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DEPARTMENT OF BIOLOGY AND BIOTECHNOLOGY
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Modulation of NKG2A and NKG2D ligand expression during monocyte differentiation

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CHAPTER 1 – INTRODUCTION

1.1 INNATE IMMUNITY AND ITS INTERPLAY WITH THE ADAPTIVE IMMUNE RESPONSES

Evolutionary conserved defence mechanisms, collectively known as innate immunity, exert their impressive protective capacity in the majority of the living organisms, down to metazoans. On the contrary, adaptive immunity, an alternative system for pathogen recognition and elimination, can be found only in vertebrates as a consequence of their ability to generate antigen-specific lymphoid cells. Genetic and cellular processes (hypervariability, rearrangement of receptor gene segments and clonal selection) generate favorable somatic variants of antigen binding-receptors by which the adaptive immune cells specifically recognize pathogens and tissue insults [1,2,3]. Although the human immune responses are divided into these two branches, the immune network is the result of the extensive cross-talk between the innate and adaptive responses. The innate immune system is considered as the first line of defense against infections; it augments the protection offered by anatomic and physiologic barriers and initiates the adaptive immune responses conferring a specific and

long lasting protection. The innate immune system consists of hematopoietic cells expressing the germline encoded pattern-recognition receptors (PRRs) such as macrophages, Dendritic Cells (DCs), granulocytes, mast cells and Natural Killer (NK) cells other than soluble factors such as cytokines and chemokines. By contrast, the adaptive immune system consists of T and B lymphocytes, expressing somatically generated antigen-specific receptors, and antibodies (Fig.1) [4,5].

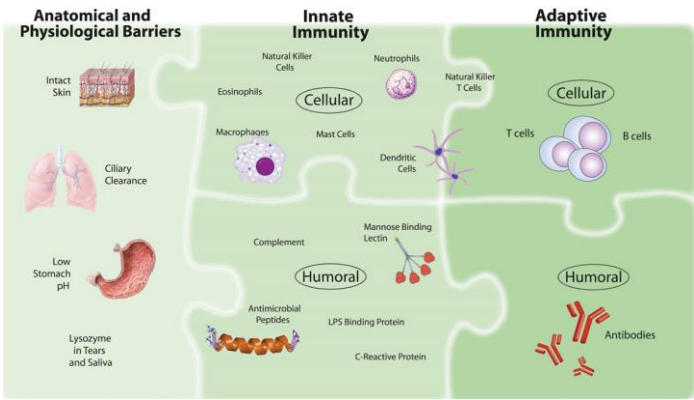

Figure 1. Integrated human immune system. The human microbial defense system can be simplistically viewed as consisting of 3 levels: (1) anatomic and physiologic barriers; (2) innate immunity; and (3) adaptive immunity. In common with many classification systems, some elements are difficult to categorize. For example, NK T cells and dendritic cells could be classified as being on the cusp of innate and adaptive immunity rather than being firmly in one camp [5].

The innate immune cells are able to discriminate foreign molecules from self by PRRs that recognize conserved molecular patterns that are shared by many bacteria or viruses (pathogen-associated molecular patterns, PAMPs). The promptness of the innate responses depends on the availability of these receptors on immune cells before exposure to the antigens. The result of the PRRs/PAMPs interaction is the activation of proinflammatory signaling pathway leading to cytokine and chemokine release [6,7]. By this mechanism, macrophages recruit neutrophils to the site of infection where they ingest, kill and digest bacteria. Although still viewed as a short-lived effector cells which provide a first line of defence against pathogen by their phagocytic role, neutrophils perform several other important functions (Fig.2) [8].

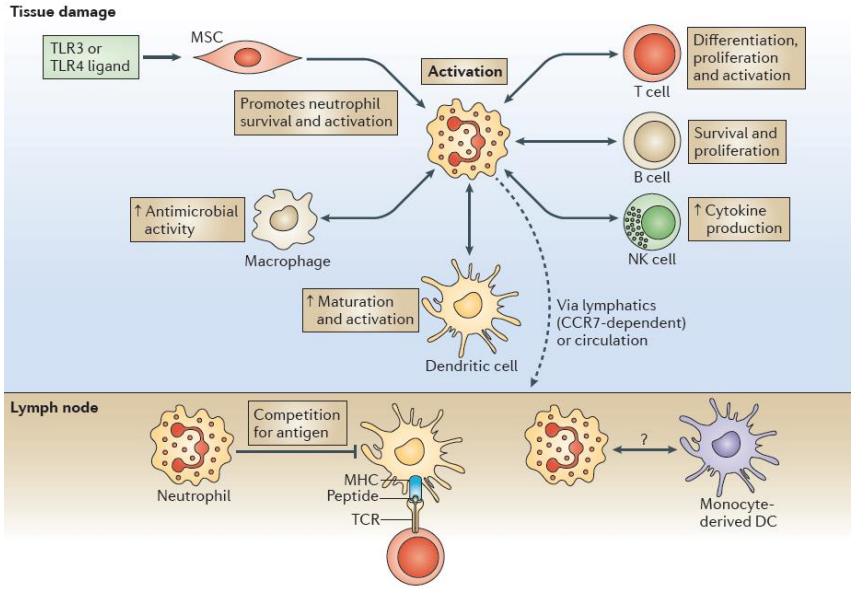

Figure 2. Neutrophils crosstalk with immune and non-immune cells in inflamed tissues and lymph nodes. Circulating neutrophils are stimulated by systemic pathogens to crosstalk with platelets and endothelial cells, and this triggers the coagulation cascade (not shown). In the presence of tissue damage, neutrophils leave the circulation and crosstalk with both resident and recruited immune cells, including mesenchymal stem cells (MSCs), macrophages, dendritic cells (DCs), natural killer (NK) cells and B and T cells. The figure shows the main outcome(s) of the effects that MSCs exert on neutrophils and of neutrophil crosstalk with other cell types. Neutrophils can also migrate to the lymph nodes either via the lymphatics (in a CC-chemokine receptor 7 (CCR7)-dependent manner, similarly to tissue DCs) or via the circulation (similarly to monocytes). In the lymph nodes, neutrophils can interact with DCs to modulate antigen presentation. TCR, T cell receptor; TLR, Toll like receptor [8].

The two main cellular types that exert supervisory functions in the immune responses are the macrophages

and dendritic cells. These mononuclear phagocytes, both derived from blood monocytes, are able to recruit the immune cells to the site of infection by chemotactic cytokines and modulate their function by several soluble or membrane-bound molecules. Moreover, they are known as "professional" antigen presenting cells (APCs) because of their capability to present antigenic molecules to T lymphocytes, so initiating the adaptive immune responses. Among APCs, only dendritic cells have the ability to induce a primary immune response in naïve T lymphocytes; therefore, they play a pivotal role in the induction of the adaptive immunity (Fig.3) [9].

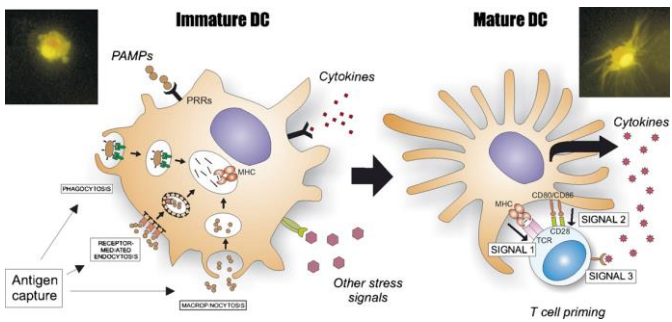

Figure 3. Functional profile of immature and mature DCs. Immature DCs efficiently sample antigens from the surrounding environment by macropinocytosis, receptor-mediated endocytosis and phagocytosis. On the other hand, they express a high diversity of receptors which enable them to recognize PAMPs, cytokines, chemokines and other stress signals. Upon maturation, they become capable to activate naïve T cells (signals 1 and 2) promoting the differentiation of newly activated T lymphocytes into effector cells (signal 3) [9].

Cytolytic effector functions against infected cells or tumor cells in the innate immunity are performed by the natural killer cells which do not bear a specific antigen receptor. Moreover, they release several immunoregulatory cytokines that impact on DCs, macrophages and neutrophils as well as on the adaptive immune response. Conversely, T and B lymphocytes act to enhance elements of the innate system by cytokine or antibody production, respectively [10]. Therefore, the tissue homeostatic maintenance and the resolution of infection are dependent on this strong interplay between the two arms of the immune system while a dysregulation of this crosstalk could lead to pathology (Fig.4).

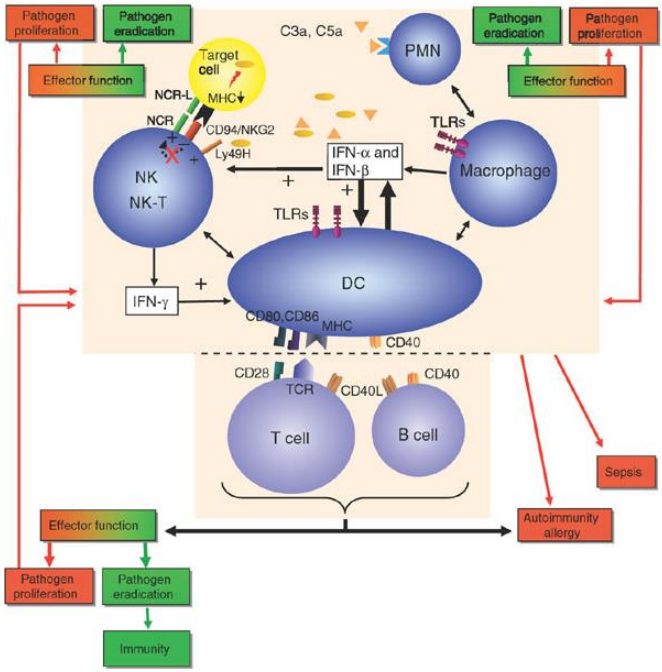

Figure 4. The interface between innate and adaptive immunity and the consequences of success or failure. Essential to the successful removal of pathogens is the early recognition of microbes by components of the innate immune system. These involve the complement system, specialized receptors expressed on NK cells and the family of TIRs that are expressed on myeloid as well as lymphoid cells and that recognize specific microbially derived molecular structures. Successful engagement of some of these pathways lead to pathogen eradication and host immunity (green). Failure to efficiently discriminate self from non-self in innate as well as adaptive immunity can lead to pathogen proliferation and ultimately sepsis (red) and may also be the cause for development and maintenance of autoimmune disease and allergy (red). CD40L, CD40 ligand; IFN, interferon; MHC, major histocompatibility complex; PMN, polymorphonuclear cells; TCR, T cell receptor; NCR, natural cytotoxicity receptor; NCR-L, natural cytotoxicity receptor-ligand [11].

1.2 Ontogeny and heterogeneity of mononuclear phagocytes

The mononuclear phagocyte system could be roughly described as a subgroup of leukocytes of myeloid lineage that circulate in the blood as monocytes to further differentiate into a range of tissue macrophages and dendritic cells (DCs). These phagocytic cells mediate essential functions in the steady state and during inflammation including phagocytosis, cytokine production, initiation of adaptive immunity, tissue genesis and homeostasis.

Recent evidence, on the basis of transplantation studies in mice, strongly indicates that monocytes are not simply transitional cells but they are highly heterogeneous, adopting different activation states and changing their function in response to microenvironmental signals. Blood monocytes originate, in vivo, from hematopoietic stem cell-derived progenitor (HSC) and downstream, the commitment steps in the bone marrow follow along the common myeloid progenitors (CMP) and the granulocyte-macrophage precursors (GMP). Within the bone marrow, the GMPs differentiate to $CX3CR1^{+}$ $ckit^{+}$ $CD115^{+}$ macrophage and dendritic cell progenitors (MDP) that

give rise to monocytes, several macrophage subsets and DCs (Fig.5) [12,13].

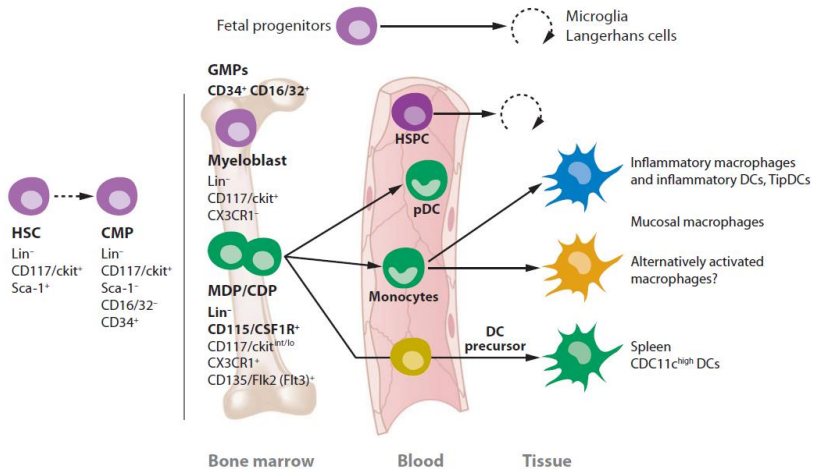

Figure 5. Differentiation of the macrophage/DC progenitor and origin of macrophage and DC subsets [13].

Two different groups have recently described the common DC precursor (CDP), originating from mouse MDP in the bone marrow, that specifically generate classical DCs (cDCs) and plasmacytoid DCs (pDCs) but not monocytes [14,15]. cDCs are professional APCs specialized to capture antigens in most tissues and further regulate T cell responses in lymphoid organs [16]. By contrast, the primary function of pDCs is the rapid and massive secretion of type I interferons (IFN-α/β) to respond to viral infection [17].

The MDPs give rise to two distinct subsets of mouse monocytes on the basis of chemokine receptor expression and the presence of specific surface molecules. The first subset, termed "inflammatory", express Ly6c ($Gr1^+$), CCR2 and L-Selectin and are selectively recruited to sites of inflammation and infection. This subset generates macrophages and the so-named Tip-DCs characterized by the production of inflammatory mediators such as tumor necrosis factor (TNF)-α and nitric oxide (NO). This group of DCs represents short-lived cells whose main function is to kill bacteria rather than to regulate T-cell function [18]. The second subset of monocytes express high levels of the chemokine receptor CX3CR1 and the integrin LFA-1 but not Ly6c ($GR1^-$), CCR2 and L-Selectin. They are termed "resident" because of the patrolling behavior in the vasculature in the steady state. This subset of monocytes rapidly extravasates after infection and contributes to early inflammatory response. However, 8h after infection, they stop to produce the inflammatory mediators while the $Gr1^+$ monocytes are now the main producers. It seems that after infection the $GR1^-$ monocyte subset initiates an M2 macrophage differentiation program that are proposed to be involved in tissue repair (Fig.6) [19,20].

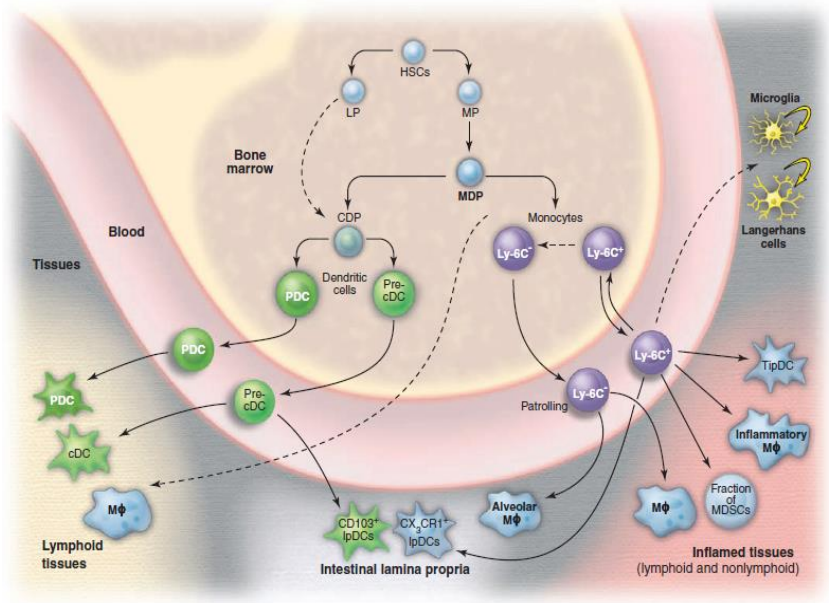

Figure 6. Differentiation of DCs and macrophages in mice. In the bone marrow, hematopoietic stem cells (HSCs) produce myeloid (MP) and lymphoid (LP) committed precursors. MPs give rise to monocyte, macrophage, DC precursors (MDPs). MDPs give rise to monocytes, some population of macrophages, and common DC precursors (CDPs). Two monocyte subsets, Ly6C$^+$ and Ly6C$^-$, leave the bone marrow to enter the blood. CDPs give rise to preclassical dendritic cells (pre-cDCs) and plasmacytoid dendritic cells (PDCs). Pre-cDCs circulate in blood and enter lymphoid tissue, where they give rise to CD8α^+ and CD8α^- cDCs, and nonlymphoid tissues, where they may give rise to CD103$^+$ lamina propria DCs (lpDCs). Under homeostatic conditions, Ly-6C$^-$ monocytes may contribute to alveolar macrophages (MΦ), and Ly-6C$^+$ monocytes can become CX_3CR1^+ lpDCs in nonlymphoid tissues. During inflammation, Ly-6C$^+$ monocytes give rise to monocyte-derived DCs, for example, tumor necrosis factor (TNF) and inducible nitric oxide synthase (iNOS)-producing dendritic cells (Tip-DCs), inflammatory macrophages, and may contribute to myeloid-

derived suppressor cells (MDSCs) associated with tumors. They are also suspected (dashed arrow) to contribute to microglia and Langherans cells in selected experimental conditions. Microglia and Langherans cells can renew independently from the bone marrow (curved arrow). HSCs can also leave their bone marrow niche (dashed arrow) and enter peripheral tissues, where they differentiate to myeloid cells during inflammation. It is unclear at this time whether LPs contribute substantially to PDCs and cDCs (dashed arrow) [20].

Although the mouse monocytes exhibit remarkable plasticity, as highlighted by in vivo studies in mouse models, the human myeloid cell differentiation and function are not fully understood as yet. However, as described in mice, human monocytes can be divided into two major subsets on the basis of surface CD14 and CD16 expression (CD14+CD16- and CD14+CD16+). The human CD14+CD16- classical monocytes, which comprise approximately 95% of human blood monocytes, express the chemokine receptor CCR2 and produce TNF, IL-1 and IL-6 in response to LPS stimulation in vitro suggesting that they are the counterparts of mouse $Gr1^{+}$ inflammatory monocytes. By contrast, human proinflammatory CD16+ monocytes, equivalent to $GR1^{-}$ mouse monocytes, express high levels of CX3CR1 and are the main producers of TNF-α [21,22,23]. The CD16+ monocytes can be further divided according to their high

or low levels of CD14 expression into two smaller subpopulations, the intermediate CD14++CD16+ and the non-classical CD14+CD16++ monocytes [24]. The former have phagocytic activity and produce proinflammatory cytokines in response to different stimuli whereas the latter are weak phagocytes and respond to viral stimuli through TLR7 and TLR8 [25]. Therefore, the differentiation and contribution to immune defence of human and mice monocyte subsets appear to be similar even though they are not fully equivalent (Table 1 [26]).

Table 1 | **Mouse and human monocyte subsets**

Subset	Markers	Chemokine receptors	Functions
Mouse			
LY6C^{hi}	CD11b^{+}CD115^{+}LY6C^{hi}	CCR2^{hi}CX$_3$CR1^{low}	Pro-inflammatory[7] and antimicrobial[3] roles
LY6C^{low}	CD11b^{+}CD115^{+}LY6C^{low}	CX$_3$CR1^{hi}CCR2^{low}	Patrolling[11]; early responses[11]; tissue repair[128]
Human			
Classical	CD14^{++}CD16^{-}	CCR2^{hi}CX$_3$CR1^{low}	Resemble LY6C^{hi} monocytes based on gene-expression arrays[7,17,140]
Intermediate	CD14^{++}CD16^{+}	CX$_3$CR1^{hi}CCR2^{low}	Pro-inflammatory roles[12,15]
Non-classical	CD14^{+}CD16^{++}	CX$_3$CR1^{hi}CCR2^{low}	Patrolling[14]; antiviral roles[14]

CCR2, CC-chemokine receptor 2; CX$_3$CR1, CX$_3$C-chemokine receptor 1.

1.3 Macrophage function and polarization

Blood monocytes are recruited into the tissue where they differentiate into macrophages in steady state and during inflammation. Macrophages can acquire functional specialization and phenotypes based on their anatomical location including Kupffer cells in the liver, osteoclasts in the skeletal system, microglial cells of the brain, alveolar

macrophages of the lung and histiocytes of the connective tissue (Fig.7) [27].

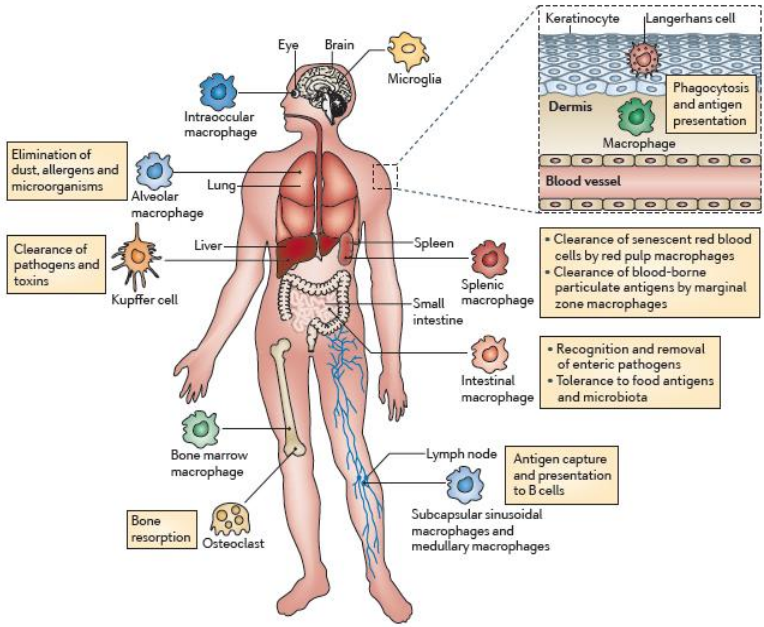

Figure 7. Tissue macrophages perform important homeostatic functions. Mononuclear phagocytes are generated from committed hematopoietic stem cells located in the bone marrow. Macrophage precursors are released into the circulation as monocytes and quickly migrate into nearly all tissues of the body, where they differentiate into mature macrophages. Various populations of mature tissue macrophages are strategically located throughout the body and perform important immune surveillance activities, including phagocytosis, antigen presentation and immune suppression [27].

Macrophages are "professional" phagocytic and antigen presenting cells but they are also involved in tissue

remodeling and repair under homeostatic and damage conditions [28]. Therefore, the macrophage lineage includes versatile and plastic cells with distinct functions that are specified by the exposure to microenvironmental signals. Mirroring the T-cell literature, two well-established polarized phenotypes have been described: *classically activated macrophages* (M1 macrophages) and *alternatively activated macrophages* (M2 macrophages) (Fig.8).

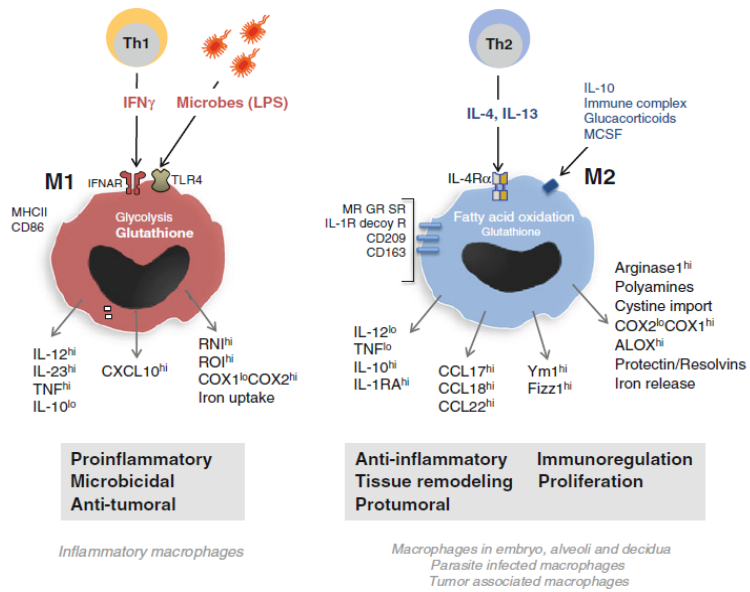

Figure 8. Schematic representation of the M1- and M2-polarized macrophages. The polarizing signals and salient molecular, metabolic and functional characteristic of these macrophages are indicated (adapted from [28]).

M1 macrophages arise in response to interferon-γ (IFN-γ) alone, typically produced by NK and T helper 1 cells, or in combination with microbial stimuli (eg LPS) or cytokines (eg TNF and GM-CSF). M1 cells have microbicidal activity, antigen presenting function and express pro-inflammatory cytokines (IL-1, TNF, IL-6). In contrast, M2 macrophages arise in response to interleukin-4 (IL-4) or IL-13, which are produced by T helper 2 cells. M2 cells express the mannose receptor CD206, arginase 1 and IL-10 among others. They are associated with anti-inflammatory and homeostatic functions linked to fibrosis and tissue repair [29]. Moreover, the macrophages that infiltrate tumor tissue, named tumor-associated macrophages (TAMs), share several properties with polarized M2 cells. They promote immunosuppression, angiogenesis, cell motility and tumor progression [30].

Accumulating evidence exists that several transcription factor and epigenetic regulation contribute to macrophage differentiation and polarization. The transcription factor PU.1 binds to genomic enhancers and controls the regulatory regions in which transcription factors operate to modulate macrophage identity. M1 stimuli like LPS, GM-CSF and IFN-γ activate the

transcription factors STAT1/2, IRF5 and NF-κB that regulate the expression of inflammatory genes characteristics of the M1 polarization state. By contrast, M2 stimuli like IL-4 and IL-13 active STAT6, PPARγ and IRF4 that mediate the M2 polarization of macrophages (Fig.9) [31].

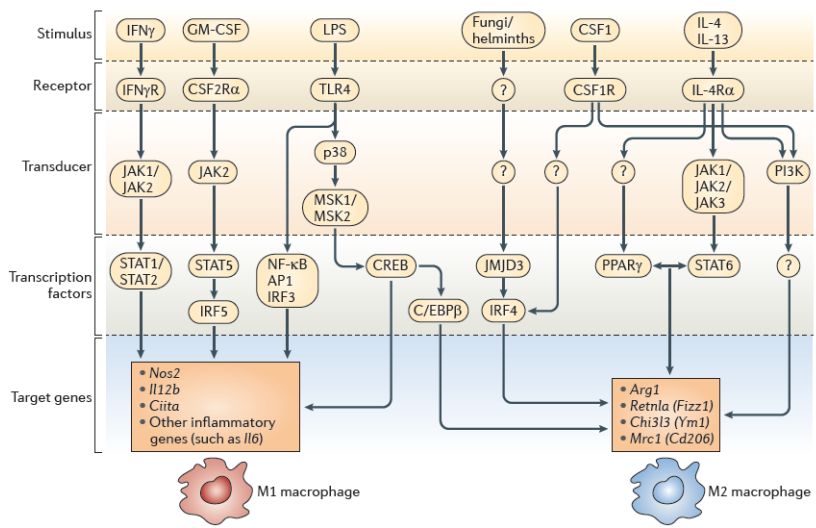

Figure 9. Signal transduction pathways to M1 and M2 macrophage polarization. Signaling pathways implicated in M1 and M2 macrophage polarization are indicated. An initial stimulus leads to the activation of sequence-specific transcription factor that eventually mediate the changes in the transcriptional output. The main genes that are characteristic of either the M1 or the M2 polarized state are also shown. Arg, arginase 1; C/EBPβ, cAMP-responsive element-binding protein; CSF, colony-stimulating factor; IFNγ, interferon-γ; IL, interleukin; IRF, interferon-regulatory factor; JAK, janus kinase; LPS, lipopolysaccharide; Mrc1, macrophage mannose receptor

1; MSK, mitogen- and stress-activated kinase; NK-κB, nuclear factor-κB; Nos2, nitric oxide synthase 2; PI3K, phosphoinositide 3-kinase; PPARγ, peroxisome proliferator-activated receptor-γ; Retnla, resistin-like-α; STAT, signal transducer and activator of transcription; TLR4, Toll-like receptor 4 [31].

1.4 Natural Killer cells and their receptor repertoire

Natural Killer (NK) cells are a subset of lymphoid cells that play a pivotal role in the innate immune responses. Differently from antigen-specific T and B lymphocytes, NK cells recognize and kill target cells, such as transformed or virus-infected cells, without prior sensitization [32].

The level of CD56 (Neuronal cell adhesion molecule) and CD16 (Fcγ receptor III) expression defines the two major NK cell subsets both arising from the bone marrow CD34+ hematopoietic progenitor cells (HPCs) (Fig.10).

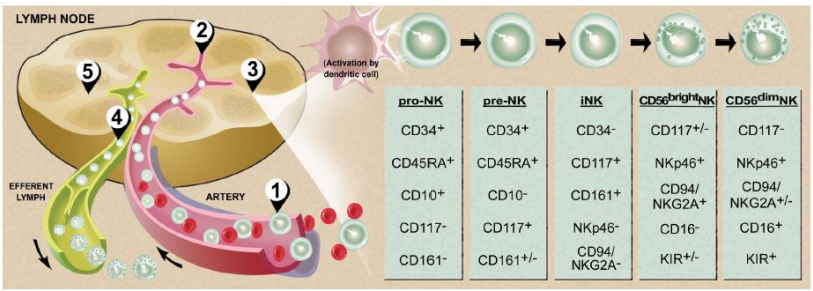

Figure 10. Model of human NK-cell development. (1) Bone marrow–derived CD34+CD45RA+ HPCs circulate in the blood and (2) extravasate across lymph node high endothelial venules to enter the parafollicular space. There, (3) pro-NK cells are activated to progress through distinct stages of maturation (far right) to create both CD56bright and CD56dim NK cells. Maturing CD56dim NK cells return to the circulation via the efferent lymph (4), whereas some CD56bright NK cells remain within the secondary lymphoid tissue to interact with DCs (5) [33].

The $CD56^{bright}$ subset, which expresses low or no levels of CD16, produces high amounts of immunoregulatory cytokines including IFN-γ, TNF-β, IL-10, IL-13 and GM-CSF. By contrast, the $CD56^{dim}CD16^{bright}$ subset, which constitutes approximately 90% of human NK cells, is mainly responsible for natural cytotoxicity against NK-sensitive targets (Fig.11) [34,35].

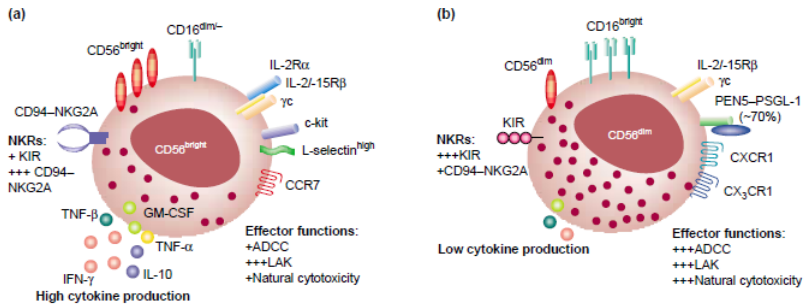

Figure 11. Scheme of human natural killer (NK)-cell subsets. (a) $CD56^{bright}$ NK cells produce high levels of cytokines following stimulation with monokines. This subset has low-density expression of CD16 and exhibits low natural cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC), but potent lymphokine activated killer (LAK) activity. $CD56^{bright}$ NK cells have high-level expression of the inhibitory CD94–NKG2A C-type lectin NK receptor (NKR) but have low level expression of killer Ig-like receptors (KIRs). This NK-cell subset expresses a number of cytokine and chemokine receptors constitutively, including the high-affinity interleukin-2 receptor (IL-2Rαβγ), c-kit and CC-chemokine receptor 7 (CCR7). The adhesion molecule L-selectin, which, in combination with CCR7, is involved in trafficking to secondary lymph nodes, is also found on $CD56^{bright}$ NK cells. **(b)** By contrast, $CD56^{dim}$ NK cells produce low levels of NK-derived cytokines but are potent mediators of ADCC, LAK activity and natural cytotoxicity, and have a more granular morphology than $CD56^{bright}$ NK cells. The $CD56^{dim}$ NK-cell subset has high-level expression of KIRs. These cells have distinct expression of cytokine (e.g. IL-2Rβγ) and chemokine (e.g. CXCR1 and CX3CR1) receptors. $CD56^{dim}$ NK cells lack L-selectin but highly express PEN5–P-selectin glycoprotein ligand-1 (PSGL-1), another adhesion molecule [Adapted from 34].

NK cells express several germ-line encoded inhibitory and activating receptors, and their activation state results from a fine balance between the inhibitory and activating signals (Fig.12) [36].

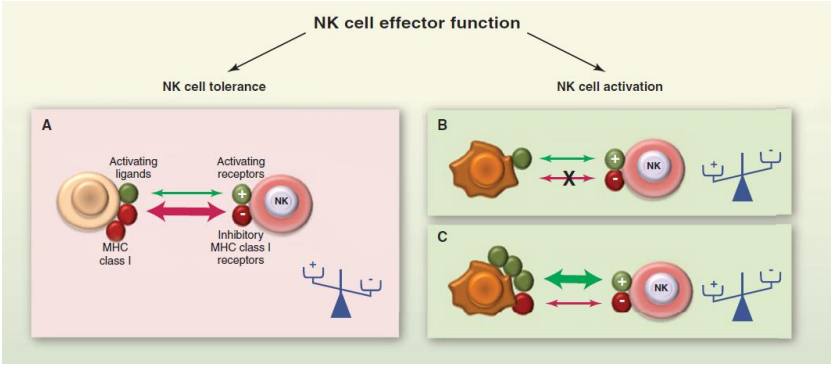

Figure 12. The dynamic regulation of NK cell effector function. NK cells sense the density of various cell surface molecules expressed at the surface of interacting cells. The integration of these distinct signals dictates the quality and the intensity of the NK cell response. NK cells spare healthy cells that express self-MHC class I molecules and low amounts of stress-induced self molecules (A), whereas they selectively kill target cells "in distress" that down-regulate MHC class I molecules (B) or up-regulate stress induced self molecules (C). +, activating receptors; –, inhibitory receptors [36].

At the moment, three main families of Human Leukocyte Antigen (HLA) class I receptors have been described: the human Killer cell Ig-like receptors (KIR) and the human Ig-like transcript (ILT), which belong to the immunoglobulin (Ig) superfamily and bind a large

number of HLA class I alleles; and the C-type lectin-like receptors (NKG2/CD94) which recognize the HLA-E molecules [37]. The inhibitory receptors belonging to these families, such as KIR2DL or KIR3DL (with a "long" cytoplasmic domain), the heterodimer NKG2A/CD94 and the ILT receptors, share one or more Immunoreceptor Tyrosine based Inhibitory motifs (ITIM) in their cytoplasmic tail that suppress the NK cell responses. By constrast, KIR2DS or KIR3DS (with a "short" cytoplasmic domain) and the heterodimer NKG2C/CD94, lack ITIM but interact with the DAP12 protein that contains the Immunoreceptor Tyrosin based Activation motifs (ITAM) [38].

The principal NK cell activating receptors include the Natural Cytotoxicity Receptors (NCR), DNAM-1 and NKG2D. The NCR family includes NKp30, NKp44 and NKp46 that belong to the Ig superfamily and form a complex with an ITAM-bearing accessory protein. The molecular ligands of NCRs still remain elusive despite their involvement in the lysis of diverse tumor target cells [39]. The other two important activating NK receptors that cooperate in the induction of tumor or virus-infected cell killing are the DNAX accessory molecule-1 (DNAM-1) and NKG2D (Natural Killer group

2, member D). The poliovirus receptor (PVR) and Nectin-2 have been identified as the DNAM-1 ligands [40] whereas NKG2D interacts with several molecules. These include the highly polymorphic Major Histocompatibility Complex (MHC)-I related chains A and B (MIC-A and MIC-B) and the UL-16 binding protein (ULBP-1,2,3,4,5,6) that are normally expressed on infected or transformed cells (Fig.13) [41].

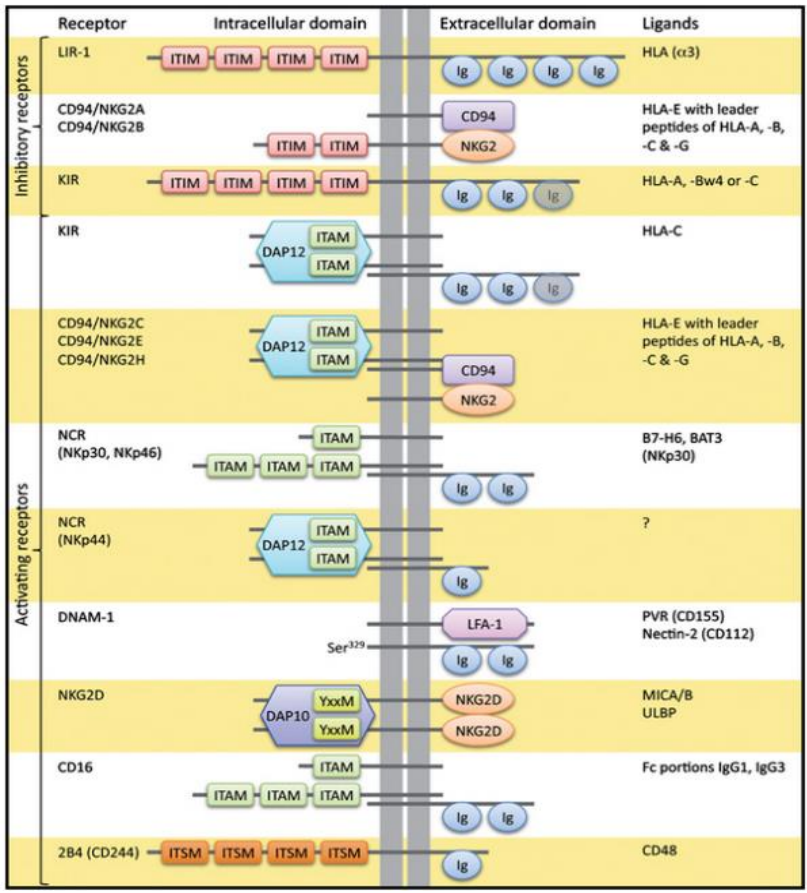

Figure 13. Major inhibitory and activating NK cell receptors and their ligands involved in AML. NK cell activation upon contact with a target cell depends on the balance between inhibitory and activating signals relayed through a variety of NK cell receptors after binding with specific ligands on the target cell. Most NK cell receptors bind their ligands through extracellular immunoglobulin-like (Ig) domains, while NKG2 hetero- or homodimers have C-type lectin-binding domains. Inhibitory receptors, such as the leukocyte immunoglobulin-like receptor-1 (LIR-1), inhibitory KIRs and heterodimers of CD94 and NKG2 isoforms A and B, have cytoplasmic tails containing multiple immune-receptor tyrosine-based inhibitory motifs (ITIMs). After ligation of the receptors these ITIMs become phosphorylated, allowing them to decrease activating cell signaling through the recruitment of phosphatase enzymes. Activating receptors include the activating forms of KIRs and CD94/NKG2 heterodimers, NCRs, DNAM-1, NKG2D homodimers, CD16 and 2B4. Many of these receptors have intracellular domains containing immunoreceptor tyrosine-based activation motifs (ITAMs), such as the transmembrane DAP12 adapter protein. As opposed to ITIMs, ITAMs facilitate activating cell signaling when phosphorylated following receptor ligation. Instead of via ITAMs, the NKG2D and 2B4 receptors promote activation through phosphorylation of the tyrosine residues of the tyrosine-x-x-methionine (YxxM) motif in the DAP10 adapter protein and in the immunoreceptor tyrosine-based switch motifs (ITSMs), respectively. For DNAM-1, signaling involves phosphorylation of Ser^{329} by protein kinase C [Adapted from 42].

Accumulating evidence indicates that elevated levels of soluble MIC-A/B [43,44,45] and ULBPs [46,47] can be found in the culture supernatants of several tumor cells as well as in the sera of patients affected by different cancers. The release of these NKG2D ligands, by

proteolytic shedding or through exosomes, promotes escape of tumors from NKG2D-mediated immunosurveillance that results from a reduction in cell surface density of the membrane-bound NKG2D ligands or a down-regulation of NKG2D receptor on the immune effector cells.

1.5 HLA class I antigen processing and presenting machinery

The HLA-ABC antigens, also called Major Histocompatibility Complex (MHC) class I, are highly polymorphic glycoproteins expressed on the cell surface of almost all nucleated cells. These classical HLA class I molecules consist of a heavy chain of about 44-45 kDa, encoded by the HLA class I genes located on the short arm of chromosome 6, and a soluble light chain (β2-microglobulin) of 12 kDa encoded by a gene located on chromosome 15. The ectodomain of the alpha heavy chain can be divided into three globular domains (α1, α2, α3). The region between the α1 and α2 domains form the antigen binding domain that accommodates antigenic peptides ranging from 8 to 11 amino acids in length

whereas the α3 domain binds the CD8 glycoprotein which is important for the recognition of antigen by cytotoxic T cells (Fig.14) [48,49].

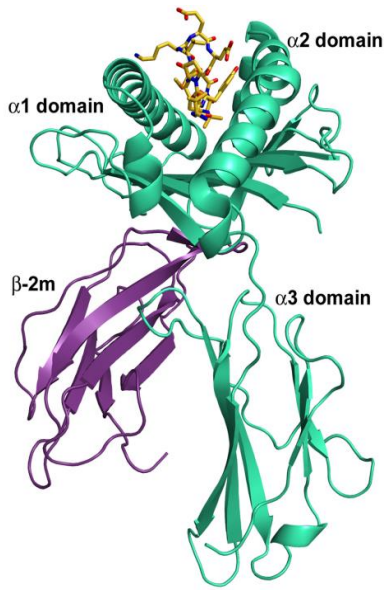

Figure 14. Overview of the structure of the HLA class I. The heavy chain is shown in cyan, β2m in purple, and the peptide in yellow [Adapted from 50].

The principal role of the classical HLA class I molecules is to bind and present intracellular antigenic peptides to T-cell receptors (TCRs) leading to killing of the infected or transformed target cells. Therefore, the high degree of HLA polymorphism increases the range of adaptive immune responses that different individuals can mount

against a broad spectrum of antigenic peptides. Moreover, several HLA class I alleles are associated with different autoimmune or inflammatory diseases probably because the peptide binding cleft, which is the more polymorphic region, could impair the peptide binding as well as the TCR recognition [51].

Loading of high affinity intracellular pathogen or self-derived peptides onto HLA class I molecules is accomplished within the endoplasmic reticulum (ER) through the so called antigen processing machinery. This is a quality control machinery that allows the expression of correctly assembled trimeric HLA-I/β2m/peptide complex on the cell surface where it displays the antigenic peptides to CD8+ CTL which, in turn, trigger a specific adaptive immune response. Not surprisingly, many transformed or virus-infected cells display several abnormalities in this pathway that allows their escape from immune surveillance [52,53,54].

The majority of antigenic epitopes contained within various intracellular proteins are generated via the constitutive multicatalytic 26S/20S proteasome complex. The peptide repertoire is however enlarged by the alternative IFN-γ-induced immunoproteasome which exhibits an altered catalytic activity on peptide

substrates. The cytosolic peptides generated by proteasome are, then, actively translocated to the ER lumen by the transporters associated with antigen processing (TAP1/TAP2) where they are further trimmed by ER aminopeptidases (ERAP-1/2). These peptidases ensure that the antigenic epitopes reach the optimal length required to fit the peptide-binding pocket of HLA class I molecules. The newly synthesized HLA class I heavy chains rapidly associate with the ER chaperone Calnexin and the thiol oxidoreductase ERp57 that facilitate their folding and interaction with the β2m. Therefore, only the correctly folded HLA-I/β2m heterodimers are released from calnexin and recruited into the Peptide-Loading Complex (PLC) which ensures that only high-affinity peptides are loaded onto HLA-class I molecules and presented to T-lymphocytes. The PLC is a quality control machinery organized around the TAP molecules by the adapter protein Tapasin (Tsn), which recruits the peptide-receptive HLA class I molecules, now associated with the chaperone Calreticulin (CRT). The complex is further stabilized by the disulfide isomerase ERp57 which forms a stable conjugate with Tsn and CRT (Fig.15).

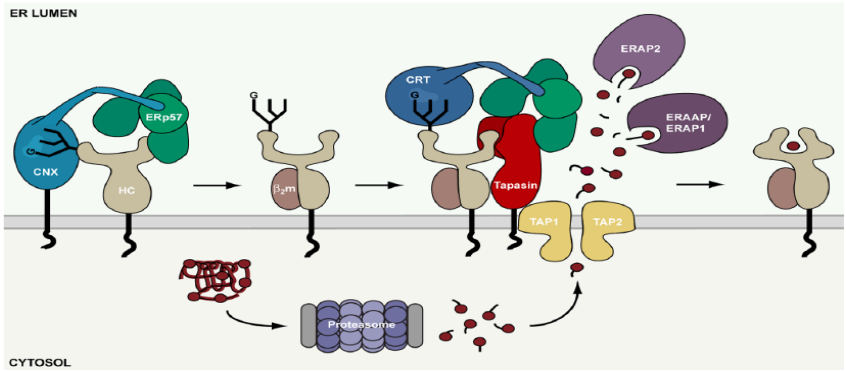

Figure 15. ER quality control and MHC class I assembly. MHC class I assembly in the ER. The folding of the MHC class I HC and formation of disulfide bonds in the α2 and α3 domains is assisted by CNX and ERp57. The HC then assembles with β2m to generate an empty heterodimer that is highly unstable for most MHC class I alleles. The HC/β2m dimer is stabilized by association with tapasin, ERp57, CRT, and TAP in the PLC, where it awaits peptide binding. TAP transports peptides generated from cytosolic proteins by the proteasome into the ER lumen where they are further trimmed by ERAAP/ERAP1 (mice and humans) and ERAP2 (humans only). Once high affinity peptides of the appropriate length are generated, they are loaded onto empty MHC class I molecule by the PLC. Peptide binding induces dissociation of MHC class I from the PLC and it subsequently traffics to the cell surface [Adapted from 55].

After the binding of a peptide, ER resident chaperones are released and the HLA class I molecules traffic via the secretory pathway to the plasma membrane (Fig.16). Here, the peptides derived from pathogen or mutated proteins are recognized by the specific TCR on CD8+ T cells triggering their cytotoxic functions, whereas those derived from self-proteins are, usually, not [55,56,57,58].

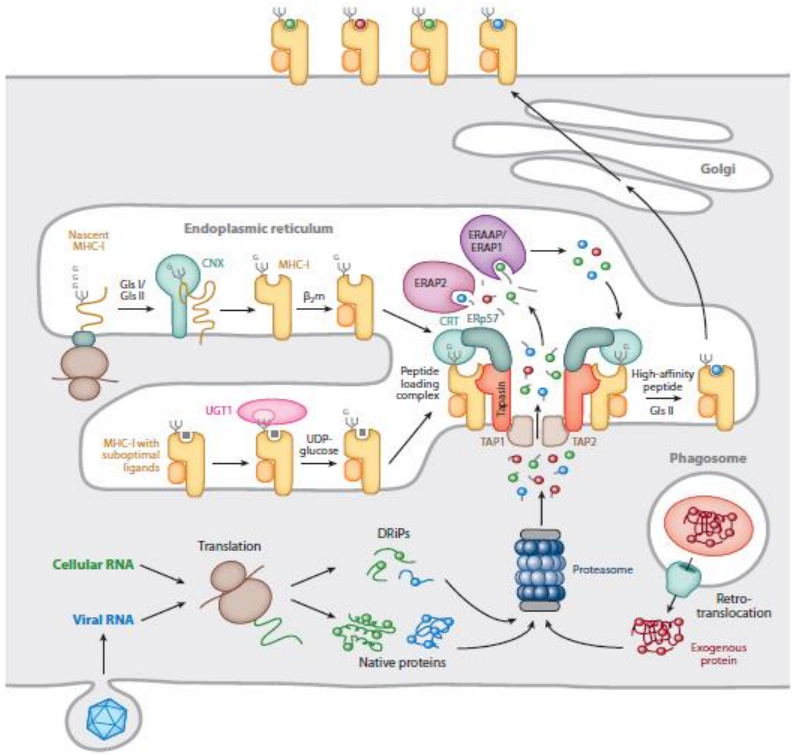

Figure 16. MHC-I biosynthesis and antigenic peptide binding in the endoplasmic reticulum (ER). Trimming of the N-linked glycan by glucosidases I and II (GlsI/GlsII) to a single terminal glucose residue (G) permits the interaction of the MHC-I heavy chain with lectin-like chaperones at several stages during folding and assembly. The initial folding events involve the chaperone calnexin (CNX) and allow subsequent assembly with β2microglobulin (β2m). The empty heterodimer, which is inherently unstable, is then recruited by calreticulin (CRT) via the monoglucosylated N-linked glycan to the peptide loading complex (PLC). The association of MHC-I/β2m heterodimers with the PLC both stabilizes the empty MHC-I molecule and maintains the binding groove in a conformation that favors high-affinity peptide loading. These functions are mediated by direct interactions between the MHC-I heavy chain and tapasin and are

supported by coordinating interactions with CRT and ERp57 in the PLC. MHC-I molecules with suboptimal peptides are substrates for UGT1, which reglucosylates the heavy chain glycan, allowing reentry of the MHC-I into the PLC and exchange for high-affinity peptides. Peptides translocated into the ER by the transporter associated with antigen processing (TAP) originate primarily from the proteasomal degradation of endogenous proteins or DRiPs. These proteins may arise from the translation of either self or foreign (i.e., viral) RNA or, in the case of cross-presentation, by translocation into the cytosol from endosomes or phagosomes. Many of the peptides that are delivered into the ER are longer than the 8–10 residues preferred by MHC-I molecules and undergo trimming by ER aminopeptidases known as ERAAP/ERAP1 and ERAP2. Finally, high-affinity peptides bind preferentially to MHC-I molecules in the PLC by a tapasin-mediated editing process; MHC-I-peptide complexes are released and then transit to the cell surface for T cell recognition by CD8+ T cells [58].

1.6 HLA-E: a non-classical HLA class I molecule

In addition to the classical class I genes (HLA-1a), the HLA class I region includes a range of "non-classical" HLA class I (Ib) molecules such as HLA-E, F and G. The exact function of most of these molecules remains poor defined; however, several recent studies suggest that they have different important immunoregulatory roles by acting as ligands for receptors of both the innate and the adaptive immune systems [59].

Among these, HLA-E has a wide tissue distribution and shares many structural features with the classical class I molecules: its heavy chain, non-covalently associated with the ß2m, is organized into three globular domains (alpha-1, 2 and 3) (Fig.17). On the opposite, HLA-E is characterized by a limited polymorphism and a relatively low cell surface expression compared to its HLA-Ia counterpart.

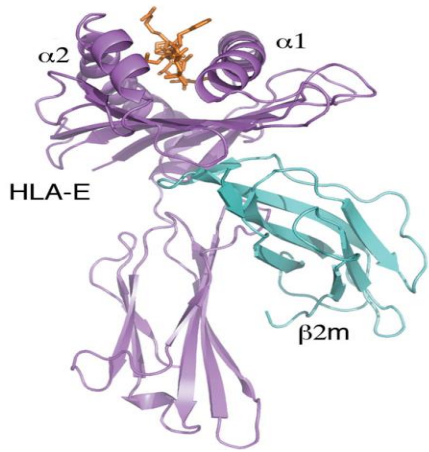

Figure 17. HLA-E structure. The heavy chain of HLA-E and ß2m are shown as violet and cyan ribbons, respectively, with the VMAPRTLFL peptide in orange sticks [Adapted from 60].

Two nonsynonymous HLA-E variants have been described, E*0101 and E*0103, that differ for an arginine ($HLA\text{-}E^{R}$) or a glycine ($HLA\text{-}E^{G}$) at position 107 of the protein, located in the α2 domain of the heavy chain. It is

also been suggested that the HLA-E^G allele has a higher binding affinity for nonamer peptides and are more stable than HLA-E^R allele resulting in a higher level of cell surface expression [61,62].

The limited polymorphism of the HLA-E peptide binding site, located between the α1 and α2 domains, may account for the restricted peptide repertoire presented by HLA-E molecules. In fact, HLA-E molecules predominantly bind a related set of nonamer peptides derived from the leader sequence of other HLA class I molecules. The signal sequence, processed by signal peptide peptidase (SPP), is released into the cytosol where it is further trimmed by proteasomes. As for the classical HLA-I molecules, their loading onto HLA-E is TAP and tapasin-dependent [63,64].

Structural analysis of existing peptide-HLA-E complexes reveals the presence of two primary anchor residue (P2/Met and P9/Leu) as well as additional subdominant anchors at position 3, 6 and 7 of the peptide binding motif accommodated into well-defined pockets in the HLA-E binding groove (Fig.18). Therefore, any variation of these molecular adaptations results in a reduction in the efficiency of peptide binding to HLA-E as well as in the affinity of the HLA-E/receptor interaction [65,66,67].

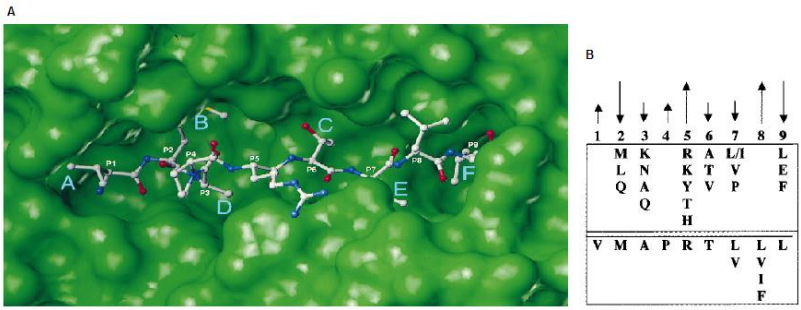

Figure 18. (A) View of the Class Ia Leader Peptide Bound in the HLA-E Groove. The heavy chain is shown in green and the pockets are labeled (A)–(E). The peptide is oriented with the N terminus at the left of the picture in the A pocket, and the Cα atoms from each residue are numbered P1–P9. **(B) HLA-E peptide-binding motif.** Numbers depict the amino acid positions of HLA-E-binding peptides. Upward and downward pointing arrows show the position of contact and anchor residues, respectively. Major and minor positions are reflected by the length of the arrow. Experimentally determined amino acids that are preferred at each anchor residue (*top box*) are shown in comparison with residues in endogenous HLA class Ia leader sequence peptides that bind HLA-E (*bottom box*) [Adapted from 65 and 66].

To date, the HLA-E-mediated immunoregulatory functions have been primary ascribed to its interaction with the CD94/NKG2x heterodimeric receptors expressed on NK and a small subset of T cells [68,69,70]. These heterodimers consist of one CD94 molecule covalently associated to inhibitory (NKG2A-B) or activating (NKG2C-E-H) NKG2 molecules. NKG2A molecules contain ITIMs in their cytoplasmic tails that lead to

inhibition of cytotoxicity, whereas NKG2C interacts with the adaptor protein DAP12, which contains ITAM, leading to activation of the immune effector cells [71,72]. However, the affinity of interaction between HLA-E/peptide complex and the heterodimer CD94/NKG2 appears higher for the inhibitory CD94/NKG2A receptor [67]. Moreover, the inhibitory signal through NKG2A prevails in NK or T cells double positive for both NKG2A and NKG2C [73,74].

Thus, because the expression and function of HLA-E is closely related to that of the other HLA class I molecules, which provide the nonamer peptides, it acts as a sensor by which innate immune NK cells monitor the integrity of the HLA class I biosynthesis and their antigen and processing pathway on healthy cells. Events such as a viral infection or cell transformation, are likely to alter the components of the HLA class I antigen processing machinery, and to lower the expression of the HLA-E molecules thus contributing to unbalance the inhibitory signals that keep the NK cells in check [75] (Fig.19). However, recent evidence exists that the previously mentioned model is not a rule. In fact, some tumor cells express HLA-E molecules on their cell surface despite the reduction in the classical HLA class I expression [76].

Moreover, virus-infected cells use a similar mechanism to escape from immune surveillance. HIV for example provides a peptide that stabilizes the cell surface expression of HLA-E thus allowing its inhibitory function, also in the absence of HLA class I permissive alleles [77]. A further case is that of the Cytomegalovirus gpUL40 protein from which a HLA-E binding peptide is produced in a TAP-independent manner that enhances the cell surface expression and the NK-inhibitory function of the HLA-E molecules [78]. Michaëlsson et al., have also described a nonamer peptide derived from the leader sequence of the Heat shock protein (HSP)-60 that competes with the class I leader sequence peptides for binding to HLA-E molecules. The HLA-E/HSP60-derived peptide complex, however, is not efficiently recognized by the inhibitory CD94/NKG2A receptor making these stressed cells more susceptible to NK [79].

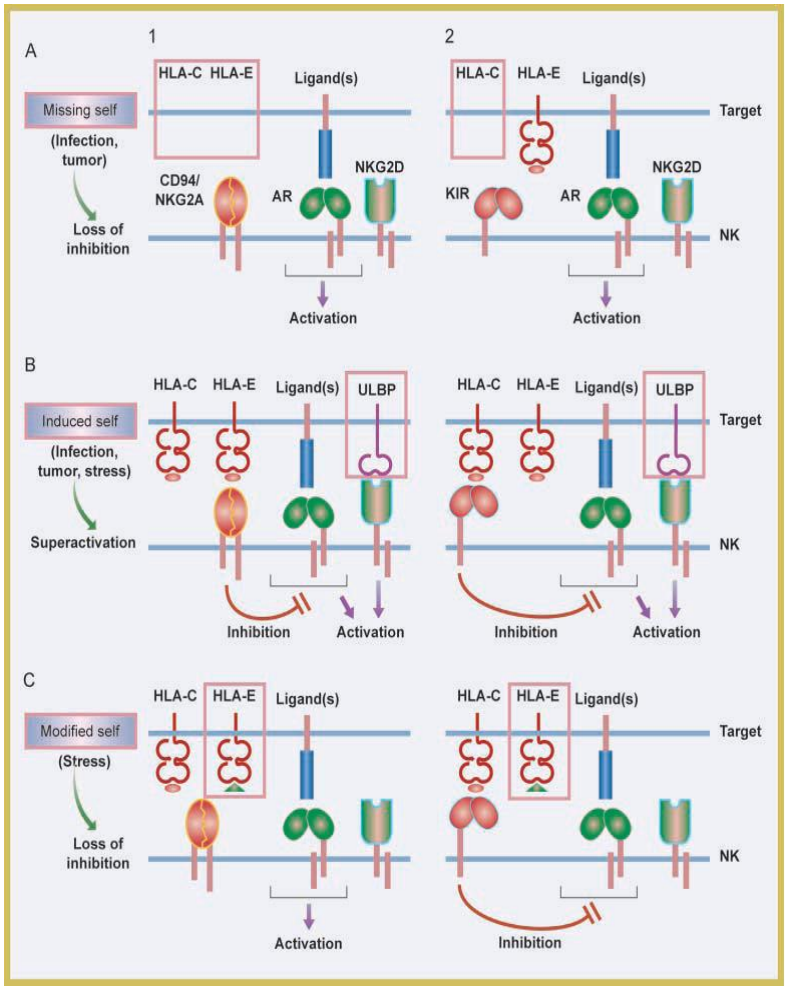

Figure 19. Outcomes of surveillance by NK cells. NK cells survey cells for changes in self, represented here by MHC class I and MHC class I–related molecules (e.g., ULBP, MICA). (1) Depicts an NK cell that is inhibited by the HLA-E-specific receptor CD94/NKG2A, and (2) depicts one that is inhibited by an HLA-C–specific KIR. The KIR family also includes inhibitory receptors with three Ig domains that bind to HLA-A and HLA-B. A representative activation receptor (AR) of the natural cytotoxicity receptor family is shown in association with an immunoreceptor tyrosine-based activation motif (ITAM)-containing subunit, such as DAP12. NK cells express multiple activation receptors on their cell surface. NKG2D is a

lectin-like receptor that is associated with the subunit DAP10. (A) "Missing self": a global down-regulation of the expression of MHC class I molecules, as seen in certain tumors or during viral infections, results in the loss of surface HLA-E. The lack of engagement of the inhibitory CD94/NKG2A leads to activation of NK cells (A1). The loss of a single class I allotype, such as HLA-C, may activate NK cells that express an HLA-C-specific inhibitory KIR (A2). (B) "Induced self": expression of ligands of NKG2D is induced on target cells due to cellular stress, transformation, or viral infections and results in an activation of NK cells despite the presence of inhibitory receptors (Superactivation). It is not known if NKG2D engagement bypasses inhibition or if it overrides inhibition by enhancing activation signals from other receptors. ULBP is a family of molecules with α1-α2 domains. NKG2D binds also to MICA and MICB, which have α1-α2-α3 domains. ULBP and MICA/B proteins are not associated with ß2-microglobulin and do not bind peptides. (C) "Modified self": stress-induced molecule hsp60 provides a signal sequence-derived peptide that binds to HLA-E and prevents recognition by inhibitory CD94/NKG2A. This results in activation of NK cells expressing CD94/NKG2A (C1) but not of NK cells expressing inhibitory KIR (C2) [75].

Despite HLA-E is well known for its ability of regulating innate immune responses, recent evidence has established a role for HLA-E also in adaptive immunity. HLA-E restricted αß CD8 T cells have been described specific for Mycobacterium tubercolosis, Salmonella enterica, Hepatitis C virus (HCV) and Cytomegalovirus (CMV) antigens allowing to speculate that HLA-E may play a role in T cell defense against viruses or intracellular bacteria [80,81,82,83]. Moreover, it has been

reported that pathogen or self-derived peptides might be presented by HLA-E to CD8 T cells with regulatory properties [84,85]. Further studies are, however, needed to investigate the immunoregulatory activities of HLA-E restricted regulatory CD8 T cells in infection and autoimmune disorders. Therefore, all these results suggest that HLA-E is a versatile molecule and its peptide-binding repertoire is much broader than initially described.

In addition, the existence of a soluble form of the HLA-E protein has also been recently identified. The release of sHLA-E molecules by melanoma cells, melanocytes and IFN-γ-activated or virus-infected endothelial cells might presumably counter or promote immune surveillance by NK or T cells, thus increasing the unresolved ambiguities of HLA-E molecules [86,87,88].

CHAPTER 2 – RESEARCH AIM

The pivotal role of macrophages in sensing and initiating an immune or inflammatory response against any insult that can alter the homeostatic balance is widely accepted. Despite macrophages rapidly exert their innate immune functions by phagocytosis, they also cooperate with both innate and adaptive immune cells leading to resolution of inflammation and restoring the tissue homeostasis. The fine interplay between hematopoietic cells and different types of tissue is mediated by ligand/receptor-mediated cell-cell interactions as well as through soluble factors. Within the immune system, the Natural Killer (NK) cells are emerging as the central effectors that provide protection against infection and cancer. The cytotoxic or regulatory functions of NK cells are tightly dependent on separate or cooperative signals derived from different receptors. Thus, the interplay existing between NK and immune/non-immune cells is strongly dependent on the expression pattern of receptors and, their ligands. The central role of macrophages and NK cells in the regulation of the human immune responses, therefore, prompt to focus on the molecular aspects of their crosstalk.

My PhD thesis project has specifically focused on the modulation of the components of the machinary leading to processing and presentation of the HLA class I antigen, with particular attention on the non-classical HLA-E molecule, during the monocytes to macrophages differentiation process. HLA-E binds to inhibitory NKG2A receptor expressed on the cell surface of a large subset of NK cells, and its expression pattern on healthy as well as damaged cells is still not fully delineated. Moreover, because almost all NK cells express the activating NKG2D receptor, the modulation of its ligands has been also investigated. The analysis was carried out on macrophages generated *in vitro* from primary human blood monocytes or human leukemic cell lines. The latters allowed us to analyze the functional outcome of the ligand quantitative variation in regulating NK cell activity as well as to compare leukemic cells with their normal counterpart in the way they modulate the expression of the NK ligands.

CHAPTER 3 – MATERIALS AND METHODS

3.1 CELL CULTURES AND STIMULATION CONDITIONS

Human peripheral blood mononuclear cells were separated on Lympholyte (Cedarlane Laboratories) by density centrifugation of heparinized blood from healthy donors. Monocytes subpopulations were purified from PBMC by positive selection with anti-CD14 mAb coupled to magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of $CD14^+$ was routinely >90% as estimated by flow cytometry using the anti-CD14 mAb (Miltenyi Biotec). Cells were seeded at concentration of 10^6 cells/ml in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 25 U/ml penicillin and 25 U/ml streptomycin (all purchased from Gibco, Invitrogen, Carlsbad, CA, USA) in cell culture plates and treated for 9 h with LPS (0.05 μg/ml; Sigma-Aldrich), PMA (80 nM; Sigma-Aldrich), or differentiated for 7 days with recombinant human GM-CSF (100 ng/ml; Miltenyi Biotec) or M-CSF (100 ng/ml; Miltenyi Biotec).

The U937 and THP1 cell lines were obtained from ATCC, cultured in RPMI 1640 supplemented with 10% FBS. U937 and THP1 cells ($5x10^5$/ml) were left untreated or treated for the indicated time with GM-CSF (25 ng/ml), 1,25-dihydroxyvitamin D3 (10 nM; Sigma-Aldrich), a combination of GM-CSF+Vitamin D3, PMA (80 nM for U937 and 100 nM for THP1) or IFN-γ (250 U/ml; BD Pharmingen).

Natural Killer cell populations were obtained by a 10-day co-culture of PBMC with irradiated (30 Gy [3000 rad]) Epstein-Barr virus–transformed B-cell line RPMI 8866 (4:1 ratio), in RPMI 1640 medium supplemented with 10% FBS and 2 mM glutamine, as previously described [89]. Cultures were used at day 10, and were routinely more than 80% NK ($CD56^+CD16^+CD3^-$), as assessed by immunostaining and flow cytometry analysis.

Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

3.2 RNA isolation and quantification by Real Time (RT) – PCR analysis

Total RNA was isolated from monocytes, U937 and THP1 cell lines using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality was monitored by running the aliquots of each sample in 1% agarose gel and by spectrophotometric analysis. HLA-E transcripts were evaluated using real-time PCR (HS00428366, Applied Biosystems). One microgram of total RNA from each sample was used to perform reverse transcription using the High-Capacity cDNA Reverse Trascription kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed in ABI PRISM 7300 Sequence Detection Systems (Applied Biosystems) using TaqMan®2X Universal Master Mix and the following TaqMan® Gene Expression Assays: HLA-E, Hs00428366; HLA-B, Hs00741005; HLA-A, Hs01058806 (Applied Biosystems). Each sample was assayed in triplicates. The thermal cycling conditions were set up sequentially as follows: denaturating at 95°C for 10 minutes and 60 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fold change of the HLA genes in the samples was calculated using the $2^{-\Delta\Delta CT}$ method. All values were

normalized to endogenous control GAPDH (Hs99999905, Applied Biosystems) and were expressed in arbitrary units.

3.3 Western blot analysis

Total protein extracts were obtained by lysing cells for 30 min at 4°C in lysis buffer (20 Mm Tris-HCl pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA) in the presence of protease and phosphatase inhibitors (Sigma-Aldrich). Culture supernatants were collected at the indicated times after activation and concentrated (10X) using Amicon Ultra Centrifugal Filters (Millipore, Bedford, MA). Proteins were resolved by SDS-PAGE and immunoblotting was performed on nitrocellulose membranes (GE Healthcare, Piscataway, Nj, USA) using the following primary antibodies: anti–HLA-E (MEM-E/02, kindly provided by Prof. Vaclav Horejsi, Prague, Czech Republic), anti–HLA-B/C (HC-10) and anti-HLA-A (HCA2) (purified from HC10 and HCA2 hybridoma supernatants), anti-Hsp60 (StressMarq Bioscience Inc, BC, Canada), anti-β2m and anti-p21 (Santa Cruz Biotecnology, Santa Cruz, CA), anti-ERp57 (kindly provided by Dr. Doriana Fruci, Rome, Italy). Anti-mouse

or anti-rabbit IgG, HRP-linked Abs (GE Healthcare) were used as secondary Abs in chemiluminescent Western blot assays using the Pierce ECL system (Thermo Scientific). Protein levels were normalized for GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) protein levels using Image J version 1.45 software (developed at the National Institutes of Health).

3.4 Flow cytometry

Cells were harvested, washed twice with PBS containing 1% BSA and 0,1% NaN_3 and then incubated on ice for 30 minutes with a saturating concentration of the primary antibody. After 3 washes, cells were incubated with an FITC-labeled goat anti–mouse IgG antibody (Jackson ImmunoResearch Laboratories) at 4°C for 30 minutes. Finally, cells were suspended in 1% paraformaldehyde in PBS. Negative controls were performed by incubation of the cells with isotype-matched control antibodies. The mouse mAbs used for this study were anti-CD11b and anti–CD14 (Miltenyi Biotec), anti-HLA-E (3D12; BioLegend), anti-MICA and anti-MICB, anti-ULBP1, 2, 3 (all from R&D Systems). Fluorescence was measured

on 10 000 cells/sample using a FACScalibur and analyzed using CellQuest software (BD Biosciences).

3.5 Cytotoxicity assays

HeLa or U937 target cells (untreated or differentiated as described above) were labelled with ^{51}Cr (PerkinElmer, MA, USA) (100 uCi/1x10^6 cells) for 1h and 15' at 37°C. When indicated, cells were pre-incubated overnight with 60 μg/ml of the HLA-B7 [VMAPRTVLL] or Hsp60 [QMRPVSRVL] signal peptides before labeling. HeLa cells were pre-incubated 20' at 37°C with supernatants from either differentiated or untreated U937 cultures. Serial dilutions of in vitro expanded NK effector cells were plated in U-bottom 96-well plates in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine and 10 mM Hepes and incubated with saturating concentration of anti-NKG2A (Z199 clone, kindly provided by Prof. Miguel Lopez-Botet), anti-CD56 mAb (C218 clone, kindly provided by Prof. Bice Perussia), or medium, for 30' at 4°C, before adding target cells. After a 4 hr incubation at 37°C, 30 μl of supernatants were transferred to 96-well Lumaplate (PerkinElmer) and released radioactivity was counted with a β-counter

instrument (TopCount, PerkinElmer, Waltham, MA). Percent specific lysis was calculated according to the formula: % specific lysis = (experimental release cpm - spontaneous release cpm)/(maximum release cpm - spontaneous release cpm) x 100. Spontaneous release never exceeded 5% of total release.

3.6 Statistical analysis

Results are shown as the mean and standard error of the mean from at least three independent repeated experiments. The level of significance (P values) between treated and untreated groups was analyzed using the Student's t-test, and $P < 0.05$ was considered significant.

CHAPTER 4 – RESULTS

4.1 Peripheral blood CD14+ monocytes up-regulate HLA-E molecules following LPS or PMA activation

Perypheral blood monocytes from nine individuals were treated with LPS (0.05 μg/ml), PMA (80 nM) or medium alone (MED) for 9h and the expression of HLA-E was evaluated using the MEM-E/02 antibody. The expression of HLA-E molecules is induced by LPS and, even more, by PMA (Fig.20 A-B). On the opposite mRNA measured by qRT-PCR was highly induced by LPS but significantly down-regulated by PMA treatment for 9h (Fig.20 C). To verify whether the kinetics of PMA induction was anticipated compared to LPS, mRNA level was evaluated at 1 and 3 hours after treatment. The level of HLA-E mRNA was indeed increased following 1h treatment and already decreased after 3h whereas the adherence on the plastic plate acted as partial stimulus since the HLA-E mRNA expression in the control (medium alone) increased during time (Fig.20 D). Therefore, the stimulus induced by PMA under the conditions used here seemed to be more effective than LPS in inducing the up-

regulation of the HLA-E molecules. This prompted us to differentiate monocytes *in vitro* to verify whether the expression of HLA-E could be a marker of differentiation.

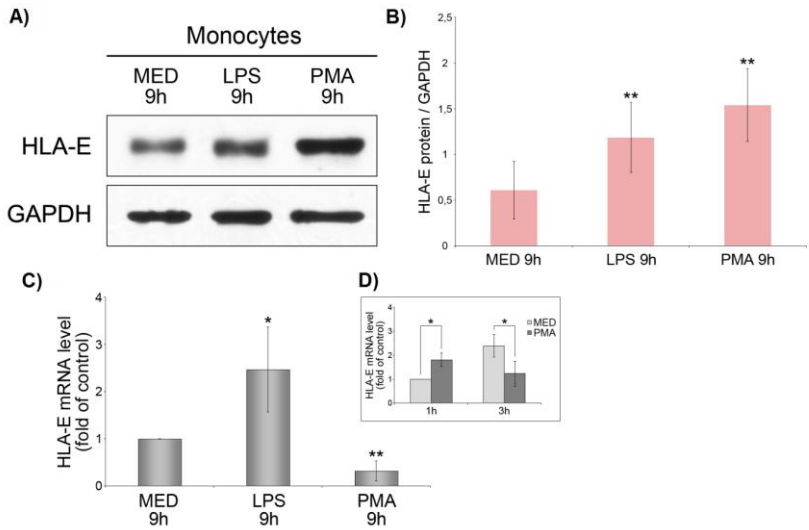

Figure 20. (A) Western blot analysis showing HLA-E in cell lysates from monocytes plated and treated for the indicated time with culture medium alone (MED), 80 nM PMA or 0.05 µg/ml LPS. Cell lysates were immunoblotted using MEM-E/02 (as anti-HLA-E mAb). GAPDH immunoblot was used as loading control. **(B)** Densitometric analysis of western blot. Immunoreactive bands were normalized to GAPDH. Histograms represent HLA-E protein levels as mean ± SD in monocytes from nine healthy donors. **(C)** qRT-PCR analysis of HLA-E mRNA in the same sample as above. Untreated control (MED) level was set to 1. **(D)** qRT-PCR showing the HLA-E mRNA level in monocytes from four healthy individuals activated by PMA or cultured with medium alone for 1 and 3 hours. Data were normalized to GAPDH and expressed as relative to control (MED 1h) arbitrarily set at 1. Statistical significance: *p<0.05 and **p<0.01 compared to untreated control (MED).

4.2 HLA-E and HLA class I antigen processing machinery components are strongly modulated during the monocyte to macrophage differentiation process

To analyze in more details the dynamics of the HLA-E up-regulation and its subsequent functions in the monocyte-differentiated cells, the human monocytic cell lines U937 and THP1 have been differentiated to monocytes or macrophages. In the former case, the hormonally active form of Vitamin D3 (1,25-dihydroxyvitamin D3) was used in combination with GM-CSF for 96h. The differentiation was monitored through the up-regulation of the monocyte-associated cell surface antigens, CD14 and CD11b, which was high in both cell lines after 96h treatment (Fig.21 A). Interestingly, while the U937 cell line, consistently with the results reported by others [90,91,92], requires both agents to reach the maximal expression of CD14 and CD11b on the cell surface (Fig.21 A), THP1 does not require the presence of GM-CSF. However, we decided to use the same protocol for both and all the analyses in U937 and THP1 cells

were carried out using 10 nM VitD3 and 25 ng/ml GM-CSF for 96h if not otherwise specified. By contrast, the differentiation into a macrophage-like phenotype was induced by PMA (80 nM) treatment for 96h, the time at which the expression of the CD11b macrophage marker was maximum and all cells still alive (Fig.21 A). As expected for macrophages, the expression of CD14 was low.

Next, we compared the expression of HLA-E proteins and a number of molecules either involved in HLA class I antigen processing and presentation or stress-induced. U937 and THP1 cells were left untreated or exposed for 96h to a combination of GM-CSF plus VitD3 or PMA alone and a western blotting analysis was performed on total-cell lysates (Fig.21 B and C). There was no substantial effect of GM-CSF+VitD3 on HLA-E expression as well as on the other molecules. By contrast, the level of HLA-E as well as ERp57, involved in antigen presentation, increased after PMA treatment for 96h. Moreover, there was not a significant up-regulation of the HLA-B/C class I and Hsp60 molecules that are known to act as peptide donors for the HLA-E proteins, in PMA-treated cells. PMA also caused a major increase in p21

expression, that is consider to play a critical role in cell cycle arrest and differentiation of PMA-treated monocytic cell lines, suggesting that the HLA-E up-regulation is a consequence of the differentiation program. This prompted us to differentiate human blood monocytes *in vitro* to verify whether the results were reproducible in a more physiological setting. Monocytes from four different donors were treated for 7 days with GM-CSF and the expression of HLA-E and the other molecules, as above, was evaluated in comparison with the corresponding *ex vivo* isolated monocytes. Immunoblotting confirmed a strong increase of the HLA-E protein in GM-CSF monocyte-derived macrophages (MDM) and similar, but less marked changes, were noted in the other molecules belonging to the HLA class I antigen processing and presentation machinery or stress-induced (Fig.21 B and C).

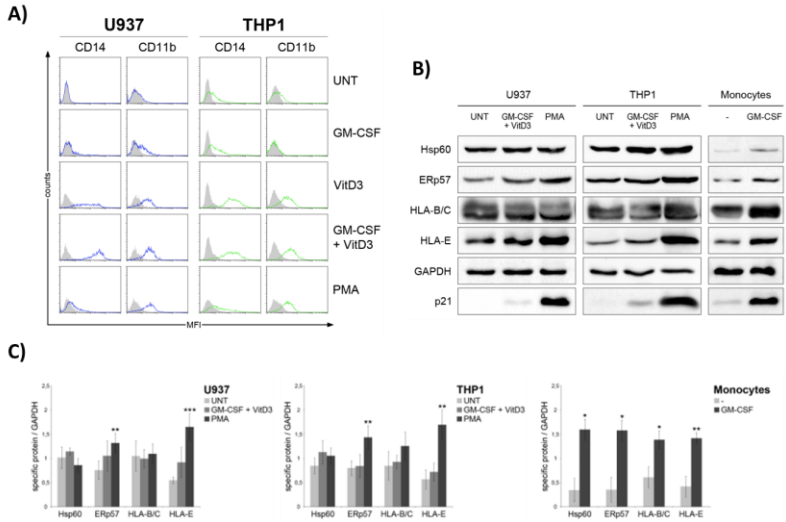

Figure 21. (A) Analysis of differentiation markers. U937 and THP1 cell lines were cultured for 96h with medium alone (MED), 25 ng/ml GM-CSF, 10 nM 1,25-dihydroxyvitamin D3 (VitD3), a combination of GM-CSF and VitD3, or 80 nM PMA. Surface expression of CD14 and CD11b was quantified by flow cytometry using APC- conjugated CD14 or CD11b mAbs or an isotype-matched control antibody (histograms in gray). **(B)** Expression of HLA-E and HLA class I antigen processing machinery components during the monocyte differentiation. Western blot analysis showing Hsp60, ERp57, HLA-B/C, HLA-E and p21 in cell lysates from U937 and THP1 cells treated for 96h with medium alone, a combination of GM-CSF and VitD3, or PMA. The same proteins were analyzed in cell lysates from freshly isolated peripheral blood monocytes or monocyte-derived macrophages differentiated by 50 ng/ml GM-CSF for 7 days. Immunoblots were reprobed with anti-GAPDH mAb to compare protein loading among different samples. **(C)** Densitometric analysis of the blots. Immunoreactive band were normalized to GAPDH. The bars represent mean values (±SD) of the analysis from four independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistically significant differences as compared to untreated control.

In order to elucidate the mechanisms producing the increase in the total HLA-E protein levels in U937 undergoing PMA-induced differentiation, which seems not paralleled by the classical HLA class I molecules, a time course of effect of PMA on HLA-E and HLA-A, B, C expression was performed. A significant increase in the HLA-E protein level was already observed 24h after PMA treatment. In parallel, as positive reference, the up-regulation of HLA-E was induced by activation of the cells with 250 U/ml IFN-γ for 48h (Fig.22 A and B). Treatment with PMA also produced the up-regulation of β2-microglobulin but with a slower kinetics (Fig.22 A and B). Interestingly, the amount of other HLA class I molecules (HLA-A, B, C) as detected by HC10 (specific for heavy chains of HLA-B, C alleles) and HCA2 (specific for HLA-A) antibodies was no significantly modified. As expected, IFN-γ activation considerably increased the levels of classical HLA class I protein (Fig.22 A and B).

A quantitative RT-PCR showed how mRNA concentration mirrored the results obtained at protein levels with a significant increase of the HLA-E specific transcript but not of that specific for classical HLA class I (Fig.22 C). Conversely, the IFN-γ induces a strong up-regulation of both HLA-E and HLA-A, -B molecules

(Fig.22 C). This observation suggests that gene transcription can be in part responsible for the increase of the HLA-E molecules in PMA-treated U937 cells.

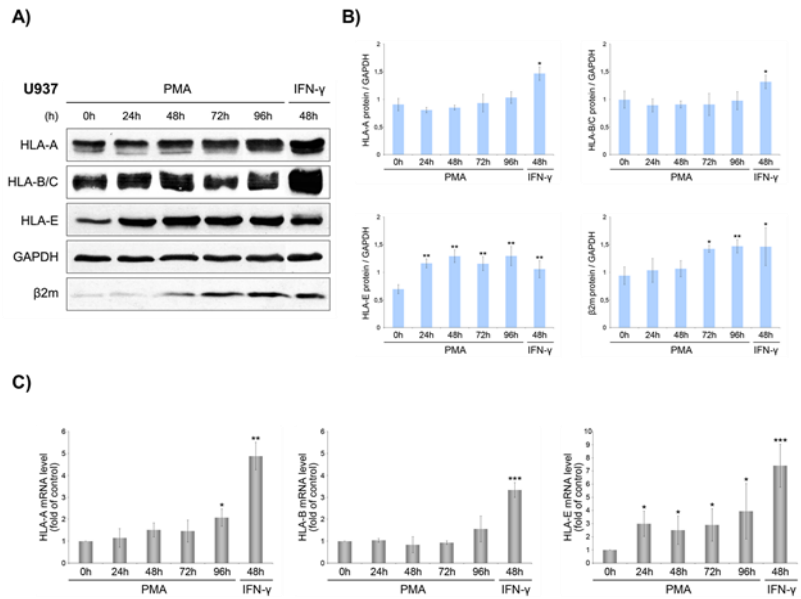

Figure 22. (A) Western blot analysis showing HLA-A, HLA-B/C, HLA-E and β2-microglobulin in cell lysates from U937 treated for the indicated time with 80 nM PMA or for 48 hours with 250 U/ml IFN-γ. Immunoblots were re-probed with anti-GAPDH mAb to compare protein loading among samples. A representative immunoblot of 4 indipendent experiments is shown. **(B)** Densitometric analysis of the blots. Immunoreactive band were normalized to GAPDH. The bars represent mean values (±SD) of the analysis from four independent experiments. **(C)** HLA-E, HLA-A and HLA-B gene transcription in PMA differentiated U937 cells. Cells were differentiated for the indicated times with 80 nM PMA; stimulation with IFN-γ has been taken as positive reference. Total RNA was purified from the respective cell pellets and analyzed by qRT-PCR for the expression of HLA-E, HLA-A and HLA-B. Results were normalized to GAPDH expression levels. Untreated

cells were arbitrarily set at 1. Values are mean ± SD of four experiments. *p < 0.05, **p < 0.01 and ***p < 0,001 represent a significant difference as compared to untreated control.

4.3 The NKG2A and NKG2D ligand expression is differently modulated on the cell surface of human monocytic cell lines or primary monocytes undergoing differentiation.

It was interesting to know whether PMA or GM-CSF-induced up-modulation of the HLA-E was paralleled by an increased expression of such molecules on the cell surface. HLA-E expression was investigated by flow cytometry with the mAb 3D12, which is considered to be specific for HLA-E, on the cell surface of either human monocytic cell lines or primary human monocyte/macrophage cells. The expression of the ligands of one of the major NK activating receptor (NKG2D) was also investigated.

Figure 23 shows that U937 and THP1 cells share a basal expression of HLA-E on the cell surface. Unexpectedly, neither the combination of GM-CSF plus VitD3 nor PMA alone further increased HLA-E cell surface expression,

which is not consistent with the immunocytochemistry analysis on the total protein extract, performed before. Moreover, both the monocytic leukemia cell lines expressed the UL16-binding proteins (ULBP-1, 2, 3), but not the MHC class I-related molecules (MICs) that are known to bind the NK-activating receptor NKG2D. Interestingly, the ULBPs were strongly down-regulated when the cells were differentiated by PMA.

All the analyses were then repeated on freshly human blood monocytes as well as on GM-CSF monocyte-derived macrophages, where the expression pattern of NKG2A and NKG2D ligands did not reproduce that obtained on the cell lines. In fact, primary monocytes expressed only a small amount of HLA-E on the cell surface and, consistently with immunocytochemistry, FACS analysis confirmed that GM-CSF-induced differentiation produced a significant increase in membrane-bound HLA-E compared with constitutive levels (Fig.23). In addition, human blood monocytes did not express the ligands for the NKG2D receptor although the ULBP-1, 2, 3 molecules were strongly up-regulated by GM-CSF (Fig.23).

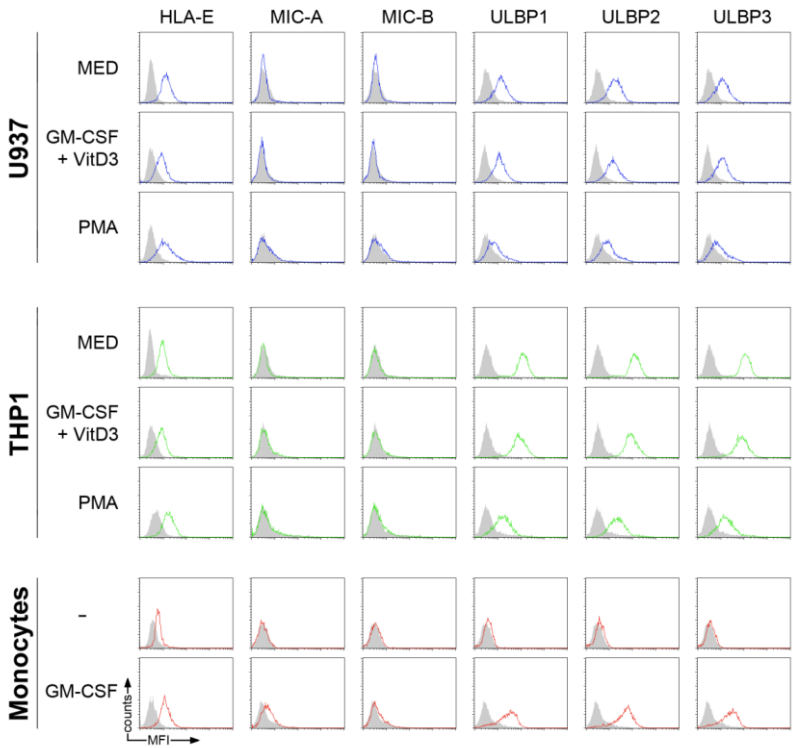

Figure 23. Cell surface expression of NKG2A and NKG2D ligands. U937 and THP-1 cells were cultured for 96h with medium alone, a combination of GM-CSF and VitD3, or PMA. Human peripheral blood monocytes were differentiated by GM-CSF for 7 days. Membrane-bound NKG2A and NKG2D ligands were analysed by flow cytometry following cell immunostaining with specific mAbs or isotype-matched control antibodies. One representative experiment out of 3 performed is shown here.

4.4 Modulation of the sensitivity to NK-mediated lysis in "untreated" or "differentiated" U937 cells

To analyze whether NK cells could kill the human monocytic cell line U937 depending on its differentiation state, CD94/NKG2A expressing polyclonal NK cells were used as effectors in cytolytic assays against U937 cells untreated or differentiated for 96h by GM-CSF+VitD3 or PMA. As shown in Figure 24A, untreated and GM-CSF+VitD3-treated cells displayed similar susceptibility to NK-mediated lysis. By contrast, upon PMA-induced differentiation, their susceptibility to NK lysis was significantly decreased. These results suggested a direct correlation between the susceptibility to NK lysis and the expression level of ULBP molecules on the cell surface of U937 cells, previously described. Moreover, their susceptibility to lysis inversely correlated with the level of expression of total HLA-E molecules, although the FACS analysis did not show any modulation in the HLA-E cell surface expression after PMA treatment.

Therefore, to evaluate the contribution of HLA-E molecules in inhibiting the NK cytotoxic activity, cytolytic assays were performed in the presence of mAbs specific for the inhibitory NK cell receptor CD94/NKG2A

or anti-CD56 mAb as control. As shown in Figure 24B, it is evident how the PMA-differentiated U937 cells were much less prone to lysis by NK cells although the anti-NKG2A antibody was effective in restoring the lysis in these cells as well as in their untreated counterparts.

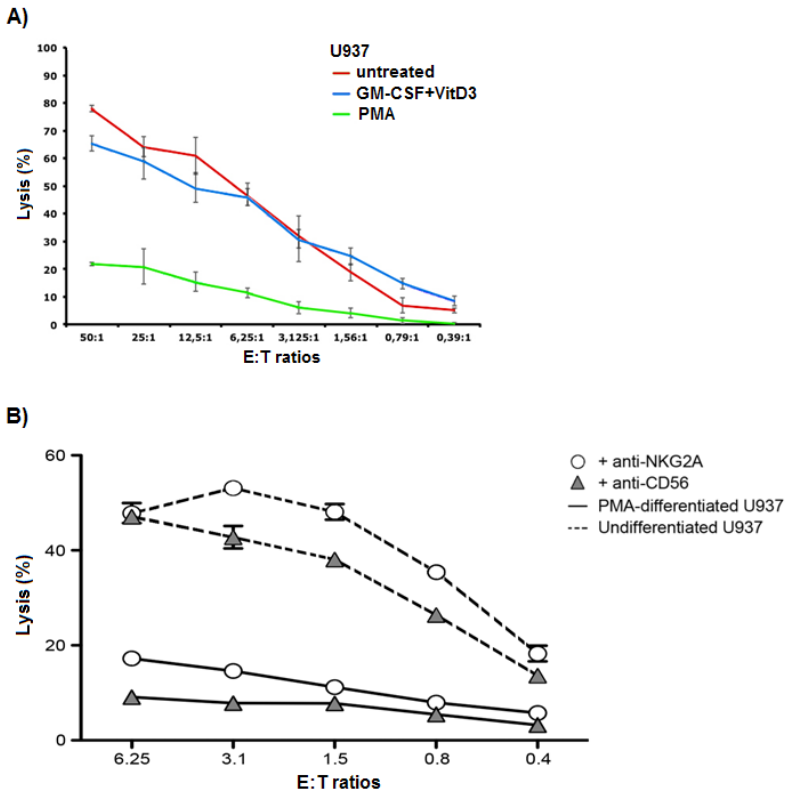

Figure 24. (A) NK cytotoxic activity against U937 cells untreated or stimulated for 96h with GM-CSF+VitD3 or PMA. **(B)** Cytotoxic activity against undifferentiated and PMA-differentiated U937 cell line, in the presence of anti-NKG2A or anti-CD56 mAb.

To address this point further, PMA-treated and control U937 cells were pulsed O.N. with HLA-E specific peptides before to be tested. Two peptides were used: the natural HLA-E ligand, HLA-B7 signal peptide, or that derived from Hsp60 that is also a good binder for HLA-E but not for the CD94/NKG2A receptor. Interestingly, the presence of HLA-B7 peptide (Fig.25 A), compared to control Hsp60 signal peptide (Fig.25 B), induced a higher protection from NK lysis of both untreated and PMA-treated cells. Again, blocking NKG2A on effector cells significantly restored cell lysis although there is again no difference between the differentiated and not differentiated target cells (Fig.25 A-B). These results confirmed previous findings that undifferentiated and PMA-differentiated U937 cells express a same amount of cell surface functional HLA-E molecules that are, however, prone to binding HLA-E-restricted peptides to ensure their optimal function.

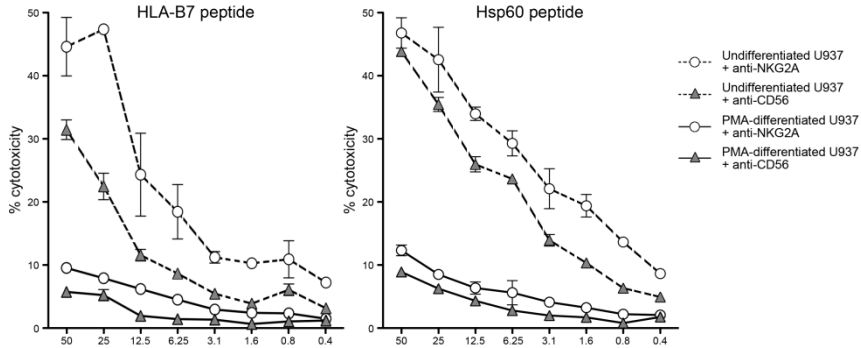

Figure 25. Cytotoxic activity against undifferentiated and PMA-differentiated U937 cell line, in the presence of anti-NKG2A or anti-CD56 mAbs. U937 cells were pulsed with HLA-B7 **(A)** or Hsp60 **(B)** peptides before to be used as target.

4.5 Macrophages release a soluble form of HLA-E molecules which does not appear to influence target cell susceptibility to NK cells

The above data indicated that the PMA induced a strong increase of the HLA-E transcript and intracellular protein levels that were not translated into a higher expression of cell surface HLA-E molecules on both U937 and THP1 human monocytic cell lines. Therefore, we asked whether part of the over-expressed HLA-E molecules might be released a soluble molecules in the

culture medium. To this aim, a Western blot analysis was performed, using the specific anti-HLA-E mAb MEM-E/02, on the supernatants of U937 and THP1 cells cultured for 96h with medium alone, a combination of GM-CSF+VitD3 or PMA. Cell lysate from U937 cells was also loaded onto a 12% polyacrylamide gel as a further control. As shown in Figure 26A, a band of lower molecular mass than the full-length protein was detected in the culture supernatants of PMA-differentiated cells, compatible with a proteinase-dependent shedding form.
Interestingly, a similar band could be detected in culture supernatants of primary human blood monocytes differentiated for 7 days by GM-CSF or M-CSF (Fig.26 B) suggesting that the release of soluble HLA-E molecules could be an event normally associated to monocyte-macrophage differentiation.

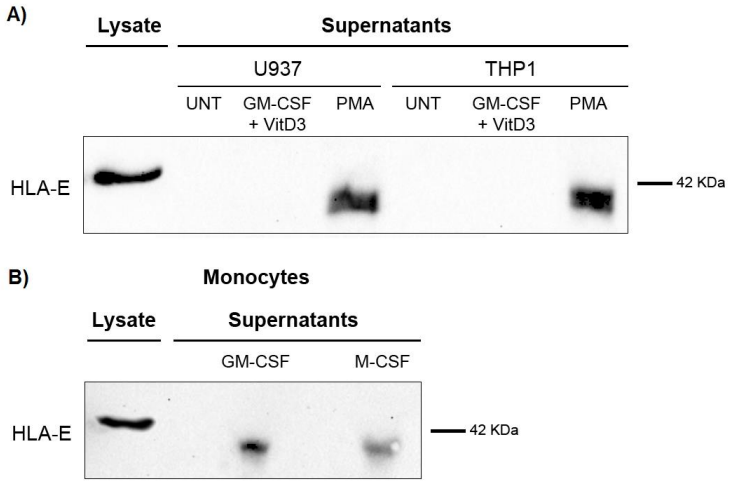

Figure 26. Production of sHLA-E by macrophages. **(A)** Cell culture supernatants were collected from U937 and THP1 cells that were cultured for 96h with a combination of GM-CSF+VitD3, PMA alone, or left untreated (UNT). sHLA-E was then detected by Western blotting in concentrated (10x) supernatants (10 µL/sample) with the mAb MEM-E/02. A cell lysate from U937 cells was loaded as a control. **(B)** Culture supernatants of the GM-CSF or M-CSF monocyte-derived macrophages were collected and analyzed, as previously described. A total cell lysate from GM-CSF-treated monocytes was loaded as a control. One representative experiment out of three performed is shown here.

Next, a cytotoxicity assay was performed to evaluate a possible protective effect of sHLA-E molecules against CD94/NKG2A-dependent NK cell lysis. The cytotoxic activity of NK cells toward HeLa cells, which do not express HLA-E molecules on the cell surface, was

measured in conventional medium or in the presence of cell-conditioned medium containing sHLA-E derived from PMA-differentiated U937 cell culture supernatant. As shown in Figure 27A, HeLa cells were efficiently killed by NK cells with no effect of the anti-NKG2A-blocking mAb, which is consistent with the absence of functional HLA-E molecules at the cell surface. This experiment also indicated that cell lysis decreased when sHLA-E was present in the culture medium, although this protection was not apparently provided by sHLA-E molecules themselves since blocking NKG2A with a specific mAb did not restore NK-mediated HeLa lysis (Fig.27 B). This result suggested that the culture supernatant of PMA-differentiated U937 cells contained one or more factors, different from sHLA-E, able to affect the NK-cell cytolytic potential.

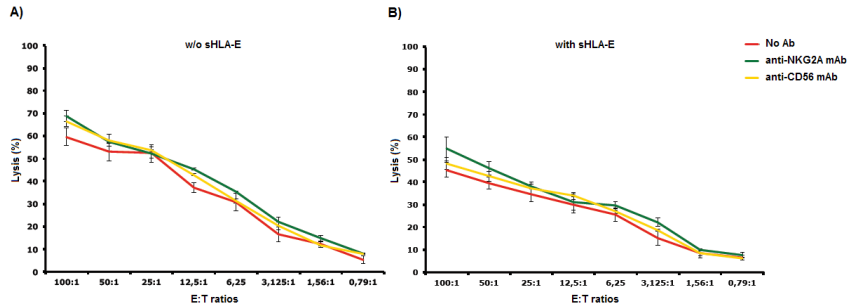

Figure 27. Effect of sHLA-E on NK cytotoxicity against HeLa cells that do not express cell surface HLA-E molecules. HeLa cells were labeled with ^{51}Cr and preincubated with **(A)** normal culture medium or **(B)** conditioned medium, containing sHLA-E, from PMA-differentiated U937 cells for 20 minutes a RT before incubation with NK cells for 4 hours at 37°C. Blocking experiments were performed after preincubation of NK cells with medium alone, anti-NKG2A or anti-CD56 mAbs.

CHAPTER 5 – DISCUSSION

HLA-E molecules are becoming more and more interesting in the immune network because of their function as ligand for canonical NK inhibitory receptor CD94/NKG2A but also, as more recently demonstrated, because they can engage the TCR on CTL [80-83] or favoring the expansion of regulatory T cells [84,85]. Despite its little polymorphism, the HLA-E gene has been found to be associated with several diseases [93-103] suggesting an active role in the pathogenic mechanisms. Nevertheless, little is still known about the regulation, the function and the peptidome of the HLA-E molecules. Here we add a little tile on the puzzle showing how these molecules are up-regulated along the monocyte/macrophage differentiation process. Interestingly, their expression profile as well as the NKG2D ligand expression pattern on primary cells do not overlap with the myeloid leukemia cell lines undergoing differentiation used here suggesting that their regulation is of pivotal importance for tumor immune surveillance.

The present findings show that human blood monocytes significantly up-regulate HLA-E molecules as well as other HLA class I antigen processing and presenting machinery components during their *in vitro*

differentiation to macrophages by GM-CSF, which is known to induce an M1-like polarized phenotype. Most importantly, the increase of intracellular HLA-E levels is translates into a higher cell surface expression, highlighting the potential role of HLA-E in macrophage immunobiology as also suggested by others for M1-polarized macrophages [104]. Furthermore, we found that GM-CSF induced up-regulation of ULBP-1, 2, 3 molecules, the ligands for NKG2D, an NK activating receptor. These results lead us to speculate about the existence also in humans of a complex crosstalk, recently described in mice, by which macrophages activate NK cells that can recognize tumor or infected cells meanwhile protecting themselves by up-regulation of HLA-E molecules [105].

However, such a scenario does not occur in both the human monocytic cell lines (U937 and THP1) used here as a model. Both these myeloid leukemia cell lines express HLA-E on the cell surface. Interestingly however, when treated with PMA that induce their differentiation to a macrophage-like cell type, the high increase of the intracellular pool of the HLA-E molecules observed is not paralleled by a substantial increase on the cell membrane. The retention of these proteins within tumor

cells is a frequently reported event and there might be many possible explanations for this. The more likely hypothesis is that the peptide repertoire changes dramatically in tumor cells and a limiting amount of optimal HLA-E peptides is available to binding. This seems however unlikely in our case since classical HLA class I, the major productive source of peptides for HLA-E molecules, as well as the β2m and ERp57 were strongly increased by treatment with PMA. So, as previously reported, tumor cells seem to activate some mechanisms that do not allow the over-expression of the HLA-E molecules on the cell membrane despite the large intracellular pool and the efficiency of the HLA class I antigen processing and presenting machinery [106].

Moreover, it has been demonstrated that HLA-E molecules are present on the cell surface in different conformations [107], some of which probably devoid of classical peptides. These findings suggest that we are only at the beginning of our understanding of the multifaceted role of these molecules which appear to be at the crossroads between protection by NK killing and activation of specific functions in T lymphocytes. It was therefore interesting to verify whether the newly synthesized molecules, maybe undetectable on the cell

surface because of their non-canonical conformation, were functional, at least in terms of protection from NK. Here we show how the PMA-differentiated U937 cells are lysed less efficiently than the undifferentiated counterpart, and this is probably due to the strong down-modulation of the ligands (ULBPs) for the activating receptor NKG2D. However, blocking the NKG2A receptor was restoring lysis in both PMA-treated and undifferentiated cells at about the same efficiency, suggesting that the interaction HLA-E – CD94/NKG2A does occur but it can probably be ascribed to a subset of the HLA-E molecules already present in the undifferentiated cells. Moreover, the use of canonical binders for HLA-E, i.e. HLA-B7 peptides, makes both targets more resistant to NK lysis indicating that the cell surface HLA-E molecules in these myeloid leukemia cell lines were prone to bind exogenously provided peptides. It remains to understand what is the fate of the increased intracellular pool of the HLA-E molecules induced by PMA-treatment.

One possibility is that, at least part of these molecules, transits through the cell membrane. This seems indeed to be the case since we show here for the first time that these cells produce a soluble form of the HLA-E protein

with a molecular mass of about 37-41 kDa. Interestingly, the same phenomenon is observed in the culture supernatants of GM-CSF monocyte-derived macrophages suggesting that the release of sHLA-E is a cellular event due to the differentiation to macrophages both for normal monocytes as well as for PMA-treated leukaemic cells. The shedding of cell surface HLA-E was also reported for melanocytes, melanoma or endothelial cells and attributed to a matrix metalloproteinase activity [86-88], suggesting that these proteases might also be responsible for the release of sHLA-E by macrophages. The generation of soluble β2m-free classical HLA class I molecules by metalloproteinase has also been described in leukemia cells [108]. Moreover, it has been suggested that the soluble β2m-free HC with bound peptide can re-associate with exogenous β2m producing a stable and probably functional complex [109].

Our data suggest that the macrophage-released sHLA-E forms do not provide protection to HLA-E negative target cells against CD94/NKG2A-dependent NK cell cytotoxicity. Therefore, the meaning of these sHLA-E molecules remains to be elucidated and will be probably dependent on their binding with the β2m and/or on the nature of the bound peptides. A further possibility is that

the number of HLA-molecules on the cell surface of macrophages is strictly regulated and an excess of these molecules could be detrimental for the fine-tuning of the crosstalk between macrophages and other cells of the immune systems such as, for example, regulatory T cells.

In conclusion, we have shown in this study how HLA-E molecules can be up-regulated during the monocyte to macrophage differentiation process and that, at least part of these molecules, are released in a soluble form. It can be hypothesized that HLA-E, besides the protective effect by NK killing, may regulate multiple functions of macrophages. Both membrane-bound and soluble HLA-E can contribute to make macrophages, which are professional APC, eventually able to interact with CTL or, in addition, T cells with regulatory functions that are restricted for HLA-E.

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