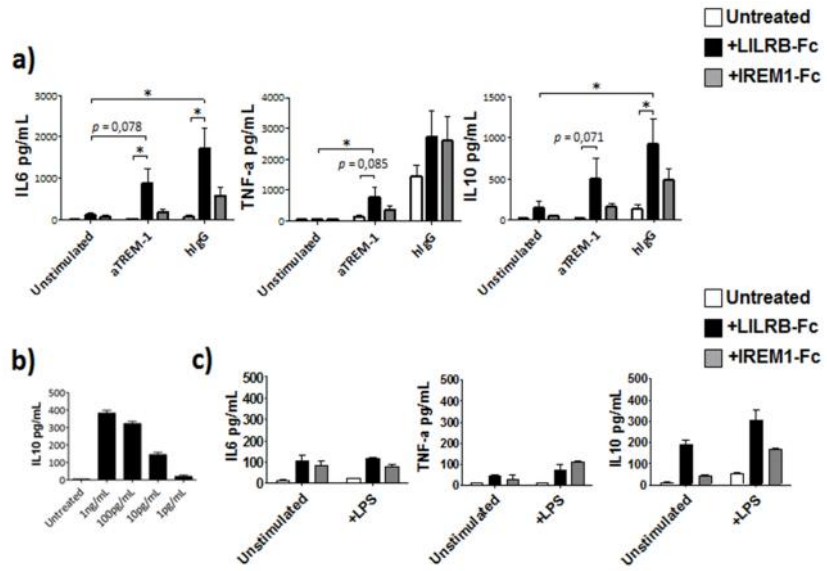

Figure 29 - Effect of the combination between ITAM-bearing receptors stimulation and LILRB1-Fc on M2 MΦ function: (a) IL-6, TNF-α and IL-10 M2 MΦ secretion upon incubation with medium alone (unstimulated) or cultured in anti-TREM-1- or hIgG-coated plates in the absence (Non-treated) or presence of LILRB1-Fc or IREM1-Fc for 24h at 37ºC. Data represents mean ± SEM of four different donors, significance was calculated using a student *t* test (* $p<0,05$). (b) Bar graphs displaying M2 MΦ IL-10 secretion upon 24h stimulation with decreasing doses of LPS. (c) IL-6, TNF-α and IL-10 M2 MΦ secretion upon incubation with medium alone (unstimulated) or LPS-stimulated (5pg/mL) in the absence (Non-treated) or presence of the LILRB1-Fc or IREM1-Fc (control fusion protein) for 24h at 37ºC.

Of note, incubation with the IREM1 fusion protein had a similar outcome. The ligand for the inhibitory receptor IREM1 has not been identified, nevertheless it is believed to be ubiquitously expressed (unpublished results). Possibly, IREM1 signaling interferes with FcγR-mediated MΦ activation and therefore the blockade of IREM1 interaction with its ligand, using the IREM1-Fc, induces an increase of MΦ cytokine secretion upon FcγR stimulation.

M2 MΦ were also stimulated trough an activating receptor independent of ITAM-mediated signaling. MΦ were treated with a previous defined sub-optimal dose of LPS (Fig. 29b) and incubated with LILRB1 fusion protein. No significant changes were observed regarding cytokine production (Fig. 29c).

The previous results show a synergism between LILRB1 blockade and ITAM-bearing receptor stimulation regarding M2 MΦ cytokine secretion. Thus, during an immune response that involves ITAM-mediated signalling, the LILRB1 interaction with HLA-I may influence the MΦ activation threshold.

3.4.2 - Role for LILRB-1/HLA-I interaction in the regulation of platelet phagocytosis by MΦ.

Platelets, or thrombocytes, are derived by budding off from megakaryocytes. This process gives origin to small anucleated cell fragments with a lifespan ranging from 5 to 9 days. It is estimated that each day around $1x10^{11}$ platelets are formed [138]. The total number of platelets in circulation must be tightly controlled, and high amounts of circulating platelets are cleared-up daily by spleen and liver MΦ phagocytosis [139]. In this section, we addressed whether platelet HLA-I expression could influence platelet uptake.

3.4.2.1 - Platelet HLA-I expression.

Knowing that platelets lack the capacity of *de novo* protein synthesis, we analyzed HLA-I surface expression during platelet aging. Platelet-rich plasma (PRP) was isolated upon whole blood collection and centrifugation. Subsequently, HLA-I and CD42b expression on platelets derived from whole blood or PRP were analyzed by flow cytometry using adapted parameters (FSC-H and SSC-H log scale) (Fig. 30a).

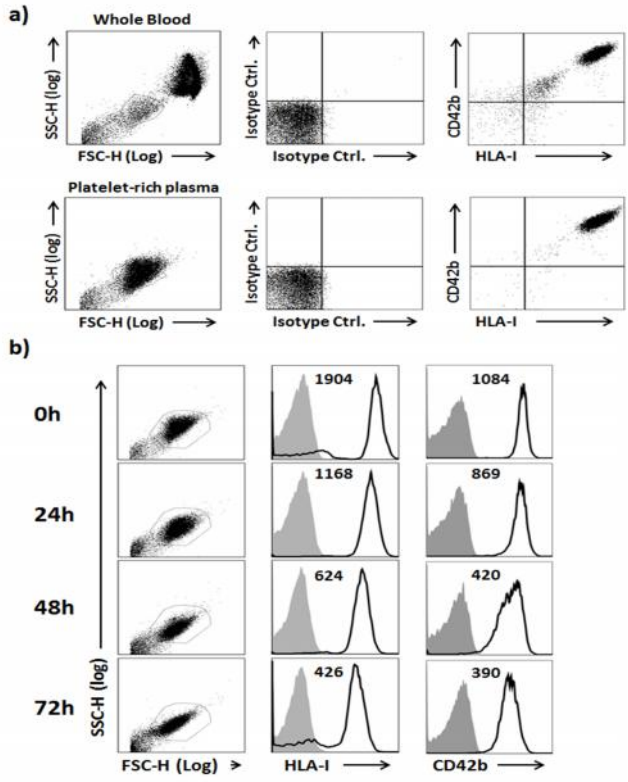

Figure 30 - Platelet HLA-I expression along storage: (a) HLA-I and CD42b expression of whole blood platelets and platelets in platelet-rich plasma (PRP) determined by immunofluorescence analysis. Data from a representative donor are shown (n=3). (b) PRP were stored for 72h in a citrate buffer pH 6,5 and both HLA-I and CD42b surface expression were measured every 24h by FACS (isotype controls - filled histograms; specific staining - bold lines. Numbers depict the positive staining geo mean).

No significant differences were detected comparing the HLA-I and CD42b expression from both whole blood and PRP derived platelets. Therefore, all the following experiments were performed using platelets from PRP.

To analyze whether platelet aging would have an influence on surface HLA-I, we stored the PRP for three days in a citrate solution with pH 6,5 and checked the HLA-I surface expression every 24h (Fig. 30b). HLA-I expression homogenously diminished during platelet aging. Nevertheless, by measuring CD42b surface expression, we observed that this protein expression was also affected along storage. It is likely that during aging several surface proteins tended to decrease from the platelet surface and therefore its interaction with phagocytes may be modified.

3.4.2.2 - Effect of HLA-I blockade on platelet phagocytosis.

In order to study the importance of HLA-I recognition on the modulation of platelet uptake we set up a method to evaluate platelet phagocytosis by M2 MΦ based on a flow cytometry analysis. To this end, platelets were stained with CFDA for 1h at 37ºC, prior incubation with MΦ followed by flow cytometry analysis (Fig. 31a).

To optimize the experimental conditions, different ratios of MΦ:platelet were compared (Fig. 31b). At 1:100 half of the MΦ incorporated platelets, whereas at 1:10 a low level of platelet-phagocytosis was detected.

Next, we used an anti-HLA-I antibody (HP-1F7) to block the interaction of platelet HLA-I with MΦ LILRB1. CFDA-labeled platelets were pre-incubated with HP-1F7 Ab prior to the co-culture with MΦ at 1:10 ratio. Comparing with the isotype control, incubation with HP-1F7 clearly enhanced platelet uptake by MΦ (Fig. 31c-d).

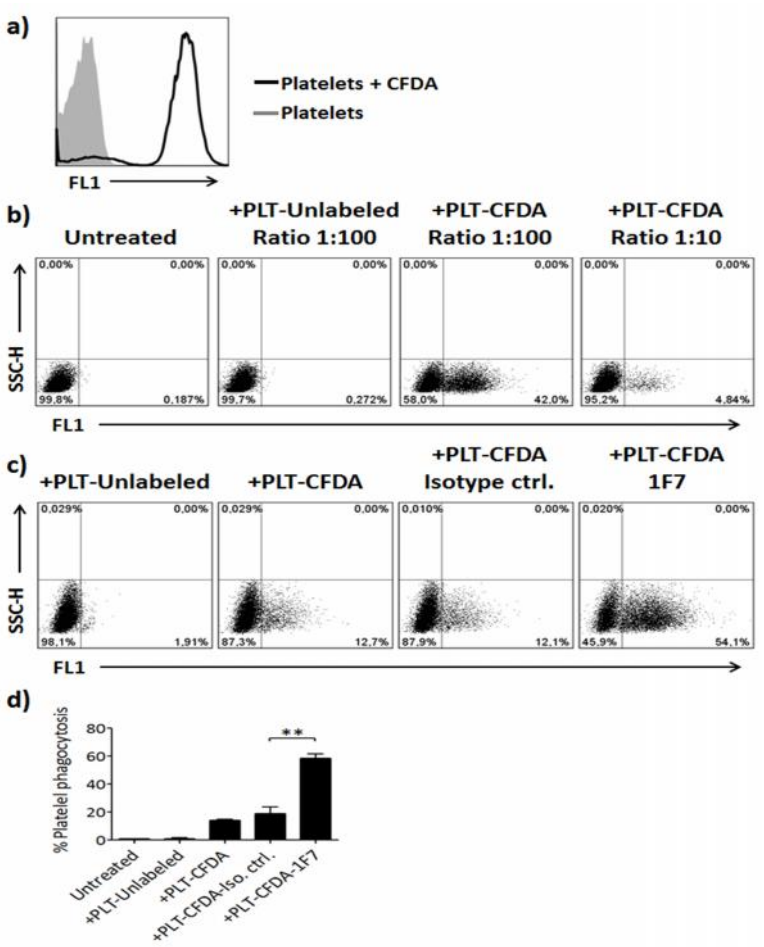

Figure 31 – PLatelet phagocytosis by M2 MΦ: (a) Fresh CFDA-labeled platelets were analysed by FACS (unlabeled platelets - filled histograms; CFDA-labeled platelets - bold lines). (b) M2 MΦ were incubated with CFDA-labeled platelets (1:100 and 1:10 ratios) at 37ºC for 1h. Platelet phagocytosis was evaluated by FACS. As negative controls we used M2 MΦ incubated with medium alone (Untreated) or unlabeled platelets. Data correspond to results from a representative donor. (c) M2 MΦ were co-cultured with CFDA-labeled platelets pre-incubated with an isotype control Ab or an anti-HLA-I mAb (HP-1F7) at 37ºC for 1h. Platelet phagocytosis was evaluated by FACS. Unlabeled platelets were used as negative control. Data correspond to results from a representative donor. (d) Bar graphs displaying % of M2 MΦ that phagocytised CFDA-labeled platelets pre-incubated with an isotype control Ab or an anti-HLA-I mAb (HP-1F7) at 37ºC for 1h. Data represents mean ± SEM of three experiments, significance was calculated using a student *t* test (** $p<0,01$).

We next evaluated whether HP-1F7 F(ab´)2 was also able to increase platelet phagocytosis. In contrast to the enhancement observed upon HLA-I blockade with the whole Ab, the addition of the HP-1F7 F(ab´)2 fragments did not promote platelet phagocytosis by M2 MΦ (Fig. 32).

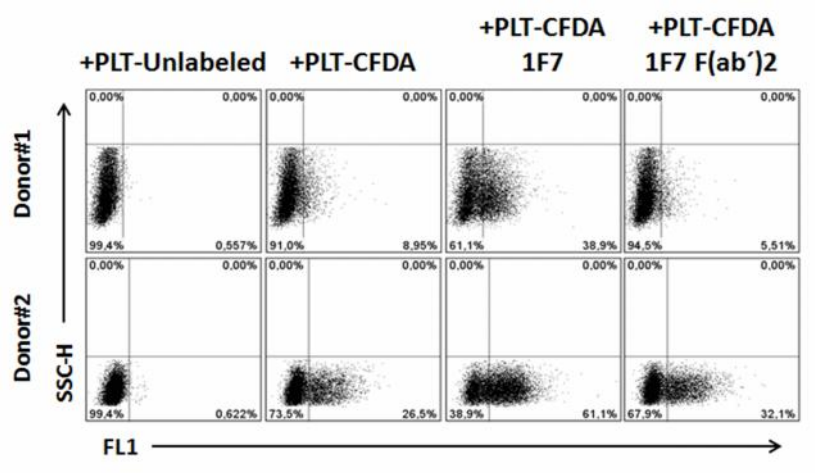

Figure 32 - Comparison between the whole and fragmented anti-HLA-I mAb capacity to induce platelet phagocytosis: M2 MΦ were co-cultured with CFDA-labeled platelets pre-incubated with an entire anti-HLA-I mAb (HP-1F7) or a fragmented anti-HLA-I mAb (HP-1F7 F(ab´)2) at 37ºC for 1h. Platelet phagocytosis was evaluated by FACS. Unlabeled platelets were used as negative control. Data from two donors are shown.

Thus, the enhanced platelet uptake induced by HP-1F7 whole antibody appeared related with the Fc capacity of promoting phagocytosis by binding to FcγR on MΦ.

3.4.2.3 - Effect of the combination between FcγR stimulation and HLA-I blockade on platelet phagocytosis.

One of the mechanisms described for aberrant platelet clearance, and associated with autoimmune disorders such as immune thrombocytopenic purpura (ITP), is mediated by antibodies [140]. Circulating platelets

sensitized by autoantibodies are recognized by spleen resident MΦ trough FcγR, inducing their phagocytosis.

Since our previous results showed the capacity of HLA-I blockade to synergize with FcγR stimulation promoting cytokine production, (Figure 29a) we analyzed whether the combination of FcγR stimulation and HLA-I blockade could modulate platelet phagocytosis by MΦ. Platelets coated with a specific Ab against the integrin alpha chain 2b (anti-CD41) were simultaneously treated with HP-1F7 F(ab´)2 before exposure to MΦ. Three anti-CD41 concentrations (1ug, 0,1ug or 0,01ug per million of platelets) were tested, in three independent experiments (Fig. 33).

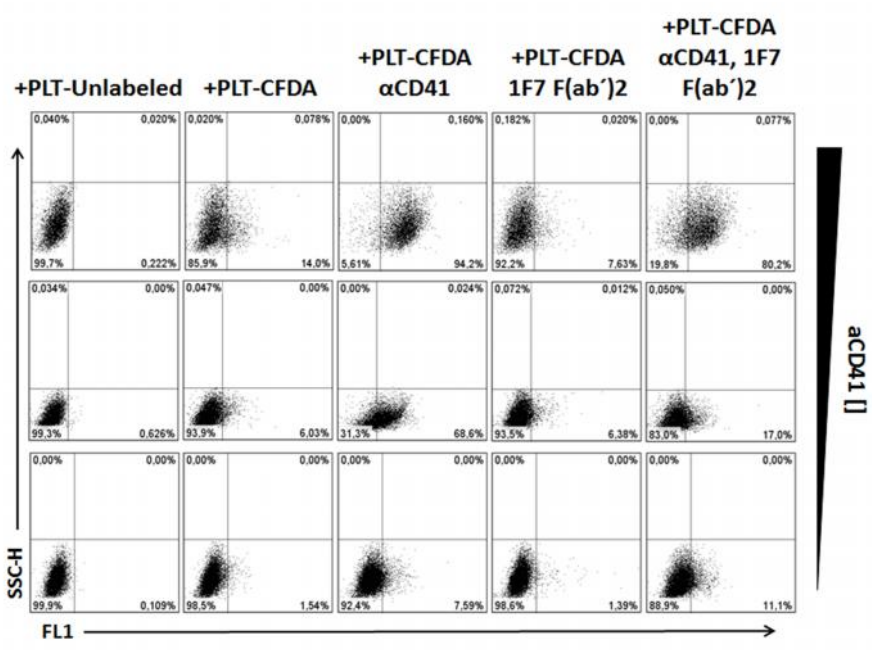

Figure 33 - M2 MΦ co-culture with platelets pre-incubated with anti-CD41 and anti-HLA-I: M2 MΦ were co-cultured with CFDA-platelets platelets pre-incubated with an anti-CD41 mAb in the absence or presence of anti-HLA-I mAb (HP-1F7 F(ab´)2) for 1h at 37ºC. Three different amounts of anti-CD41 were used (1µg; 0,1µg or 0,01µg per million of platelets). Platelet phagocytosis was evaluated by flow cytometry. Unlabeled platelets were used as negative control. Data correspond to results from a representative donor for each tested anti-CD41 concentration.

At 1ug of anti-CD41, HLA-I blockade had no effect on platelet phagocytosis. By decreasing the anti-CD41 concentration, there were lower phagocytosis rates. Yet, the combination of anti-CD41 and HP-1F7 F(ab´)2 did not result in any increase on platelet uptake by MΦ.

Thus, despite HLA-I surface expression decreased along platelet aging, we could not detect any influence of HLA-platelet levels on their recognition and phagocytosis by M2 MΦ.

4 - Characterization of the nature of LILRB1-ligands in human MΦ.

We previously observed that LILRB1-Fc bound preferentially to M2 MΦ as compared with monocytes and M1 MΦ (Fig. 16). These results kept our attention because the different cell types were derived from the same donors (no allelic variation) and expressed similar surface levels of HLA-I as detected by flow cytometry. Thus, the molecular basis for the selective interaction of LILRB1-Fc with MΦ was addressed.

4.1 – Influence of HLA-G expression on the LILRB1 interaction with MΦ.

Previous reports proposed that myelomonocytic cells, such as MΦ and dendritic cells, can express surface HLA-G [121,141]. However, this issue remains controversial mainly due to uncertainties on the specificty of the available anti HLA-G mAbs.

Knowing that HLA-G displays the highest affinity for LILRB1, together with the existence of numerous HLA-G isoforms and the plasticity and microenvironment-dependence of MΦ differentiation programs, we decided to study the expression of this non-classical HLA-I molecule on M1 and M2 MΦ to evaluate a possible relation between HLA-G expression and LILRB1 binding.

To address this issue we used the anti-HLA-G mAbs Mem-G9, G233 and 4H84. Some characteristics of each mAb are summarized (Table 3).

Antibody	HLA-G conformation	Recognized isoforms	Refs.
Mem-G9	Native	HLA-G1/5	[142,143]
G233	Native	HLA-G1/5	[144]
4H84	Native and Unfolded	All isoforms	[145,146]

Table 3 - HLA-G conformation and isoforms recognized by different anti-HLA-G mAbs.

First we performed flow cytometry analysis to confirm the capacity of the anti-HLA-G mAbs Mem-G9 and G233 to recognize surface HLA-G molecules by using the transfectant cell line .221-G1m, expressing the HLA-G1 isoform without stabilizing HLA-E, and the parental cell line .221 (Fig. 34). The ability of both Abs to recognize the HLA-G tranfectant cell line (.221-G1m) but not the HLA-I deficient one (.221) was confirmed.

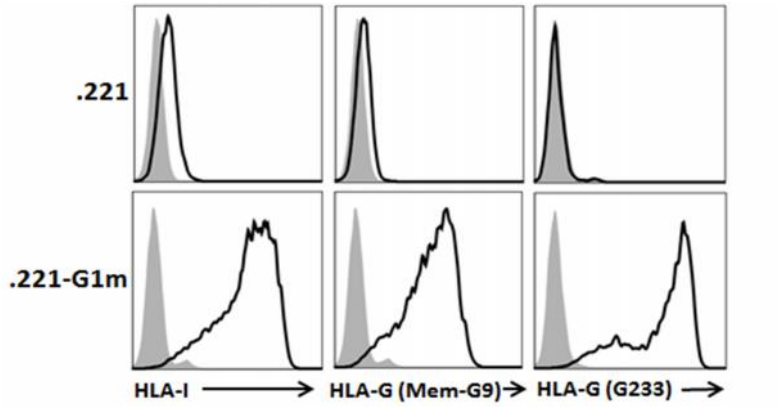

Figure 34 – Analysis of HLA-G expression by the anti-HLA-G Abs Mem-G9 and G233: .221 and .221-G1m cells were stained with the anti-HLA-G Abs Mem-G9 or G233. The anti-HLA-I Ab (HP-1F7) was used as a positive control of HLA-G recognition. The binding capacity of these Abs was confirmed by FACS analysis (isotype controls - filled histograms; specific staining - bold lines).

We next analysed the HLA-G surface expression and the association with LILRB1-Fc binding on M1 and M2 MΦ by comparing the staining with the two Abs recognizing native HLA-G1/5 isoforms (Mem-G9 and G233). According to Mem-G9 staining donors were divided in two groups: i) donors with detectable Mem-G9 staining (n=4) in M2 MΦ, and ii) donors with negative Mem-G9 binding (n=5) (Fig. 35a).

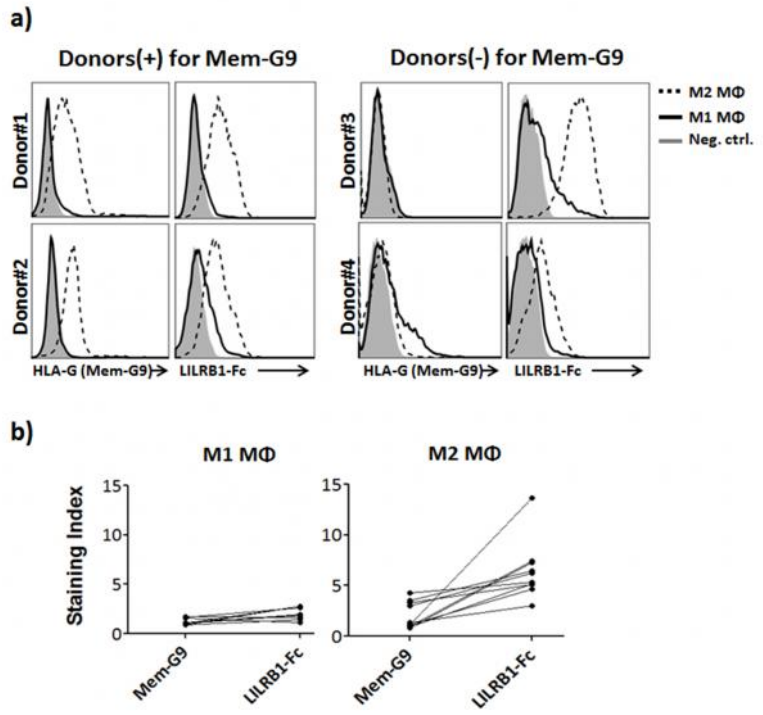

Figure 35 - LILRB1-Fc recognition and surface HLA-G expression measured by the Mem-G9 Ab: M1 and M2 MΦ surface HLA-G (Mem-G9) expression and LILRB-Fc engagement were measured by flow cytometric analysis. (a) Data correspond to results from two representative donors of each Mem-G9 (+) and (-) staining group (isotype controls - filled histograms; M1 MΦ specific staining - black bold line and M2 MΦ specific staining - black dotted line). (b) Graph displaying correlation between M1 (left) and M2 (right) MΦ LILRB1-Fc binding and Mem-G9 Staining Index. Data correspond to results from nine donors.

Taking into account the overall Mem-G9 staining, no association between Mem-G9 binding and LILRB1-Fc engagement was observed (Fig. 35b).

When G233 mAb was used to monitor HLA-G expression, we detected a modest and comparable binding of the Ab to both M1 and M2 MΦ (Fig. 36a). Moreover, like for Mem-G9 staining, no correlation with LILRB1-Fc recognition was observed (Fig. 36b).

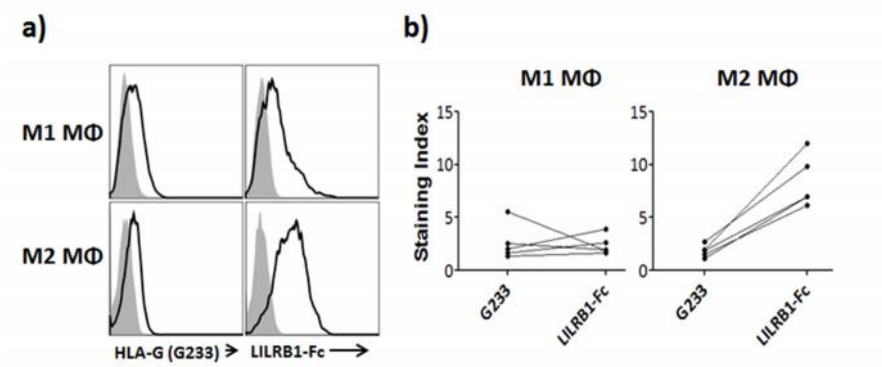

Figure 36 - LILRB1-Fc recognition and surface HLA-G expression measured by the G233 Ab: M1 and M2 MΦ surface HLA-G (G233) expression and LILRB-Fc engagement were measured by flow cytometry analysis. (a) Data correspond to results from a representative donor (isotype controls - filled histograms and specific staining - black bold line). (b) Graph displaying correlation between M1 (left) and M2 (right) MΦLILRB1-Fc binding and G233 Staining Index. Data correspond to results from five donors.

We next evaluated HLA-G expression on MΦ by western blot analysis using the 4H84 mAb. As positive and negative controls we used the .221-G1m and .221-B7 cell lines respectively, as well as the human monocytic leukaemia cells THP-1, which displayed a moderate G233 staining similar to primary MΦ(Fig. 37a). As previously reported, in the .221-G1m lane, we detected two band of Mr ~39KDa and ~80KDa , corresponding respectively to HLA-G monomers and dimers) [147]. The 4H84 antibody did not detect any protein neither in the .221-B7 lysate nor in the parental cell line .221 (Fig. 37b). No bands corresponding to known HLA-G isoforms were detected in THP-1, M1 nor M2 MΦ cell lysates with 4H84, although a ~18KDa band was displayed in the M2 MΦ lysates (Fig. 37b). Considering the characteristics of all HLA-G isoforms, only HLA-G3 might display a

molecular weight similar to the detected protein (22KDa). Yet, it is unlikely that LILRB1-Fc could recognize this molecule because HLA-G3 (truncated isoform containing the HLA-G á1 domain) lacks the LILRB1 binding site.

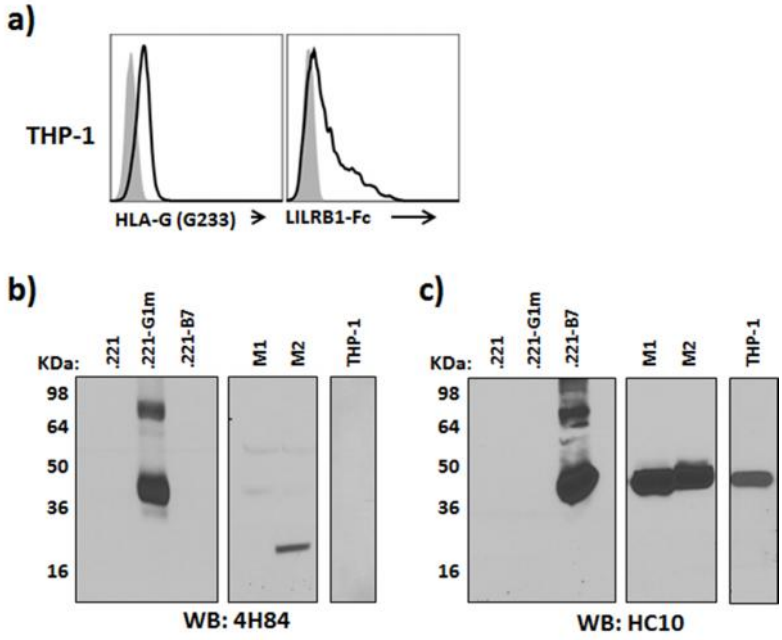

Figure 37 - Biochemical analysis of HLA-G expression: (a) Flow cytometry analysis of THP-1 cells immunostained with the G233 mAb and LILRB1-Fc (isotype controls - filled histograms and specific staining - black bold line). (b) .221, .221-G1m, -221-B7, M1 MΦ, M2 MΦ and THP-1 whole-cell lysates were resolved in a non-reducing 10% SDS-PAGE and immunoblotted with the the anti-HLA-G Ab 4H84. Molecular weight (kDa) was determined by the migration of a protein standard. (c) .221, .221-G1m, -221-B7, M1 MΦ, M2 MΦ and THP-1 whole-cell lysates were resolved in a non-reducing 10% SDS-PAGE and immunoblotted with the anti-HLA-I Ab HC10.

All cell lysates were run in parallel and blotted with the anti-HLA-I antibody HC10. This Ab does not recognize HLA-G heavy chain and thus, as expected, no band was detected on .221-G1m lane. A band corresponding to ~45KDa (classical HLA-I monomer) was observed in the .221-B7, M1 MΦ, M2 MΦ and THP-1 lanes (Fig. 37c).

Taking into account these results, the hypothesis that the differential LILRB1-Fc interaction with M1 and M2 MΦ could be related with the expression of HLA-G was ruled out.

4.2 - Influence of HLA-I conformation on the recognition of MΦ by LILRB1-Fc: a role for HLA-I dimers.

Previous reports have been shown that LILRB1 displays higher affinity for HLA-I dimers in comparison with monomers [88,89]. Among HLA-I molecules, it is known that HLA-G and HLA-B27 exhibit a greater capacity to form dimers due to unique extracellular cysteine residues, namely C42 and C67 respectively [87,148]. Nevertheless, it has been recently shown that other HLA-I alleles can also dimerize depending on their intracellular cysteine content [149].

To study the possible relation between HLA-I dimer expression and LILRB1 engagement beyond HLA-G and HLA-B27 alleles, we evaluated the LILRB1-Fc binding to different HLA-I alleles transfected in .221 cells, selected according to the presence or absence of Cys residues in their cytoplasmic domain sequence (Fig. 38a). Biochemical analysis demonstrated that the HLA-I dimerization capacity was associated with the presence of C325 or C339 in HLA-B7, -B27, A-2 and A-3 alleles; in fact, bands corresponding to HLA-I dimers were not detected in -B35 and -B51 alleles lacking these intracellular cysteines, (Fig. 38b). HLA-I dimerization was validated by the disappearance of the high molecular weight band (~90KDa) upon analyzing HLA-B7 cell lysates in SDS-PAGE under reducing conditions (Fig. 38c).

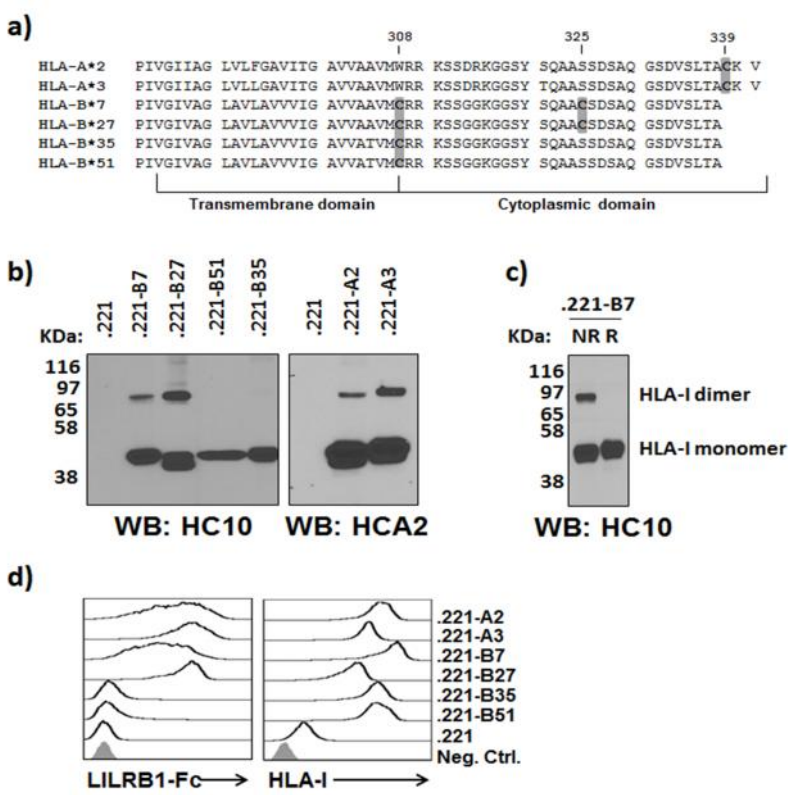

Figure 38 - Presence of HLA-I cytoplasmic C325 and C339 correlates with HLA-I dimer formation and LILRB1 recognition: (a) Multiple sequence alignment of HLA-I transmembrane and cytoplasmic domains with cysteine residues highlighted (information obtained from www.ebi.ac.uk). (b) Whole-cell extracts of .221 tranfectants were immunoblotted with HC10 (left panel) or HCA2 (right panel) mAbs. (c) HC10 immunoblot of .221-B7 whole-cell extracts resolved under non-reducing and reducing SDS-PAGE. (d) HLA-I surface expression and LILRB1-Fc binding to .221 cells transfected with different HLA-I alleles analyzed by flow cytometry (isotype controls - filled histograms and specific staining - black bold line).

Interestingly, LILRB1-Fc binding correlated with HLA-I alleles able to dimerize, as shown by the recognition of -B7, -B27, -A2 and -A3 but not -B35 and -B51 (Fig. 38d) regardless of HLA-I surface expression levels.

To evaluate whether HLA-I dimer expression in M1 and M2 MΦ could explain the differential LILRB1 binding we performed western blot analysis. Despite the clear detection of HLA-I monomers, we could not detect any high molecular weight band corresponding to HLA-I dimer in HC10 and HCA2 blots of cell lysates from steady state M1 and M2 MΦ (Fig. 39).

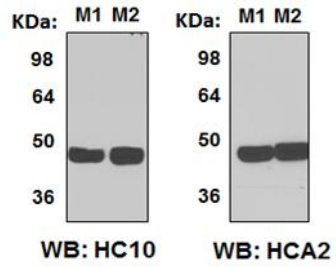

Figure 39 - Biochemical analysis of HLA-I expression on M1 and M2 MΦ: Whole-cell extracts of M1 and M2 MΦ were immunoblotted with HC10 (left panel) or HCA2 (right panel) mAbs.

4.3 - Influence of type I IFN on LILRB1-Fc binding and HLA-I dimer formation.

Since the only difference between M1 and M2 MΦ, obtained from a given donor, were the polarization factors (GM-CSF or M-CSF) present in the medium during the *in vitro* differentiation, we next studied whether different soluble factors (cytokines) could modulate LILRB1-Fc binding to MΦ. Indeed, the increase of LILRB1-Fc binding to non-infected cells exposed to HCMV prompted the inclusion of type I IFN as exogenous cytokine capable of modulating HLA-I expression and possibly HLA-I dimer formation and LILRB1-Fc engagement.

M2 MΦ were treated with the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokines IFN-α, IFN-β and IL-6 for 24h and subsequently LILRB1-Fc binding was monitored by flow cytometry (Fig. 40a). LILRB1-Fc

binding was clearly increased by IFN-α and IFN-β, whereas IL-10 and IL-6 did not modify HLA-I expression and induced a slight decrease of LILRB1 binding (not significant). As expected, the treatment with type I IFN also increased HLA-I surface levels. Yet, the magnitude of this effect was not proportional to the increase of LILRB1-Fc engagement (Fig. 40b).

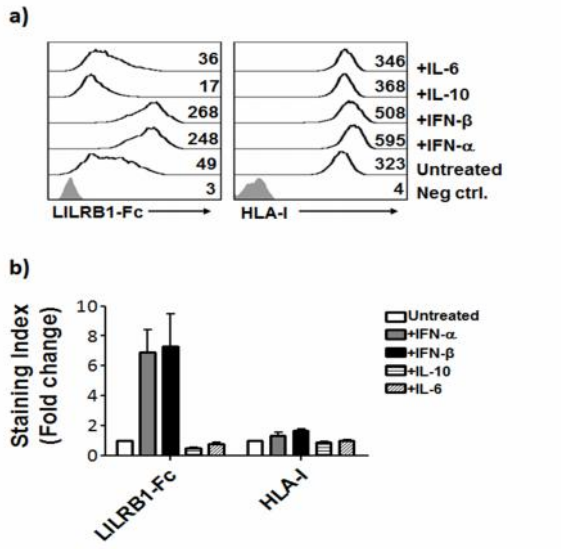

Figure 40 - Modulation of LILRB1 binding to M2 MΦ by different cytokines: M2 MΦ were treated with IFN-α (1000U/mL), IFN-β (1000U/mL), IL-10 (10ng/mL) and IL-6 (25ng/mL) for 24h before LILRB1-Fc fusion protein and anti-HLA-I Ab immunostaining. (a) Data correspond to results from a representative donor (isotype controls - filled histograms and specific staining - black bold line. Numbers depict the specific staining Geo Mean). (b) LILRB1-Fc binding and HLA-I expression. Staining Index fold change of cytokine-treated in comparison to untreated M2 MΦ. Data correspond to the mean ± SEM of four different donors.

We next addressed whether type I IFN treatment could modulate HLA-I dimerization. M1 MΦ, M2 MΦ and THP-1 cells were incubated with medium alone (untreated) or supplemented with IFN-β for 24h followed by flow cytometry analysis of HLA-I surface expression and LILRB1-Fc fusion protein binding (Fig. 41a and b). HLA-I dimer presence was monitored by WB with specific mAbs (Fig. 41c).

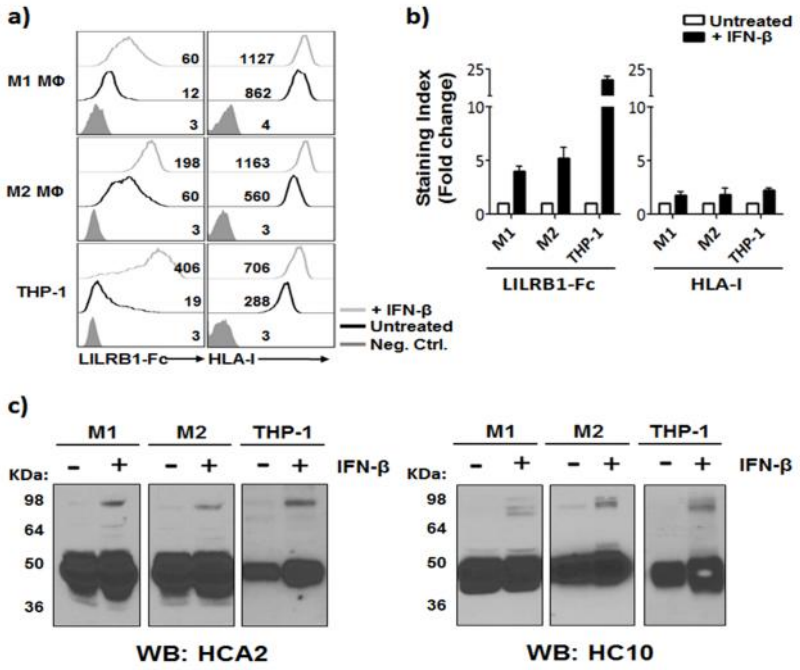

Figure 41 - LILRB1-Fc binding associates with HLA-I dimerization in myelomonocytic cells upon type I IFN treatment: M1 MΦ, M2 MΦ and THP-1 cells were incubated with IFN-β for 24h prior to flow cytometry and biochemical analysis. (a) LILRB1-Fc binding and HLA-I surface expression of both untreated and IFN-β-treated cells. Data correspond to results from a representative donor. Numbers depict the specific staining Geo Mean). (b) Staining Index fold change of LILRB1-Fc binding and HLA-I expression in IFN-β-treated versus untreated cells. Data represents mean ± SEM of three different donors. (c) Western blot analysis of HLA-I expression in whole-cell lysates from non-treated (-) or IFN-β treated (+) M1 MΦ, M2 MΦa nd THP-1 cells, run in non-reducing SDS-PAGE and immunobloted with HCA2 and HC10 mAbs.

As shown before, under basal conditions, LILRB1-Fc interacted preferentially with M2 MΦ as compared to M1 MΦ and THP-1 cells. However, after IFN-β treatment binding of the fusion protein was increased in all cell types. Once again, upon IFN-β treatment, we observed a higher fold change of LILRB1-Fc binding compared to the increased HLA-I surface expression. Biochemical analysis revealed that IFN-β-treated cells expressed higher levels of HLA-I dimers (detected with both HC10 and HCA2 Abs).

Overall, these results suggest that type I IFN enhances LILRB1-Fc interaction with myelomonocytic cells promoting HLA-I dimerization. Nevertheless, the mechanism underlying the differential LILRB1-Fc binding to M1 and M2 MΦ remains uncertain.

4.4 - Intracellular redox environment and HLA-I conformers.

Based on previous evidences indicating intracellular redox status differences between M1 and M2 MΦ [150] and the importance of the redox environment on HLA-I conformation [151,152], we next studied whether the IFN-β induction of HLA-I dimer formation was related with an alteration on the intracellular oxidative status. THP-1 cells were incubated with IFN-β or diamide, a thiol-oxidizing agent that reacts very rapidly with the anti-oxidant glutathione and therefore promotes an oxidative environment, for 2 or 24h prior to flow cytometric ROS detection by DCFH-DA oxidation. We could observe an increase in ROS detection upon 2h treatment with diamide, yet no changes in LILRB1-Fc binding were detected (Fig. 42a). Moreover, no significant changes on ROS production were observed upon IFN-β treatment whereas LILRB1-Fc binding was only increased at 24h. Western blot analysis showed that in contrast with IFN-β, HLA-I dimers were not detected in diamide-treated cells (Fig. 42b).

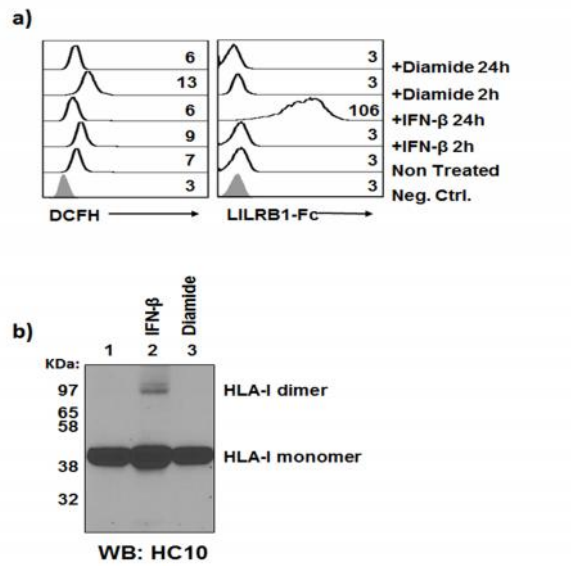

Figure 42 - Diamide treatment does not alter LILRB1-Fc binding capacity nor HLA-I dimer formation: THP-1 cells were treated with IFN-β (1000U/mL) and diamide (1mM) for 2 or 24h followed by flow cytometric and WB analysis. (a) Flow cytometry measurement of ROS production (DCFH-DA labeling) and LILRB1-Fc binding to THP-1 treated cells donor (isotype controls - filled histograms and specific staining - black bold line. Numbers depict the specific staining Geo Mean). (b) Whole-cell lysates of non-treated (lane 1), IFN-β treated (lane 2) or diamide treated (lane 3) THP-1 cells at 24h, resolved in non-reducing SDS-PAGE and immunoblotted with the HC10 antibody.

These results demonstrated that IFN-β induction of LILRB1-Fc engagement and HLA-I dimerization on THP-1 cells was not associated with ROS production. Moreover, the alteration of the intracellular oxidative environment promoted by diamide treatment did not influence the LILRB1-Fc engagement. Further experiments using different cell types and reagents should be performed before ruling out the association between the HLA-I dimerization events and the intracellular redox status.

Chapter 5

DISCUSSION

LILRB1 regulates steady-state MΦ activation threshold:

Human MΦ exert several functions that are crucial for homeostasis and host defence. Besides pathogen recognition, phagocytosis and antigen presentation these cells are also involved in apoptotic cell elimination and tissue repair. MΦ express different inhibitory surface receptors that regulate their function. The majority of these receptors use a common mechanism to interfere with proximal signalling cascades initiated by activating receptors (Tab. 1). Upon ligand binding, a tyrosine residue within an ITIM motif is phosphorylated providing a docking site to tyrosine or inositol phosphatases, resulting in a local and transient inhibition of activating signal cascades [153]. Among them, the LILR family includes inhibitory (LILRB) and activating (LILRA) receptors. The best-characterized LILR members are LILRB1 (ILT2) and LILRB2 (ILT4) inhibitory receptors. In this study, we focused on the capacity of these HLA-I-specific inhibitory receptors to regulate MΦ function in different contexts.

Surface expression of LILRB1 and particularly LILRB2 was lower in M1 compared to M2 MΦ and monocytes, suggesting that down-modulation of both HLA-I specific inhibitory receptors encompassed pro-inflammatory MΦ differentiation. LILRB1 and LILRB2 have been reported to broadly recognize different HLA-I molecules mainly based on the use of HLA-I transfected cell lines or surface plasmon resonance [71,83,86], whereas information regarding LILRB1 and LILRB2 interaction with HLA-I expressed by primary cells is quite limited. To address the function of these receptors in MΦ, constructs containing the extracellular region of LILRB1 and LILRB2 fused to human IgG1 Fc were used. Our data revealed a preferential binding of LILRB1-Fc to M2 MΦ as compared to monocytes and M1 MΦ derived from the same donor. In contrast, we could not detect LILRB2-Fc interaction with primary myelomonocytic cells despite their binding to 221-HLA-B7 transfectants. These observations comparing

different cell types derived from the same individual highlight the lack of correlation between surface HLA-I levels and LILRB1-Fc binding, indicating that additional variables unrelated to total HLA-I expression levels and to allelic polymorphisms influence the capacity of LILRB1 to recognize primary cells in *trans*.

Considering that M2 MΦ displayed high surface levels of LILRB1 and LILRB2 and the ability to engage LILRB1-Fc, we selected this cell type to analyse the functional consequences of disrupting LILRB1/HLA-I interactions. Incubation of M2 MΦ either with a blocking HLA-I-specific mAb (HP-1F7) or LILRB1-Fc induced IL-6 and low levels of IL-10 secretion but not TNF-α production. As extensively shown in studies on NK-cell biology, these data indirectly supported that HLA-I-interaction with the LILRB1 inhibitory receptor regulates the basal MΦ activation threshold by repressing constitutive activating signals. The nature of triggering receptor(s) sustaining this response is uncertain but putative candidates include surface molecules signalling through ITAM-containing adaptors and establishing low-affinity interactions with endogenous ligands (e.g. members of the FcR, SIGLEC, LILRA, TREM, CEA and IREM families). In this regard, a tonic ITAM-mediated calcium signalling and basal tyrosine phosphorylation of MΦ ITAMs in FcRγ and DAP12 adaptors has been previously reported [154]. On the other hand, the molecular events leading to the selective secretion of IL-6 triggered upon disruption of LILRB1/HLA-I interaction in MΦ, and the involvement of downstream effector signalling pathways activated by ITAM-coupled receptors deserve attention.

In addition to interacting in *trans* with HLA-I expressed on other cells, *cis* LILRB-mediated recognition of HLA-I was previously reported and associated to a constitutive SHP-1 recruitment [90]. In the same line, the ability of HLA-I-LILRB2 *cis*-interaction to regulate mast cell activation was

reported [155]. Our blocking experiments with anti-LFA-1 mAb indirectly support that constitutive *cis*-interactions between LILRB1 and HLA-I molecules predominantly repress tonic MΦ activation.

A special attention was devoted to understand the preferential binding of LILRB1-Fc fusion protein to M2 MΦ, as compared to monocytes and M1 MΦ obtained from the same donor and bearing comparable surface levels of HLA-I. Despite that HLA-G expression on myelomonocytic cells remains a controversial issue [156,157], the hypothesis that a differential expression of this HLA class Ib molecule, which constitutes a high affinity LILRB1 ligand, might account for these observations was considered. Yet, this possibility was ruled out as no consistent evidence supporting HLA-G expression in MΦ related with LILRB1 binding could be obtained based on flow cytometry and biochemical analyses using different mAbs.

The possible relation between expression of HLA-I dimers and LILRB1 recognition was also addressed. The ability of HLA-G dimers to enhance LILRB1 engagement and its inhibitory function [88,89] as well as the dimerization of other HLA-I molecules through intracellular cysteines [149] were previously reported. Despite the clear relation between the detection of HLA-I dimers and LILRB1-Fc binding to HLA-I transfectants and type I IFN-treated myelomonocytic cells, HLA-I dimers were undetectable in cell lysates from quiescent MΦ. Considering the occurrence of steady state LILRB1 and HLA-I *cis*-interactions in MΦ, it seems plausible that differences in HLA-I conformation between M1 and M2 MΦ may condition the availability of sites for LILRB1-Fc *trans*-recognition. Indeed, a putative role for LILRB1-HLA-I *cis*-interactions as regulators of *trans* ligand-receptor recognition has been recently proposed [158]. Finally, additional experiments ruled out that basal differences in the intracellular redox status between M1 MΦ and M2 MΦ might explain the distinct LILRB1-Fc binding patterns [150][152].

Regarding the functional implications of LILRB1-HLA-I interaction, it is of note that the involvement of the inhibitory receptor could not be formally established since no cytokine production was detected upon blocking with HP-F1 Fab, alone or in combination with an anti-LILRB2 (27D6) mAb, in contrast to the effects observed in the presence of an anti HLA-I mAb or a LILRB1-Fc fusion protein. The antagonistic effect of HP-F1 well-established in other experimental sytems does not imply a direct recognition of the LILRB1 interaction site with HLA-I, and likely involves a steric hindrance effect. This would explain that the lower size and avidity of monovalent HP-F1 Fab fragments hamper their ability to efficiently compete with the LILRB1-HLA-I *cis*-interactions. Of note, down-regulation of surface LILRB1 by siRNA did not promote cytokine secretion, suggesting that even low surface levels of the receptor interacting in *cis* with HLA-I might be sufficient to control MΦ basal activation. Though unlikely, the possibility that MΦ might express an unidentified HLA-I-specific inhibitory receptor, overcoming the effect of LILRB1 and LILRB2 blockade cannot be formally ruled out.

Overall, our data indicate that LILRB1 engagement by HLA-I in *cis* constitutes an important regulatory mechanism of MΦ activation in steady state conditions, controlling the tonic signalling by activating receptors. It is conceivable that a defective LILRB1 expression / function might be associated to a pro-inflammatory state. In this regard, DC isolated from patients with systemic lupus erythematosus (SLE) exhibited lower LILRB1 surface expression in comparison with control individuals [159]. Additional studies are warranted to validate this hypothesis.

Non-classical HLA-I conformers as ligands for LILRB1 in myelomonocytic cells:

A novel finding disclosed in this study is the relationship between the effect of type I IFN, HLA-I dimer formation and LILRB1-HLA-I interaction. By using transfectants expressing individual HLA-I alleles, the preferential LILRB1 binding to HLA-G dimers was extended to other HLA-I alleles. In the case of HLA-B7, -B27, -A2 or -A3, HLA-I dimer detection by WB coincided with the presence of intracellular cysteines at positions 325 or 339, whereas alleles lacking both C325 and C339 (HLA-B35 and -B51) only expressed the HLA-I monomer conformation. This observation is consistent with a previous report proposing that cytoplasmic cysteines are required for LILRB1 recognition of HLA-B7 [160]. Remarkably, comparison of cytoplasmic sequences published at (www.ebi.ac.uk) showed that all HLA-A alleles contained the C339 residue, and therefore HLA-I dimer formation appears as a general mechanism potentially occurring in all individuals, regardless of HLA polymorphisms. On the other hand, Jones et al. described that LILRB1 and LILRB2 binding to the products of >90 different HLA class I alleles was related to polymorphisms within the α3 region and HLA I class conformation [80].

Despite the LILRB1-Fc recognition in transfected cells of HLA-I alleles forming dimeric complexes, these were undetectable in primary MΦ and in the THP1 cell line under basal conditions, and became only perceived after type I IFN pre-treatment. IFN-β induced an up-regulation of surface HLA-I expression encompassed by an increased LILRB1-Fc binding and the detection of HLA-I dimers. Whether such enhanced LILRB1 recognition of type-I IFN-treated MΦ reflects simply the increase in HLA-I expression which provides new binding sites not blocked by *cis* LILRB1 interactions, or type I IFN actively promotes HLA-I dimerization are non-mutually exclusive interpretations. In summary, these observations indicate that the LILRB1-

HLA-I interaction may be regulated by environmental factors triggering type I IFN production.

LILRB1-HLA-I regulation of MΦ responses to tumor cells:

The role of LILRB1-HLA-I interaction on the MΦ response was evaluated in different *in vitro* experimental systems mimicking scenarios in which HLA-I expression is altered, namely cell transformation and viral cell infection. Down-regulation of surface HLA-I allows pathological cells to evade recognition by cytotoxic T-lymphocytes (CTL) [161,162], but renders them susceptible to NK-cells [131,163] impairing the control by HLA-I specific inhibitory receptors (i.e. KIR receptors, NKG2A and LILRB1) [164]. In a first approach, we used the 721.221 HLA-I-deficient EBV-transformed B-lymphoblastoid cell line, transfected with different HLA-I molecules, as a model to evaluate the regulatory capacity of HLA-LILRB1 *trans* interaction in the recognition of tumor cells by MΦ.

M2 MΦ recognition of HLA-deficient 721.221 cells promoted the release of IL-6 secretion in the absence of TNF-α and IL-10 production, indirectly supporting the existence of MΦ activating receptors for molecules expressed on B-LCLs capable of overcoming the homeostatic regulation by HLA-I/LILRB1 *cis*–interactions. In this model, the activation induced by the 721.221 cell line was prevented by the expression of either HLA-G or HLA-I alleles with dimer forming capacity (HLA-B7). Moreover, a relation between LILRB1-Fc binding to .221-HLA-I transfectants and the repression of MΦ activation regardless of total HLA-I surface expression levels was perceived.

Solid tumors may be infiltrated by a heterogeneous immune cell population, including different subsets of lymphoid and myeloid cells [165]. The presence of tumor-associated MΦ (TAM) is associated with a poor

prognosis in a variety of cancer types [166]. It has been described that TAM adapt to the microenvironment, switching from a M1-like to M2-like phenotype during the three phases of tumor progression (elimination, equilibrium and escape) [167]. M2-polarized TAM can contribute to tumor growth through different mechanisms including extracellular matrix remodelling, angiogenesis, promotion of cancer cell proliferation and suppression of adaptive immune anti-tumor responses through IL-10 and TGF-β production [168]. On the other hand, alterations of MHC class I expression and conformation may allow solid and hematopoietic tumor to escape from the immune system. HLA-I molecules can be present at the tumor cell surface as conventional monomers, HLA-I dimers and free heavy chain (FHC) conformers [169–171]. Our results indicate that HLA-I alterations on tumor cells interfering with LILRB1-dependent sensing may promote the release of IL-6 by MΦ. The production of IL-6 by TAMs has been associated with an enhanced invasive activity of transformed cells [172], and IL-6 has been recently pinpointed as a homeostatic determinant of the MΦ alternative activation [173].

Overall, our data indicate that MΦ can sense and respond to HLA-I alterations on tumor cells. Whether the production of IL-6 in TAM is activated in response to tumor HLA-I down-regulation deserves attention.

Role of LILRB1-HLA-I in the regulation of MΦ responses to HCMV-infected cells:

HCMV encodes several proteins (i.e. US2, US3, US6, US10 and US11) that impair HLA-I assembly and egress from the endoplasmic reticulum (ER) to the cell membrane, promoting a down-modulation of surface HLA-I expression, which interferes with antigen presentation to CD8+ T cells [93,94]. As a consequence the engagement of inhibitory receptors specific for HLA-I molecules is hampered, and NK cell activation is triggered by NKRs

specific for ligands, either constitutively expressed or induced upon infection. To counteract this vulnerability, HCMV has developed a variety of strategies to inhibit the expression of ligands for activating receptors or to maintain inhibitory receptors specific for HLA-I engaged [174,175]. Among them, HCMV encodes for UL-18, a viral HLA-I homologue with a high affinity for LILRB1 [84,92,176]. Our results indicate that M1 MΦ were capable of responding to HCMV-infected autologous M2 MΦ by secreting IL-6 in the absence of TNF-α or IL-10. Technical limitations precluded to precisely address the contribution to HLA-I down-modulation in this effect using mutant viruses; yet, LILRB1-Fc binding to HCMV-infected M2 MΦ was undetectable pointing out the absence of available ligands for LILRB1 on the infected cell surface, including UL18. These results are in line with previous reports describing a limited surface expression of the viral HLA-I surrogate and highlight the uncertainty about the mechanism(s) underlying its role in immune evasion [97,135,177].

On the other hand, the fraction of non-infected fibroblasts in the culture treated with HCMV displayed an enhanced LILRB1-Fc binding, likely promoted by HCMV-induced IFN-α [133]. It is tempting to speculate that the expression of binding sites for LILRB1 in the non-infected MΦ exposed to the virus might contribute to protect them from NK cells and/or from differentiated CD8 T cells along antigen cross-presentation.

Role of LILRB1-HLA-I in the regulation of MΦ activation in response to ITAM-bearing receptors:

The disruption of LILRB1-HLA-I upon simultaneous engagement of activating receptors such as TREM-1 or FcγR in M2 MΦ resulted in the synergistic secretion of IL-6 , IL-10 and TNF-α, indicating that *cis* HLA-I recognition also regulates MΦ cytokine secretion induced by high-avidity

ITAM-dependent signaling. As an important difference with the previously explored systems, the interference with HLA-I-LILRB1 interaction resulted in a broader spectrum of cytokine production.

To address the physiological implications of these observations, we explored the putative influence of LILRB1-HLA-I interactions on platelet uptake by MΦ. Upon their generation by megakaryocyte fragmentation, circulating platelets display a short lifespan, being removed by liver and spleen MΦ after 5-9 days of circulation. *De novo* protein synthesis by these cell fragments is compromised and platelet surface protein content is progressively altered with time. In fact, as previously shown [178], a reduction of HLA-I surface expression along platelet aging/storage was confirmed. Yet, in our experimental system we could not detect any modification on basal or antibody-mediated platelet phagocytosis by M2 MΦ after blocking LILRB1-HLA-I interactions with a specific F(ab`)2, suggesting that it this mechanisms is not central in regulating platelet turnover.

Overall, our data support that LILRB1 interaction with HLA-I not only modulates the steady state MΦ activation threshold but also the magnitude of MΦ response upon high-avidity ITAM-mediated stimulation. Even though we did not achieve an experimental demonstration, it is likely that LILRB1-HLA-I interaction may participate in the regulation of different MΦ effector functions besides cytokine secretion (i.e. phagocytosis). Additional studies addressing this hypothesis should be performed in order to fully elucidate the physiological role of LILRB1 in the regulation of MΦ function.

According to our results, the following model to interpret the regulation of MΦ function by LILRB1 can be proposed (Fig. 43).

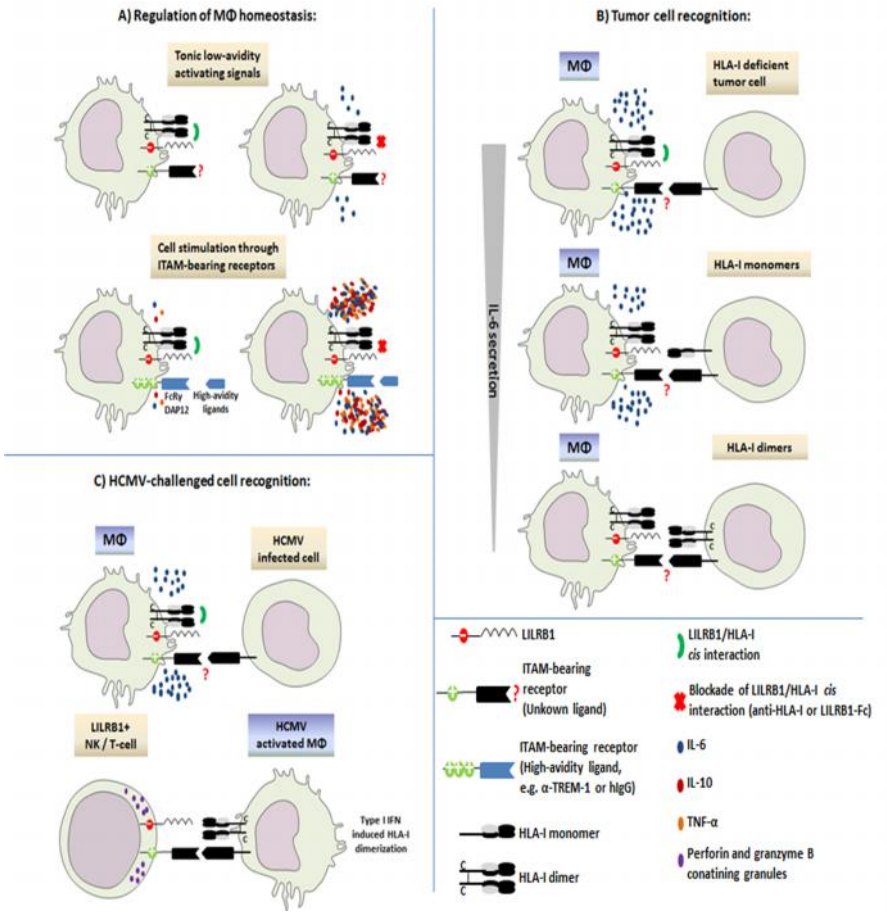

Figure 43 - MΦ function regulation by LILRB1-HLA-I interaction:

A) Regulation of MΦ homeostasis. B) Tumor cell recognition. C) HCMV-challenged cell recognition.

A) The first scenario illustrates the "missing-self" recognition by steady-state or activated MΦ. LILRB1 engagement by HLA-I in *cis* negatively modulates activating signals triggered by ITAM-bearing receptors. The avidity of receptor/ligand interaction determines the outcome of MΦ response. When LILRB1-HLA-I interaction is prevented, low-avidity constitutive ligands induce IL-6 secretion while high-avidity ligands promote a wider and magnified cytokine production.

B) Pathological events leading to malignant cell transformation and the promotion of HLA-I surface expression alterations may be sensed by surrounding MΦ. Regardless of the regulation by LILRB1-HLA-I *cis*-interaction, MΦ secrete IL-6 in response to HLA-I deficient tumor cells. Expression of non-classical HLA-I or non-conventional HLA conformers by tumor cells can preclude MΦ activation.

C) HCMV-infected cell recognition by non-infected autologous MΦ results in the secretion of IL-6, overcoming LILRB1-HLA-I *cis*-interaction. LILRB1 does not bind to any ligand HLA-I/UL18 on the infected cells surface. Immune responses triggering type I IFN secretion (i.e. viral infections), will promote HLA-I expression and dimer formation on surrounding cells encompassed by enhanced LILRB1 recognition. This secondary event induced by type I and perhaps also by type II IFNs in HLA-I MΦ expression, could preserve non-infected myelomonocytic cells from LILRB1+ NK and $CD8^+$ T effector cells.

Chapter 6

CONCLUSIONS

1. Monocytes and M2 MΦ expressed higher surface levels of LILRB1 than M1 MΦ.

2. LILRB1-Fc differentially bound to primary myelomonocytic cell types independently of total HLA-I expression levels, allelic polymorphisms and expression of HLA-G, which was undetectable.

3. LILRB1-Fc preferentially recognized HLA-I molecules containing cytoplasmic C325 or C339 residues which might allow dimer formation.

4. Type I IFN enhanced LILRB1-Fc binding to myelomonocytic cells, correlating with an increase of total HLA-I expression and the detection of HLA-I dimers.

5. Anti HLA-I mAb and LILRB1-Fc induced M2 MΦ production of IL-6 in the absence of TNFα. This response was unaffected by blocking LFA-1, indirectly supporting that LILRB1-HLA-I *cis* interactions regulate the steady state activation threshold in M2 MΦ.

6. IL-6 secretion by M2 MΦ in response to the HLA-I-defective 721.221 cell line was selectively regulated by the expression/transfection of HLA-I molecules recognized by LILRB1.

7. M1 MΦ secreted IL-6 in response to autologous HCMV-infected M2 MΦ, in which HLA-I expression was down-regulated.

8. Interfering with the LILRB1-HLA-I interaction enhanced M2 MΦ cytokine production triggered by TREM-1 and FcγR activating receptors.

9. Platelet uptake by M2 MΦ was not influenced by LILRB1-HLA-I interaction.

10. Altogether our data support that LILRB1 engagement, which depends on HLA-I conformation, regulates steady state MΦ functions and their response to activating stimuli.

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ABBREVIATIONS:

APC	Antigen presenting cells
AS	Ankylosing spondylitis
BAC	Bacterial artificial chromosome
BCR	B cell receptor
β2m	Beta-2 microglobulin
Btk	Bruton tyrosine kinase
CCL	Chemokine C-C motif ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CLEC	C-type lectin domain family
CR	Complement receptor
CRD	Carbohydrate recognition domain
CXCL	Chemokine CXC motif ligand
DAMP	Danger-associated molecular patterns
DAP-12	12kDa DNAX adaptor protein
DC	Dendritic cell
Dok	Downstream of tyrosine kinase
Erk	Extracellular signal-regulated kinase
EVT	Extravillous trophoblasts
FcγR	Fc-gamma receptor
FHC	Free heavy chains
FRET	Fluorescence resonance energy transfer
GM-CSF	Granulocyte-macrophage colony stimulating factor
HCMV	Human cytomegalovirus
HLA	Human leukocyte antigen
IC	Immunocomplexes
IE	Immediate early
IFN	Interferon
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
IL	Interleukin
ILT	Immunoglobulin-like transcript
IREM	Immune receptor expressed by myeloid cells
ITAM	Immune receptor tyrosine-based activating motif
ITIM	Immune receptor tyrosine-based inhibitory motif
KIR	Killer immunoglobulin-like receptor

LAT	Linker of activated T cells
Lck	Lymphocyte-specific protein tyrosine kinase
LCL	Lymphoblastoid cell line
LDL	Low density lipoprotein
LFA	lymphocyte function-associated antigen
LILR	Leukocyte immunoglobulin-like receptor
LPS	Lipopolysaccharide
MΦ	Macrophage
mAb	Monoclonal antibody
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompability complex
MICL	Myeloid inhibitory C-type lectin-like
MOI	Multiplicity of infection
MyD88	Myeloid differentiation primary response gene (88)
NF-kB	Nuclear factor- kB
NK	Natural killer
NKG2	Natural killer group 2
NLR	Nod-like receptor
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PE	Phosphatidylethanolamine
PECAM	Platelet/endothelial cell adhesion molecule
PH	Pleckstrin Homology
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PRP	Platelet-rich plasma
PRR	Pattern-recognition receptor
PS	Phosphatidylserine
PSR	Phosphatidylserine receptor
RIG	Retinoic acid inducible gene
ROS	Reactive Oxygen Species
SH-2	Src homology-2 domain
SHIP	SH-2 domain containing inositol phosphatase
SHP	SH-2 domain-containing protein tyrosine phosphatase
SIGLEC	Sialic acid binding Ig-like lectins
siRNA	small interfering RNA
SIRP	Signal regulatory protein
SR	Scavenger receptor
syk	Spleen tyrosine kinase
TAM	Tumor-associated macrophage
TCR	T cell receptor

TLR	Toll-like receptor
TM	Transmembrane
TNF	Tumor necrosis factor
TREM	Triggering receptor expressed by myeloid cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
UL	Unique long
US	Unique short
ZAP-70	ξ – chain Associated Protein 70

Annex 1

MANUSCRIPTS

1. **Diogo Baía**, Aura Muntasell, Jordi Pou and Miguel López-Botet; *LILRB1 interaction with primary myelomonocytic cells is related with HLA class Ia dimerization enhanced by type I interferon.*
   (Submitted)

2. **Diogo Baía**, Aura Muntasell, Jordi Sintes, Jordi Pou and Miguel López-Botet*; LILRB1 interaction with HLA class I molecules regulates IL-6 production by steady state M2 macrophages.*
   (In preparation)